

**DIELECTROPHORETIC ISOLATION, QUANTIFICATION, AND
CHARACTERIZATION OF NANOPARTICLES FOR EARLY CANCER DETECTION
AND TREATMENT**

By

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CERTIFICATE OF APPROVAL

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Dedication

*To my mother and father –
Thank you for everything.*

Epigraph

“If you’re going through hell, keep going.”

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List of Abbreviations

AADT	amino acid depletion therapy
AC	alternating current
AF	Alexa Fluor®
ALL	acute leukemic lymphoma
Asn	asparagine
B6a	albino C57Bl/6
B7H6	B7 homolog 6
BG	background
BSA	bovine serum albumin
CA19-9	carbohydrate antigen 19-9
CEA	carcinoembryonic antigen
CEMIP	cell-migration-inducing hyaluronan binding protein
cfDNA	cell-free DNA
CHIP	clonal hematopoiesis of indeterminate potential
CLDN3	Claudin 3
CM	Clausius-Mossotti
CS	chitosan
CT	computed tomography
CV	coefficient of variation
cyc-DEP	cyclic immunofluorescence imaging on dielectrophoretic chip
cycIF	cyclic immunofluorescence

CZI	Carl Zeiss Image
DBCO-TEG	dibenzylcyclooctyl-triethylene glycol
DCM	dichloromethane
DDS	drug delivery system
DEP	dielectrophoresis; dielectrophoretic
DEP-FFF	dielectrophoretic field-flow-fractionation
DLS	dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DMT	(+)-Dimethyl L-tartrate
DNA-PAINT	DNA-based point accumulation for imaging in nanoscale topography
DPBS	Dulbecco's Phosphate-Buffered Saline
DS	docking strand
EC	ethylene carbonate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunoassay
ESCRT	endosomal sorting complexes required for transport
EUS-FNA	endoscopic ultrasound-guided fine needle aspiration
EV	extracellular vesicle
FASN	fatty acid synthase
FBS	fetal bovine serum
FFPE	formalin-fixed paraffin-embedded

FPLC	fast protein liquid chromatography
γH2AX	phospho-histone H2A.X
HCl	hydrochloric acid
IM	intramuscular
IS	imaging strand
IV	intravenous
LED	light-emitting diode
MRI	magnetic resonance imaging
MUC5AC	mucin 5AC
MUC16	mucin 16
MVE	multivesicular endosome
MVB	multivesicular body
nDEP	negative dielectrophoresis
NK	Natural killer
NKG2D	Natural killer group 2 member D
NKp30	Natural killer protein 30
NP	nanoparticle
NSG™	NOD scid gamma
NTA	nanoparticle tracking analysis
oligo	oligonucleotide
oligo cyc-DEP	oligonucleotide cyclic immunofluorescence imaging on dielectrophoretic chip
PBS	phosphate buffered saline

PDAC	pancreatic ductal adenocarcinoma
pDEP	positive dielectrophoresis
PDI	polydispersity index
PEG	polyethylene glycol
PFA	paraformaldehyde
PGK1	phosphoglycerate kinase 1
PK	proteinase K
PLC	peritoneal lavage cytology
PLGA	poly(lactic-co-glycolic acid)
P/S	penicillin-streptomycin
PSA	prostate specific antigen
PVA	polyvinyl alcohol
RFP	red fluorescent protein
ROI	region-of-interest
SEM	scanning electron microscopy
SiLGA	silica-coated poly(lactic-co-glycolic acid)
SNR	signal-to-noise ratio
TAA	tumor-associated antigen
TE	Tris-ethylenediaminetetraacetic acid
TEM	transmission electron microscopy
TIFF	Tag Image File Format
TMOS	tetramethyl orthosilicate
UV	ultraviolet

Abstract

Dielectrophoretic Isolation, Quantification, and Characterization of Nanoparticles for Early Cancer Detection and Treatment

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The detection of cancer at its early stages requires the analysis of new biomarkers that are specific to the tumor and have less background than conventional biomarkers. Nanoparticles released by tumors into circulation are an important source of new biomarkers that have the potential to be sensitive enough for early stage cancer detection. These nanoparticles include exosomes, which are secreted by tumor cells at higher levels than healthy cells, contain biomolecular cargo from the tumor cells themselves, and perform critical functions in carcinogenesis. Analysis of tumor-derived exosomes could therefore provide disease-, tissue-, and stage-specific information, making them attractive targets for liquid biopsy assays; however, isolation of exosomes

from the blood remains a major challenge that often requires large sample volumes and time-intensive protocols, hindering clinical translation. Therefore, there is an unmet clinical need for the quick isolation of cancer-derived nanoparticles from low-volume liquid biopsies for analysis of biomolecular content in an effort to develop effective platforms for early cancer detection. There is also a need for novel nanoparticle drug delivery systems to treat early-stage and non-resectable tumors once they are discovered.

The present research utilizes a microfluidic, dielectrophoretic chip to collect exosomes and other extracellular vesicles directly from small volumes of undiluted blood plasma for immunofluorescence labeling of protein biomarker content. A custom image-processing algorithm was designed and created to automate the quantification of fluorescently labeled biomarkers collected on the chip for comparisons of target protein content across samples, eliminating analytical user bias. An internal standard was developed to help correct for differences in biomarker collection efficiency across chips, further reducing error. Characterization of these techniques established a lower limit of detection of the system and a dynamic range over which fluorescence increased linearly with exosome concentration and with image acquisition exposure time. These image quantitation methods were utilized to develop new techniques to multiplex proteomic profiling of endogenous extracellular vesicles and cell-derived nanoparticle collected on the dielectrophoretic chip *via* cyclic immunofluorescence, confirming both complete quenching between successive rounds of staining and the impact of round order on fluorescence intensity. On-chip cyclic immunofluorescence increased multiplicity by a factor of three, demonstrating successful interrogation of multiplex proteomic panels in

small-volume liquid biopsies. Furthermore, to develop chemotherapy treatment strategies appropriate for these discovered early stage cancers, a hybrid silica-coated poly(lactic-co-glycolic acid) nanoparticle was developed to make enzymatic cancer treatment less immunogenic. These particles prevented proteolysis and slowed the initial release of enzymatic cargo. This drug delivery system could improve amino acid depletion therapy in patients with early-stage non-resectable tumors. Together the detection and treatment strategies developed here could lead to new clinically relevant techniques to help improve overall survival of cancer patients.

Chapter 1. Introduction to early cancer detection and treatment

1.1 Cancer impact on society

Cancer is currently the second leading cause of death in the United States. Recent models estimate that the year 2021 will witness 1.9 million new cases of cancer and over 600,000 cancer-related deaths across the nation¹. At present rates, approximately 40% of the country's population will develop cancer during their lifetimes¹. With the combined five-year relative survival rate at around 65%¹, just under 15% of the current population of the United States (over 46 million people) will ultimately die from cancer within five years of diagnosis.

Perhaps surprisingly, these statistics mark substantial progress in the decades-long fight against cancer. In the early 1960s, combined five-year relative survival rates of cancer in the United States were less than 40% for White people and less than 30% for Black people¹. Though overdiagnosis, length bias, lead-time bias, and smoking cessation programs all contributed to increased survival rates^{1,2}, therapeutic advances and earlier detection drove the increase in combined five-year relative survival rates¹. Data suggests that novel approaches for early detection, diagnosis, and treatment will continue to improve patient prognosis as they have historically.

1.2 Early detection improves survival

Current strategies for early detection rely on routine screening of high-risk populations with genetic predispositions, potential comorbidities like early-onset diabetes, or elevated exposure to carcinogens. The purpose of routine cancer screening is to refer at-risk individuals for diagnostic procedures prior to symptomatic presentation and tumor metastasis, thereby prompting diagnosis of malignant tumors at earlier stages when therapeutic intervention is most effective. Cancer diagnosis at early stages indisputably correlates with improved treatment efficacy and survival^{1, 3-5}.

One-year survival for adults diagnosed with cancer between 2012 and 2016 highlight the importance of early detection⁵. The United Kingdom's Office of National Statistics estimated that one-year patient survival of colorectal cancer was 98% when diagnosed at stage 1 and 44% at stage 4. For lung cancer, 87% of patients survived one year after stage 1 diagnosis while only 19% survived that long after stage 4 diagnosis. One-year survival of liver cancer was 69% when diagnosed at stage 2, dropping to 39% at stage 3. These data show an increase in survival relative to the period spanning 2006 to 2010 in part due to earlier detection. With the current rate of early cancer diagnosis at approximately 57%⁶, the United Kingdom must make substantial progress over the next six years to meet its goal of diagnosing 75% of cancers at stage 1 or 2 by the year 2028⁷. This goal will require developing new technologies for early cancer detection.

1.2.1 Cancer detection methods

For at-risk individuals with access, screening reduces the morbidity of several types of cancer⁸. Mammography⁹⁻¹¹, Pap smears¹²⁻¹⁵, fecal occult blood testing/colonoscopy¹⁵⁻¹⁷, and low-dose computed tomography^{15, 18} (CT) have improved patient outcomes for populations at risk of breast, cervical, colon, and lung cancers, respectively. Unfortunately, approaches to cancer screening are far from perfect. Many screening tests require expensive equipment, trained technicians, and specialized knowledge to acquire and interpret results. Financial burden, physical access to medical facilities, time required for testing, procedural invasiveness, and lack of awareness can reduce compliance with screening recommendations.

Several cancers, like pancreatic ductal adenocarcinoma (PDAC), lack accepted screening techniques and must be detected incidentally or through symptomatic presentation. Magnetic resonance imaging (MRI) and CT have coincidentally identified pancreatic cysts in patients presenting symptoms unrelated to PDAC^{19, 20}. After incidental identification of pancreatic cysts²¹ or symptomatic presentation^{22, 23} (e.g. weight loss, jaundice, or abdominal pain), biopsies of pancreatic tissue are collected using highly-invasive endoscopic ultrasound-guided fine needle aspirations (EUS-FNA) for diagnosis of PDAC^{24, 25}. EUS-FNA is ultimately unneeded for at least 60% of referred patients²⁶. Furthermore, 2.7% of referred patients will develop diseases or complications, and 0.2% of patients will die, as a direct result of EUS-FNA biopsy²⁷. Screening techniques must be developed to prevent unnecessary tissue biopsies, identify tumors at earlier stages, and improve diagnosis, prognosis, and treatment.

Most invasive diagnostic and post-progression tissue biopsies for non-small-cell lung cancer utilize either bronchoscopy or percutaneous needle biopsy, which are associated with downstream health complications and substantial financial burden²⁸. Within thirty days of the procedure, approximately 65% of biopsies resulted in hospitalization for complications primarily related to pneumothorax, difficulty breathing, or chest pain. In addition to the extra physical burden on patients, downstream health complications increase the cost of already expensive procedures. Diagnostic biopsies that prompt complications within thirty days are on-average 56% more expensive per patient than those without complications (\$24,968 versus \$15,988). Post-progression biopsies prompting complications within thirty days are on-average 142% more expensive per patient than those without complications (\$30,293 versus \$12,494). These costs are compounded by the fact that almost half of primary lung cancer patients undergo more than one tissue biopsy before diagnosis²⁹. As with EUS-FNA for PDAC, lung tissue biopsies also present potential long-term health complications. Upon insertion or retraction, needles used to biopsy lung tissue can dislodge tumor cells throughout the body³⁰, which occasionally seed additional tumor sites^{31, 32} that could prompt long-term health complications with additional costs. The overall invasiveness, complications, and associated costs of lung tissue biopsy make it unsuitable for routine screening of at-risk individuals, which could partially explain why 79% of lung cancers are diagnosed at late stages²⁹ and why lung cancer is the deadliest malignant neoplastic disease in the United States¹.

1.2.2 Blood-based liquid biopsies for cancer screening

Blood-based liquid biopsies offer logistical advantages over tissue biopsies and other screening techniques. Blood collection is commonplace, quick, minimally invasive, necessitates minimal training, and requires no specialized equipment. Blood collection, processing, and analysis are standard procedures in point-of-care testing at almost all medical facilities. These logistical advantages could feasibly improve access to cancer screening for racial minorities and the socioeconomically disadvantaged, whom will face increasing and disproportionate financial barriers to diagnosis and treatment as cancer-related direct medical costs are projected to exceed \$245 billion per year by 2030¹.

In addition to logistical considerations, blood has several technological and physiological advantages over tissue biopsies. Unlike the specialized equipment and training required to biopsy pancreatic tissue using EUS-FNA²⁴, complex technology is not required for blood collection. Furthermore, as effective assays for blood-based screening emerge, the underlying technology could likely be leveraged or modified to screen for additional types of cancer. The primary physiological advantage of blood is that it physically contacts every organ in the body, including neoplastic tissues as small as one millimeter in diameter⁴. For this reason, blood may also provide a more complete picture of overall tumor heterogeneity than tissue biopsies, which only sample small areas of the tumor. Theoretically, a series of blood-based assays conducted on a single blood sample could provide information about every tissue in the body, which would otherwise be obtained through a combination of tissue biopsy, MRI/CT, x-rays, palpation, colonoscopy, and other invasive procedures. The utility of blood-based liquid

biopsy as a catch-all for cancer screening is further increased by the likelihood that effective early detection strategies may rely on measuring physiological changes over time. Routine blood tests are more feasible to implement than repeated imaging and tissue biopsy.

The primary practical challenges of blood-based assays are: 1) identifying tissue- and disease-specific targets; and, 2) reliably isolating targets in quantities sufficient for detection. Some blood-based cancer screening tests, like the prostate-specific antigen (PSA) test for prostate cancer^{33, 34}, are widely accepted despite major flaws. PSA is a highly tissue-specific protein that plays several roles in the progression of prostate cancer³⁵⁻³⁹; however, changes in circulating PSA levels do not necessarily indicate cancer. Benign prostatic hyperplasia, urinary tract infections, bacterial prostatitis, physical activity, ejaculation, stimulation of the prostate gland, and use of certain medications can affect circulating PSA levels. These confounding variables cause high rates of false-positive test results, which prompt unnecessary biopsies and overtreatment^{40, 41}. Considering these complications, PSA testing is no longer specifically recommended by major medical organizations. Instead, at-risk populations are merely encouraged to meet with healthcare providers to make an informed decision about PSA testing¹. While effective at reducing prostate cancer mortality, PSA is insufficiently disease-specific to be a viable target for blood-based prostate cancer screening.

One blood test for PDAC monitoring targets carbohydrate antigen (CA) 19-9⁴²⁻⁴⁴. While elevated levels of CA 19-9 can indicate advanced PDAC, a variety of diseases or other conditions are also known to increase circulating CA 19-9 levels^{42, 45, 46}, including:

pancreatitis, cirrhosis, acute cholangitis, and other cancers. Furthermore, not all PDAC patients exhibit elevated serum levels of CA 19-9⁴⁵. While multi-target panels that look for CA 19-9 and additional markers like mucin (MUC) 5AC and MUC16⁴⁷, carcinoembryonic antigen (CEA)⁴⁸, and cell-migration-inducing hyaluronan binding protein (CEMIP)⁴⁹ can increase assay sensitivity and/or specificity, CA 19-9 is not recommended as a blood-based biomarker for PDAC screening, despite its clinical use, due to its lack of tissue- and disease-specificity^{42, 43, 50}. Problems involving the clinical use of PSA and CA19-9 highlight the need to develop effective blood-based assays for cancer screening that rely on multi-omic target panels.

1.2.3 Challenges of detecting tumor-derived cell-free DNA

Next-generation blood tests target a variety of circulating biomarkers including DNA. Sequencing of tumor-derived cell-free (cf) DNA, or circulating tumor (ct) DNA, from blood-based liquid biopsies can reveal oncogenic mutations; however, traditional methods of recovery usually target the smaller apoptotic DNA fragments, typically between 160 and 200 base-pairs long⁵¹⁻⁵³. Background noise levels from healthy tissue can reach as high as 10^4 healthy DNA fragments per tumor-derived fragment⁵⁴⁻⁶⁰, which makes cancer detection difficult.

In addition to the low signal-to-noise ratio (SNR), the mere presence of mutated DNA does not necessitate the downstream translation of proteins that directly drive malignant tumor development, growth, and progression. Therefore, while certain mutations are requisite for tumor development, they do not always indicate malignant

neoplastic growth. For example, it can be difficult for ctDNA analysis to differentiate tumors from clonal hematopoiesis of indeterminate potential (CHIP)⁶¹, which involves the relatively normal and highly prevalent accumulation of genetic mutations in blood cells as one ages⁶²⁻⁶⁴. Confusion between tumor- and CHIP-derived mutations^{65, 66} could lead to unnecessary cancer treatment for people with CHIP⁶⁷, whom are more likely than people without CHIP to develop neoplasms after chemotherapy⁶⁸. While not pervasive, this confusion can entirely negate the purpose of ctDNA-based cancer screening and indirectly initiate tumor growth.

Part of this confusion is that plasma ctDNA represents a variable yet often small fraction of all plasma cfDNA, especially in patients with low tumor burden^{61, 69}. Some estimate fewer than two mutant templates per milliliter of blood plasma from patients with certain early-stage cancers^{70, 71}, an inherently low SNR that can potentially prompt unreliable sampling⁷⁰ and hinder early detection. Another setback for ctDNA analysis is that a variety of different tumors may exhibit the same oncogenic mutation(s), which is problematic for identifying unique neoplastic tissues⁷². Therefore, genomic analysis of ctDNA, if detected, might identify the presence of cancer without indicating the exact neoplastic region in the body.

1.2.4 Other challenges for blood-based liquid biopsy detection

Despite these challenges, genomic- and methylation-based ctDNA analysis undoubtedly has its place in blood-based liquid biopsy, especially when incorporated into multi-omic, multiplex panels like CancerSEEK⁷³. Genomic information about

mutations in ctDNA enabled high assay specificity, while added proteomic information improved tissue-specificity and overall assay sensitivity. Similar improvements to assay sensitivity were achieved when *KRAS* mutations in the plasma cfDNA of PDAC patients were analyzed in conjunction with multiple protein biomarkers⁷⁴. Other next-generation assays for blood-based liquid biopsy focus on a variety of biomarkers, like GRAIL's Circulating Cell-Free Genome Atlas Study for cell-free nucleic acids⁷⁵⁻⁷⁷, PanSEER for cfDNA methylation⁷⁸, CellMax Life FirstSight for circulating tumor cells (CTCs)⁷⁹⁻⁸¹, and a variety of metabolic panels⁸²⁻⁸⁶.

Most, if not all, of these blood-based assays lack the sensitivity and/or specificity necessary for implementation as cancer screening platforms at the population level. To further improve assay sensitivity and specificity, new sources of circulating cancer-derived biomarkers must be considered as they may provide tumor-specific information with low background from healthy tissue. One promising category of biomarkers are nanoparticles (NPs) shed by tumors into circulation throughout their development. While new methods of NP recovery and analysis that are suitable for the clinic would be necessary, cancer-derived NPs like exosomes and other extracellular vesicles (EVs) deserve further consideration.

1.2.5 Tumor-derived exosomes as a source of circulating biomarkers

Exosomes and other EVs carry biomarkers with great potential for early cancer detection. Exosomes are oversecreted by tumor cells⁸⁷ and contain tumor-associated antigens (TAAs) that indicate cellular origin. In order to aid clinical intervention of early-

stage solid tumors with malignant potential, there is an urgent need to develop inexpensive blood-based assays that target cancer-derived NPs like exosomes for routine screening of high-risk populations.

Exosomes are a subset of EVs defined by their size (30 – 150 nm), protein content, and biogenesis⁸⁸. Active exosome secretion originates with multivesicular endosomes (MVEs), which form from inward invaginations of the cell membrane. Each MVE then undergoes its own sequence of inward invaginations, ultimately forming a multivesicular body (MVB) that contain several intraluminal vesicles. Intraluminal vesicles encapsulate nucleic acids, proteins, lipids, cytokines, and enzymes from the cytosol. When MVBs fuse with the cell membrane, intraluminal vesicles are released from the cell and become exosomes. Importantly, all cells actively secrete exosomes, which are found in all bodily fluids including plasma⁸⁸.

Proteins and other biomolecules associated with exosomes reflect their biogenesis and cellular origin. By definition, exosome membranes contain molecules involved with endosomal sorting complex required for transport (ESCRT)^{89, 90}, endosome-specific tetraspanins (CD63, CD9, etc.)^{91, 92}, and ESCRT accessory proteins (TS101, ALIX, etc.)⁸⁹. Each exosome contains biomolecules unique to their cellular origin that were present in the cytosol or on the surface of the cell membrane during biogenesis^{93, 94}. This feature implies functionality. Once believed to be a mode of cellular waste removal^{95, 96}, exosomes are now seen as messengers for intercellular communication⁹⁷, delivering biomolecules from the original cell to other cells throughout the body⁹⁸. Several specific mechanisms of exosome-cell interaction have been

identified⁹⁹, and associated biomolecular cargo remains active after exosomal delivery¹⁰⁰.

Rates of exosomal biogenesis are higher in tumor cells than in healthy cells¹⁰¹⁻¹⁰³, suggesting that tumors may partially depend on exosomes for carcinogenesis. Immunoregulation¹⁰⁴⁻¹¹¹, microenvironmental reprogramming¹¹², metastasis¹¹³⁻¹¹⁶, and angiogenesis¹¹⁷⁻¹²⁰ are all moderated in part by tumor-derived exosomes. TAAs on tumor-derived exosomes may have prognostic and/or diagnostic value as changes in exosome profiles could indicate shifts in carcinogenesis, reflecting disease progression and malignant potential. Importantly, SNRs are sufficient for interrogation of exosomal TAAs and other proteomic cargo. Data suggests that exosome fractions from cancer patients often contain higher overall protein content than those from healthy donors^{103, 121}. Protein content from plasma exosomes could have predictive value in certain cancers^{103, 122}. In all, high-multiplicity proteomic profiling of tumor-derived exosomes could potentially provide disease-, tissue-, and stage-specific information from blood-based liquid biopsies.

1.2.6 Traditional exosome isolation techniques are not suitable for the clinic

Efficient isolation is one of the main barriers prohibiting the clinical implementation of exosome profiling for diagnostic applications. “Gold-standard” techniques for the isolation of EVs like exosomes from blood, plasma, and serum include ultracentrifugation¹²³⁻¹²⁵, precipitation¹²⁴⁻¹²⁶, immunoaffinity capture^{124, 125, 127, 128}, chromatography¹²⁴⁻¹²⁶, and filtration^{124, 125, 129}. Many isolation techniques require sample

volumes of at least several hundred microliters to one milliliter and can take several hours to perform. Others utilize proteins on subsets of EVs or exosomes for immunocapture-based collection, which may exclude particles containing the target protein of interest because biomarker profiles are not uniform across all exosomes. This potential bias in collection can be problematic when profiling relatively low-abundance TAAs affiliated with tumor-derived subpopulations of exosomes and other EVs. While these isolation techniques work well in laboratory settings, laborious, time-intensive, and non-standardized protocols for processing large volumes of blood hinder the clinical utility of circulating exosomes for cancer screening at the population level^{42, 124}. For example, research has shown that circulating exosomes are present during early-stage PDAC^{130, 131}, yet complicated isolation protocols prohibit the study of cohorts sufficiently large to prove the clinical utility of exosome profiling for screening⁴². As Konoshenko *et al.* argue, the ideal standard isolation method should be quick, cost-effective, simple, devoid of elaborate equipment, and amenable to a variety of sample types¹²⁴. In that regard, new dielectrophoretic microchip technology offers several advantages as a platform to isolate exosomes and other EVs from blood-based liquid biopsies.

1.3 Dielectrophoresis (DEP)

1.3.1 DEP for the isolation of cancer-derived NPs

Dielectrophoretic (DEP) chip technology isolates NPs directly from undiluted, minimally processed liquid biopsies like blood plasma. Biomarker isolation occurs within

minutes of applying DEP forces. The design of the microfluidic chip allows DEP isolation of biomarkers from sample volumes as low as twenty-five microliters, which permits minimally-invasive sample collection. Importantly, isolation of targeted biomarkers is label-free because DEP depends largely on material properties and particle morphology rather than the presence of specific proteins, which is necessary for isolation techniques that utilize immunoaffinity binding or capture (e.g. immunoprecipitation beads, ELISAs, flow cytometry, etc.). One unique aspect of the chip is its ability to induce the electrokinetic motion of bioparticles in high-conductance media without diluting the sample. This feature enables exosome collection directly from blood plasma without the need for dilution, thereby maximizing biomarker concentrations for enhanced detection. Importantly, on-chip biomarker detection *via* immunofluorescent labeling minimizes fluid transfer loss. Furthermore, samples collected using DEP do not need to be fixed, eliminating the need for toxic compounds. In addition to on-chip detection, biomarkers can be eluted into clean buffer and withdrawn out of the microfluidic chamber for downstream, off-chip analysis. In particular, the low-volume requirement and the prospect of downstream analysis are attractive features for the characterization of rare retrospective cohorts. Overall, the simplicity of the platform is promising for scalability and automation, which would be necessary for clinical adoption.

This particular DEP chip technology is used primarily to collect nanoscale lipid vesicles (e.g. exosomes and other EVs) and nanoscale DNA fragments (e.g. high-molecular-weight cfDNA) from undiluted blood plasma for on-chip immunofluorescence labeling of protein content. Collection of multiple types of nanoparticulate biomarkers

simultaneously from a low-volume, undiluted liquid biopsy could feasibly enable multi-omic analysis, which could prove to be the ultimate key to early cancer detection.

The design of the DEP chip used in this work consists of a planar array of platinum microelectrodes embedded at the base of a microfluidic chamber, as shown in **Figure 1.1**. The particular design of the planar microelectrode array generates a nonuniform electric field under applied voltage. Local maxima in the applied electric field gradient are located around the circumferences of each microelectrode in the planar array. A low-volume liquid biopsy is withdrawn into the microfluidic chamber *via* flow and then held static above the microelectrode array. A proprietary hydrogel layer protects biomarkers from degradation, enabling DEP collection directly from undiluted high-conductance biofluids like plasma. Positive DEP (pDEP) forces are induced in targeted biomarkers that are suspended in the liquid biopsy. Under pDEP, targeted biomarkers move toward the electrode circumferences, where they are held during washing. Fluorophore-conjugated antibodies are withdrawn into the chamber, labeling protein content of the biomarkers. Exosomes and other EVs are not fixed prior to immunolabeling, eliminating the need for toxic compounds. Differences in fluorescence levels at the electrode circumferences relative to adjacent regions indicate the presence of content at the regions of collection, suggesting associations between targeted biomarkers and specific proteins or nucleic acids.

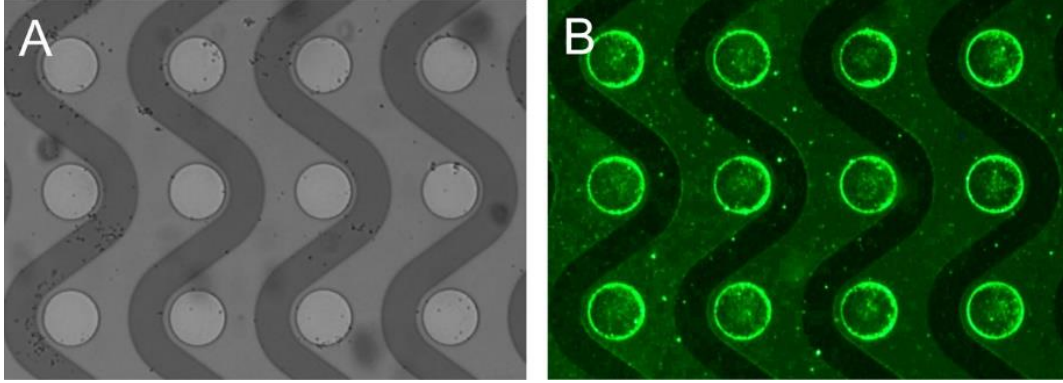


Figure 1.1. Electrode array on DEP microfluidic chip. a) Bright field image showing twelve planar microelectrodes (each 60 μm diameter) at the base of the fluidic chamber on a DEP chip. b) Exosomes from the plasma of breast cancer patients are collected at the circumference of each planar microelectrode using DEP forces. Exosomes are immunofluorescently labeled on-chip with anti-CD63 conjugated to AlexaFluor 488. Figure used with permission¹³².

1.3.2 Definitions of DEP and the DEP force

A DEP force is exerted on a particle that is placed in an electric field gradient, or nonuniform electric field¹³³. Motion of the particle induced by the DEP force is called dielectrophoresis¹³⁴⁻¹³⁶. The strict definition of the DEP force has two requirements:

1. There must be contrast between the electrical polarizability of the particle and the electrical polarizability of the suspending medium; and,
2. There must be a non-uniform electric field.

These requirements are represented in the mathematical definition of the DEP force:

$$\vec{F}_{DEP} \equiv \vec{p} \cdot \nabla \vec{E}_0$$

The first requirement of contrast in electrical polarizability is represented by a net, nonzero electric dipole moment ($\vec{p}$) across the particle. The second requirement is represented by a gradient in the applied electric field ($\nabla \vec{E}_0$), indicating that field strength varies across the space in which the electric field is applied.

Applying a voltage across a set of electrodes will generate an electric field. The applied electric field polarizes molecular dipoles in the particle and in the medium. The overall induced electric dipole moment across the particle represents the net interaction of molecular dipoles generated in the particle and medium. Therefore, it is application of the electric field, and not the electric field gradient, that induces an electric dipole moment associated with the particle.

The induced electric dipole moment then responds to the gradient in the electric field, which can be induced by particular electrode geometries. A DEP force is exerted on a particle when its induced electric dipole moment interacts with an electric field gradient. The directionality of the DEP force arises from a term called the Clausius-Mossotti (CM) factor¹³⁷, which is central to the induced electric dipole moment. The induced electric dipole moment can be represented in terms of the volume and polarization of the particle. Polarization is the density of constituent or molecular electric dipole moments that are induced within the particle in the presence of an applied electric field. For a solid (single-phase) spherical particle of volume (V_{sphere}) and polarization ($\vec{P}_{sphere}$), the induced electric dipole moment is:

$$\vec{p} = V_{sphere} \vec{P}_{sphere}$$

The primary material property governing electrical polarizability is the dielectric constant, or relative permittivity. Differences in relative permittivity between the particle and the suspending medium amount to contrast in electrical polarizability. To determine the polarization of the particle, one must assume that both the electric potential and the electric force flux are continuous at the boundary between the particle and the medium. These assumptions allow one to solve the Laplacian for electric potential, yielding an expression for polarization...:

$$\vec{P}_{sphere} = 3\varepsilon_0\varepsilon_m(CM)\vec{E}_0$$

...where ε_0 is a universal constant called the vacuum permittivity, ε_m is the dielectric constant of the medium, $\vec{E}_0$ is the applied electric field, and CM is the Clausius-Mossotti factor relating the dielectric constants of the particle (ε_p) and medium (ε_m):

$$CM = \frac{\varepsilon_p - \varepsilon_m}{\varepsilon_p + 2\varepsilon_m}$$

Therefore, the induced electric dipole moment for a solid (single-phase) spherical particle is...:

$$\begin{aligned}\vec{p} &= V_{sphere} \vec{P}_{sphere} \\ \vec{p} &= \frac{4}{3} \pi R^3 (3\epsilon_0 \epsilon_m (CM) \vec{E}_0) \\ \vec{p} &= 4\pi \epsilon_0 \epsilon_m R^3 (CM) \vec{E}_0\end{aligned}$$

...where R is the radius of the particle.

1.3.3 The CM Factor determines DEP directionality

Whereas the directionality of the induced dipole moment is determined by the directionalities of the CM factor and the applied electric field, the directionality of the DEP force is determined only by the CM factor. The CM factor indicates the contrast between the relative permittivity of the particle and the medium, which is reflected in its sign. If the particle is more polarizable than the medium ($\epsilon_p > \epsilon_m$), the CM factor and the DEP force are positive. A positive DEP force directs particle motion up the electric field gradient *via* positive dielectrophoresis (pDEP). If the medium is more polarizable than the particle ($\epsilon_m > \epsilon_p$), the CM factor and the DEP force are negative, and particle motion is directed down the electric field gradient *via* negative DEP (nDEP). The DEP force on a solid (single-phase) spherical particle can be described in terms of the CM factor...:

$$\vec{F}_{DEP} \equiv \vec{p} \cdot \nabla \vec{E}_0$$

$$\vec{F}_{DEP} = 4\pi\epsilon_0\epsilon_m R^3(CM)\vec{E}_0 \cdot \nabla \vec{E}_0$$

Given the following substitution...:

$$\vec{E}_0 \cdot \nabla \vec{E}_0 = (\vec{E}_0 \cdot \nabla) \vec{E}_0 = \frac{\nabla(\vec{E}_0 \cdot \vec{E}_0)}{2} = \frac{\nabla|\vec{E}_0|^2}{2}$$

...the equation for the DEP force becomes:

$$\vec{F}_{DEP} = 2\pi\epsilon_0\epsilon_m R^3(CM)\nabla|\vec{E}_0|^2$$

All terms in this representation of the DEP force are necessarily positive except for the CM factor, which can be positive or negative. This observation reiterates that the CM factor dictates the directionality of the DEP force.

The CM factor governs that the directionality of the DEP force is a consequence of material properties. Specifically, the electrode polarity of an applied voltage does not determine the direction in which a particle moves under DEP. Therefore, DEP forces can be generated using direct current or alternating current; however, the frequency of the applied alternating current can affect a material's dielectric properties, thereby altering a particle's DEP motion.

1.3.4 DEP under applied alternating current

Under alternating current, a material's dielectric constant is a complex number (ε^*) dependent on the applied frequency (ω) and electrical conductivity (σ):

$$\varepsilon^*(\omega) = \varepsilon - i \frac{\sigma}{\omega \varepsilon_0}$$

The CM factor, represented explicitly in terms of dielectric constants, becomes a function of frequency when the voltage that generates the electric field is applied using alternating current:

$$CM^* = \frac{(\varepsilon_p^* - \varepsilon_m^*)}{(\varepsilon_p^* + 2\varepsilon_m^*)}$$

$$CM(\omega)^* = Re[CM(\omega)] + i * Im[CM(\omega)]$$

$$Re[CM(\omega)] = \frac{\omega^2(\varepsilon_p - \varepsilon_m)(\varepsilon_p + 2\varepsilon_m) + (\sigma_p - \sigma_m)(\sigma_p + 2\sigma_m)}{\omega^2(\varepsilon_p + 2\varepsilon_m)^2 + (\sigma_p + 2\sigma_m)^2}$$

$$Im[CM(\omega)] = \frac{\omega(\sigma_p - \sigma_m)(\varepsilon_p + 2\varepsilon_m) - (\varepsilon_p - \varepsilon_m)(\sigma_p + 2\sigma_m)}{\omega^2(\varepsilon_p + 2\varepsilon_m)^2 + (\sigma_p + 2\sigma_m)^2}$$

Therefore, the DEP force under alternating current also exhibits a frequency-dependence:

$$\langle \mathbf{F}_{DEP}(\omega) \rangle = 2\pi\varepsilon_m\varepsilon_0 R^3 Re[CM(\omega)] \nabla |\mathbf{E}_{rms}|^2$$

Importantly, the directionality of the real component of the CM factor for a particle and its suspending medium can change depending on the frequency of the applied alternating current. This change results from the fact that the electrical conductivities of a particle and its medium increasingly dominate the real component of the CM factor as frequency decreases, while relative permittivity increasingly dominates as frequency increases:

$$Re[CM(\omega \rightarrow 0)] = \frac{\sigma_p - \sigma_m}{\sigma_p + 2\sigma_m}$$

$$Re[CM(\omega \rightarrow \infty)] = \frac{\varepsilon_p - \varepsilon_m}{\varepsilon_p + 2\varepsilon_m}$$

At sufficiently high frequencies, electrode polarity alternates too quickly for molecular dipoles to alternate in response. This scenario resembles molecular dipoles under direct current, in which molecular dipoles do not change polarity because electrode polarity does not alternate. The real component of the CM factor at high frequencies reduces to the expression for the CM factor under direct current, which is exclusively a function of relative permittivity of the particle and its suspending medium. Unlike under direct current, electrode polarity alternates slowly enough at low frequencies for molecular dipoles and electrical charges to alternate in response. Electrical conductivity, which characterizes a material's ability to conduct electricity or the motion of electrical charges, therefore becomes relevant at low frequencies.

1.3.5 Cross-over frequency

The frequency of the applied alternating current implicates additional material properties (*i.e.* the electrical conductivities of the particle and its suspending medium) in the directionalities of the CM factor and the DEP force. Some combinations of particles and media will prompt pDEP at certain frequencies and nDEP at other frequencies. For instance, a particle with electrical conductivity less than and relative permittivity greater than those of its suspending medium might experience nDEP at low frequencies and pDEP at high frequencies. The frequency at which the DEP force changes directionality (*i.e.* from nDEP to pDEP or vice versa) is called the crossover frequency (ω_c). The equation for the crossover frequency of a solid (single-phase) spherical particle is:

$$\omega_c = \sqrt{\frac{(\sigma_p - \sigma_m)(\sigma_p + 2\sigma_m)}{(\varepsilon_p - \varepsilon_m)(\varepsilon_p + 2\varepsilon_m)}}$$

Particles of different compositions that are suspended in a given medium often exhibit unique crossover frequencies. A particle composed of multiple phases (*e.g.* aqueous core and solid shell, or silica-coated polymeric NPs) can exhibit multiple crossover frequencies because the presence of extra materials and their associated geometries introduce additional boundary conditions when solving the Laplacian of the polarization vector, ultimately changing the expression for the CM factor. Furthermore, some particles do not exhibit a crossover frequency and experience pDEP or nDEP at all frequencies.

1.3.6 *DEP versus electrophoresis*

Comparing DEP to electrophoresis highlights the utility of using alternating current to isolate bioparticles from biofluids. Electrophoresis is defined as the electrokinetic motion of charged particles induced by an electric field. Importantly, DEP does not require that the particle carry electrical charge, only that the particle is electrically polarizable. Furthermore, DEP requires the electric field to be nonuniform, whereas electrophoresis does not. Importantly, DEP permits the presence of an electrical charge and electrophoresis permits the presence of an electric field gradient. An uncharged, electrically polarizable particle in a uniform electric field will experience neither electrophoresis nor DEP. A charged, electrically polarizable particle in a nonuniform electric field will experience both electrophoresis and DEP.

Most bioparticles carry negative electrical charge, suggesting that application of a nonuniform electric field gradient would induce both electrophoresis and DEP. This can be undesirable because Coulombic forces associated with electrophoresis might oppose the DEP force, depending on material properties including the electrical charge of the particle. This potential opposition can partially or entirely negate DEP by slowing, stopping, or reversing particle motion.

Applying alternating current to create a nonuniform electric field that induces DEP in a charged particle can effectively isolate the DEP force from the Coulombic force. As demonstrated, DEP can occur under direct or alternating current due to the dependence of the CM factor on the material properties of the particle and its suspending medium. Over time, the Coulombic force on a charged particle under

alternating current nets to zero. Furthermore, alternating current introduces frequency as an experimental parameter to induce pDEP in a subset of bioparticles while simultaneously inducing nDEP in the rest. This contrast is a consequence of the material and morphologic properties that dictate crossover frequency.

1.3.7 Potential applications for DEP in liquid biopsy

Several applications leverage the frequency dependence of the DEP force under alternating current to target biomarkers. Neoplastic cells have been targeted in DEP field-flow-fractionation (DEP-FFF)¹³⁸⁻¹⁴², which enable dynamic label-free cell-sorting by applying DEP forces to alter the trajectory of targeted cells under flow. Subpopulations of circulating cellular biomarkers are binned as similar trajectories terminate at distinct collection ports on the chip. Count data for each subpopulation can be obtained from on-chip imaging. Additionally, DEP-FFF offers unique sample pre-processing for downstream proteomic interrogation using techniques like fluorescence imaging, flow cytometry, droplet digital PCR, and sequencing.

As apparent in the equation for the DEP force, the magnitude of the force increases as the size of the particle it acts upon increases¹³⁵. This size dependence explains why DEP-FFF platforms primarily isolate cells or microparticles rather than NPs and small EVs like exosomes. DEP isolation of nanoscale biomarkers often occurs in static fluid because the DEP forces are smaller and generally cannot compete with flow forces.

1.3.8 DEP chip technology for NP isolation

The primary mode of operation for the technology used in this work induces pDEP in nanoparticulate biomarkers, which move through static fluid toward maxima in the local electric field gradient. At these maxima, DEP forces hold targeted biomarkers stationary while flow forces wash away off-target particles and bulk fluid, thereby isolating targets. Targeted biomarkers can be labeled in subsequent on-chip immunofluorescence assays, yielding information about proteomic and nucleic acid content. This DEP platform has been used to collect and immunolabel exosomes from cancer patient plasma. On-chip immunolabeling showed significant proteomic differences between patient populations, highlighting the potential for clinical utility of DEP for blood-based liquid biopsy. Circulating cfDNA has also been isolated from cancer patient plasma for on-chip immunolabeling and downstream analysis of point-mutations. As with CancerSEEK and other detection panels^{73, 74}, the multi-omic functionality of this DEP platform could be critical in developing assays with high sensitivity and specificity.

As previously mentioned, the minimal sample preparation and overall simplicity of DEP chip technology makes scalability and automation feasible. Additional steps along the path to clinical translation of DEP chip technology are addressed in this thesis, including efforts to standardize the quantification of fluorescence levels from on-chip immunoassays and increase the multiplicity of proteomic profiling from a single small-volume sample. These steps should establish a lower limit of detection, minimize analytical user bias, and improve the sensitivity and specificity of DEP-based assays.

1.4 Considerations for the treatment of early-stage cancer

Improvements to early detection necessitate treatment strategies that are appropriate for early-stage cancer. Once a tumor has been detected, it must be removed without causing undue harm to the patient. While early-stage solid tumors (*i.e.* stage 1 and 2) with malignant potential are dangerous and need to be treated, they are localized and pre-metastatic by definition. As such, traditional systemic chemotherapy is not always the most appropriate course of treatment. In fact, the majority of early-stage tumors are treated by surgical resection; however, earlier detection does not ensure tumor resectability. Some localized, non-metastatic solid tumors are inoperable based on their location in the body, like certain brainstem gliomas¹⁴³ and pancreatic tumors with hepatic arterial¹⁴⁴ or perineural invasion¹⁴⁵. Treating these tumors with systemic chemotherapy can have potential long-term health complications. For instance, doxorubicin can induce severe dose-dependent and potentially fatal cardiotoxic side effects, complicating treatment for many cancer patients¹⁴⁶⁻¹⁴⁸. Other common chemotherapeutics like cisplatin and oxaliplatin exhibit dose-limiting adverse side effects related to nephrotoxicity and neurotoxicity, respectively¹⁴⁹.

Some of these tumors can be treated with radiation therapy; however, like traditional systemic chemotherapy, most forms of radiation induce long-term health complications, which include lymphedema, carotid artery stenosis, radiation fibrosis, and radiation-induced heart disease¹⁵⁰. Hematologic malignancies cannot be surgically removed because they are not localized to a resectable region in the body. While radiation can be administered to treat leukemias and lymphomas, systemic

chemotherapy is the primary mode of treatment. With sufficient advances in early detection and drug delivery, systemic chemotherapy might one day be recommended to a patient that tests positive for cancer without indication of the malignant tissue. This scenario is most feasible for early-stage tumors that are smaller than the resolution limit of diagnostic imaging. Therefore, novel strategies for the systemic delivery of anti-tumor agents are necessary to realize the full benefit of early cancer detection.

It should be noted that resection and chemotherapy are not always mutually exclusive treatment strategies. Perioperative and adjuvant chemotherapeutics are often delivered to destroy malignant cells at the boundary of the resection site, reducing the likelihood of recurrence¹⁵¹⁻¹⁵³. This is especially true for tumors that are too large for complete resection, which must be treated using a combination of tumor debulking and systemic treatment¹⁵⁴⁻¹⁵⁶. In a manner opposite to tumor debulking, “downsizing” systemic chemotherapy can reduce an inoperable tumor burden so substantially that surgical resection becomes viable¹⁵⁷. Importantly, downsizing chemotherapy is often used to treat metastatic regions, which touches on an often-overlooked secondary aim of early cancer detection.

While a primary goal of early detection is the identification of early-stage resectable tumors, another critical aim is finding metastatic disease earlier⁴. Earlier detection of metastasis could ultimately enable tumor resection, but generally not without systemic chemotherapy. It follows that earlier detection of metastases may improve patient response to treatment; however, neither the early detection of primary tumors nor the earlier detection of metastatic disease will eliminate the need for and adverse effects of systemic chemotherapy. Therefore, the maximum benefit of early

detection will be achieved when novel therapies and delivery vehicles improve systemic chemotherapy.

Regardless of the circumstances limiting tumor resectability, NP drug delivery systems (DDSs) offer promising strategies to reduce the toxicity of systemic chemotherapy¹⁵⁸ and should be considered a powerful complement to early detection. The underlying concept is that NP DDSs encapsulate therapeutic cargo while in circulation, protecting that cargo from the immune system and limiting off-target exposure of the cargo to healthy tissues as it travels throughout the body. Once NP DDSs reach the tumor, most NP DDSs are designed to release their payloads. Targeted delivery, selective uptake, and controlled release are primary aims of NP DDSs to improve the systemic delivery of chemotherapeutics. Thorough evaluation of these properties requires characterization of DDS pharmacokinetics and biodistribution, though it can be challenging to recover NP DDSs from the blood in order to monitor circulation half-life. Fortunately, DEP is well-suited to collect NP DDSs from plasma¹⁵⁹. Therefore, through characterization of pharmacokinetic properties, DEP can be used as a tool to optimize particle design and improve the therapeutic potential of NP DDSs in treating nonresectable tumors.

response to DDSs that treat nonresectable tumors.

1.5 Contributions

With regard to blood-based liquid biopsies for cancer screening, there are unmet clinical needs for the quick and label-free separation of EVs like exosomes from undiluted plasma and for the subsequent detection of TAAs on tumor-derived EVs. Microfluidic DEP chip technology meets both needs by isolating tumor-derived EVs directly from undiluted blood plasma within minutes and enabling detection of TAAs through on-chip immunofluorescent labeling; however, the clinical translation of DEP chip technology requires standardizing quantitative techniques for on-chip detection of tumor-derived EVs as well as increasing the per-assay capacity for interrogating TAAs. To that end, this thesis presents the robust quantification and multiplexing of on-chip immunofluorescence assays that target tumor-derived EVs. Furthermore, the advent of next-generation early detection technologies necessitates novel strategies to treat patients with early-stage tumors. To that end, this thesis presents a DEP-responsive hybrid NP designed to enhance the efficacy of active enzymes in amino acid depletion therapies (AADT). The work presented here highlights essential roles for endogenous and synthetic NPs in the ongoing fight against cancer.

In this thesis, I design and characterize two complementary techniques for quantifying fluorescence levels of biomarkers that are collected from small-volume blood-based liquid biopsies using DEP and immunolabeled on-chip. Optical quantification techniques are employed in the development of high-multiplicity on-chip immunoassays. DEP-responsive hybrid NPs were synthesized to enhance enzyme-

based therapies, suggesting novel applications for DEP chip technology in monitoring therapeutic efficacy and patient response to treatment.

In Chapter 2, I developed an automated image processing algorithm and implemented an internal standard to quantify fluorescence levels of biomarkers collected and immunolabeled on-chip. The algorithm differentiated regions-of-interest (ROIs) from locally adjacent background (BG) regions and removed optical artifacts from calculations of fluorescence intensity. Full automation of the algorithm eliminated analytical user bias and reduced user burden. In conjunction with the algorithm, the internal standard measured the collection efficiency, or relative strength, of each DEP chip and adjusted biomarker levels accordingly. Characterization of these techniques established the lower limit of detection and a dynamic range over which fluorescence measurements were linear with exposure time and biomarker concentration.

Chapter 3 utilized the optical quantification techniques from Chapter 2 to design high-multiplicity on-chip cyclic immunofluorescence (cycIF) assays for proteomic profiling of liquid biopsy biomarkers collected using DEP. Samples were labeled with a panel of six primary antibody-fluorophore conjugates, introduced as pairs across three rounds of staining and two rounds of combined photobleaching and chemical quenching. Quantification techniques confirmed complete quenching of fluorescence in between rounds of immunolabeling and indicated that levels of certain target proteins varied by round order. Primary antibody-oligo conjugates and complementary oligo-fluorophores were explored as a second technique for on-chip cycIF. Ethylene carbonate effectively removed complementary oligo-fluorophores between sequential rounds of imaging, as confirmed using the algorithm from Chapter 2.

In Chapter 4, I focused on developing treatments for early-stage tumors after they have been detected. Due to long-term health complications, traditional systemic chemotherapy is rarely appropriate for treating early-stage tumors. The design of hybrid silica-coated PLGA (SiLGA) NPs as biocompatible drug delivery vehicles for enzyme-based cancer treatment shows promise for applications in early-stage cancer treatment. Encapsulation of proteins and enzymes within SiLGA NPs shielded cargo from proteolysis *in vitro* while retaining a high degree of enzymatic activity. SiLGA encapsulation also slowed the initial burst release of enzymatic cargo after intramuscular (IM) delivery in mice. Given their biocompatibility, ability to encapsulate hydrophobic and/or hydrophilic molecules, and surface functionalizability, SiLGA NPs offered a variety of novel therapeutic strategies for early cancer treatment. DEP chip technology enabled the collection of SiLGA NPs from plasma, suggesting the potential for monitoring pharmacokinetics and biodistribution during response to treatment.

Finally, Chapter 5 presents the conclusions, summary, and future directions pertaining to these studies. This chapter includes discussions about DEP automation, fluorophore libraries for improved multiplex profiling, hyperspectral image analysis, temporal blood-based biomarker phenotyping, indicators of immune escape, and DEP detection of peritoneal metastasis. Future experiments to characterize asparaginase-loaded SiLGA NPs are outlined as well.

Chapter 2. Automated fluorescence quantification of extracellular vesicles isolated using DEP

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*In this study, I designed the algorithm, harvested EVs from cell culture media, conducted experiments, analyzed data, assembled figures, and wrote the manuscript.

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2.1 Abstract

Tumor-secreted exosomes and other extracellular vesicles (EVs) in circulation contain valuable biomarkers for early cancer detection and screening. A chip-based technique that uses a microelectrode array to generate DEP forces has previously been used to collect cancer-derived NPs directly from whole blood and plasma. This technique enables direct recovery of NPs from whole blood and plasma. The biomarker payloads associated with collected particles can be detected with immunostaining and quantified. Accurately separating the fluorescence intensity of stained biomarkers from background (BG) levels becomes a challenge when analyzing the blood from early-stage cancer patients in which biomarker concentrations are low. To address this

challenge, I developed two complementary techniques to standardize the quantification of fluorescently immunolabeled biomarkers collected and concentrated at predictable locations within microfluidic chips. The first technique was an automated algorithm for the quantitative analysis of fluorescence intensity at collection regions within the chip compared to levels at adjacent regions. The algorithm used predictable locations of particle collection within the chip geometry to differentiate regions of collection and BG. I successfully automated the identification and removal of optical artifacts from quantitative calculations. I demonstrated that the automated system performs nearly the same as a human user following a standard protocol for manual artifact removal with Pearson's r -values of 0.999 and 0.998 for two different biomarkers ($n = 36$ patients). I defined a usable dynamic range of fluorescence intensities corresponding to 1 to 2000 arbitrary units (a.u.). Fluorescence intensities within the dynamic range increased linearly with respect to exposure time and particle concentration. The second technique is the implementation of an internal standard to adjust levels of biomarker fluorescence based on the relative collection efficiency of the chip. Use of the internal standard reduced variability in measured biomarker levels due to differences in chip-to-chip collection efficiency, especially at low biomarker concentrations. The internal standard did not affect linear trends between fluorescence intensity and exposure time. Adjustments using the internal standard improved linear trends between fluorescence intensity and particle concentration. The optical quantification techniques described in this chapter can be easily adapted for other lab-on-a-chip platforms that have predefined regions of particle collection and rely on fluorescence detection.

2.2 Introduction

Tumors release various types of cancer-related biomarkers into circulation starting from early stages and continuing throughout their development.¹⁶⁰⁻¹⁶³ Liquid biopsy techniques are designed to detect these biomarkers (e.g. including DNA, RNA, and proteins) in blood samples. Compared to invasive and localized tissue biopsies, liquid biopsies are advantageous as a cancer-screening tool. Blood collection is minimally invasive and offers the potential to detect biomarkers released across the entire tumor lesion, which can more accurately depict tumor heterogeneity.

Many cancer-specific biomarkers are associated with lipid-based NPs called EVs, which are secreted by tumor cells¹⁶⁴ in elevated quantities. Exosomes are one type of EV that are difficult to recover from blood due to their small size (50 to 150 nm diameter)^{41, 165, 166} and low buoyant density. Recovery of exosomes is a fundamental challenge for quantifying their associated biomarkers. Traditional “gold-standard” methods to recover exosomes for biomarker analysis include ultracentrifugation and filtration-based separations, which are time-consuming and labor-intensive.¹⁶⁷⁻¹⁷¹ New lab-on-a-chip systems for NP separation efficiently recover EVs from blood plasma.^{132, 159, 172}

DEP is the motion of an object due to DEP forces. When an object suspended in fluid is subjected to a non-uniform electric field, contrasting dielectric properties of the object and its surrounding media induce DEP forces on the object. In particular, DEP¹⁷³ applied to the microfluidic environment can preferentially concentrate tumor-derived NPs to known locations within the chip^{132, 159, 172} as shown in **Figure 2.1**. Blood-derived

plasma samples are loaded into the microfluidic chamber on top of a planar microarray of circular electrodes. When an AC voltage is applied across electrodes, this particular microarray geometry creates a strong electric field gradient that exerts DEP forces on EVs and other endogenous NPs in the fluid sample. These NPs are collected at maxima in the electric field gradient known as “high-field” regions (**Figure 2.1a**), which are located at the circumferences of each electrode in the planar microarray as predicted by theoretical simulations (**Figure 2.1d**) and confirmed by scanning electron microscopy (**Figure 2.1g – h**). NPs are held in place by pDEP forces during washing, which depletes the bulk plasma protein. Subsequent immunostaining and fluorescence imaging of the collected NPs enables detection and quantification of their associated biomarkers. The collection of endogenous NPs at the electrode circumferences increases their local concentration, and thereby the fluorescent signal, enhancing the SNR.

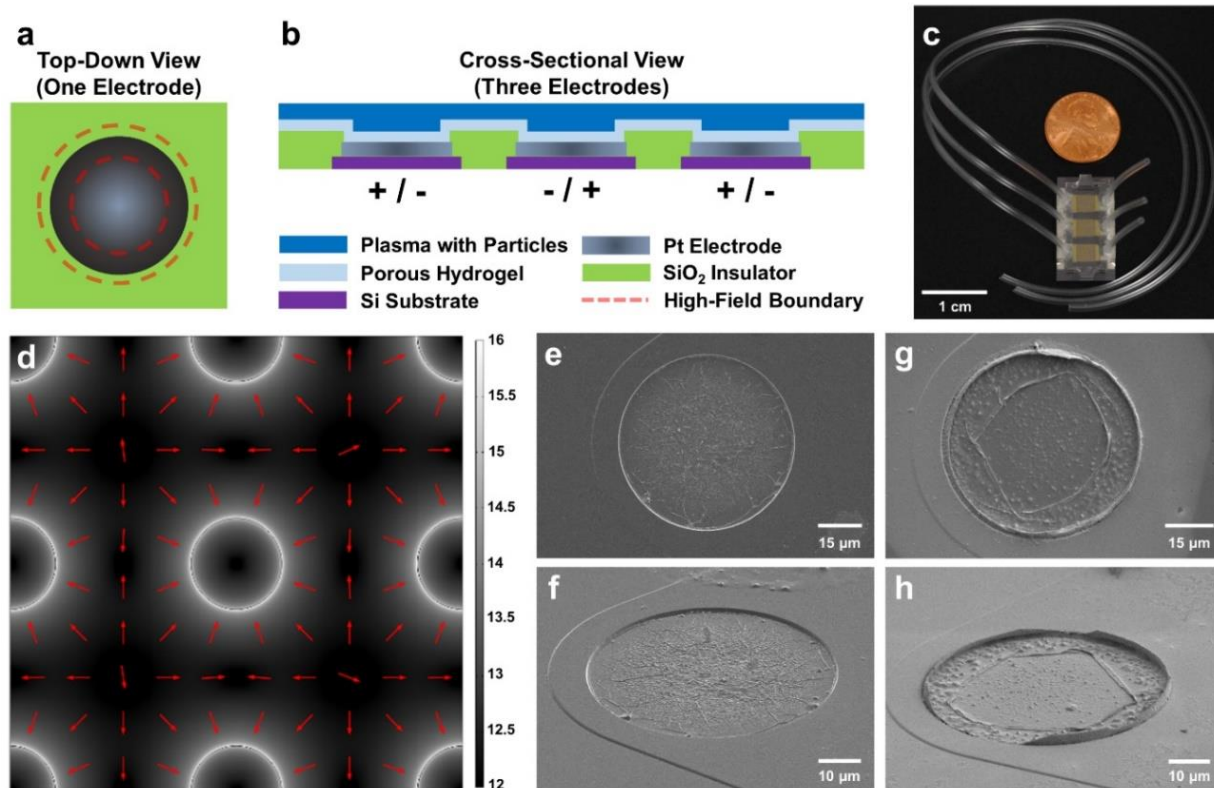


Figure 2.1. NPs were collected in the “high-field” regions around the electrode edges under pDEP. **a)** Top-down view of a circular electrode in the planar microarray of a DEP chip. EVs and other NPs were collected at the electrode edge by DEP forces (area between dashed red lines). **b)** Cross-sectional area of three electrodes in the planar microarray of a DEP chip. A layer of silicon dioxide insulated platinum electrodes, which were patterned on top of a silicon substrate. A porous hydrogel layer on the surface of the chip separated electrodes from direct contact with blood-derived plasma. **c)** Tubing was inserted into three microfluidic chambers on a DEP chip to prepare the device for experimentation. Each microfluidic chamber housed thousands of microelectrodes. The DEP chip is shown beside a penny for scale. **d)** A simulation of the gradient of the square magnitude of the electric field demonstrated a pattern of high-field regions (white) and low-field regions (black). Numbers by the greyscale heat map indicated base-10 exponents spanning magnitudes of 10^{12} to 10^{16} V^2/m^3 . NPs responsive to pDEP forces were predicted to move up the gradient along the direction of the red arrows from low- to high-field regions. High-field regions coincided with the circumferences of electrodes in the planar microarray. **e – f)** Respectively, scanning electron micrographs (SEMs) of a top-down view and an angled view (tilt angle = 59°) show typical electrodes on a DEP chip. DEP was applied to filtered 0.5X PBS in the absence of added NPs. The scale bar in **e)** applies to the vertical and horizontal dimensions along the image plane, while the scale bar in **f)** describes the horizontal dimension only. **g – h)** SEMs of top-down and angled views (tilt angle = 59°) show typical electrodes on a DEP chip that was used to collect NPs from plasma. NPs collected at the circumferences of the electrodes, agreeing with the simulation shown in panel d. The scale bar in **g)** applies to the vertical and horizontal dimensions along the image plane, while the scale bar in **h)** describes the horizontal dimension only.

Applying this technique to early cancer detection presents a unique set of challenges stemming from characteristically low concentrations of biomarkers present in circulation at early stages of disease.¹⁷⁴⁻¹⁷⁶ In order to address the challenge of inherently low SNRs, it is critical to measure the level of BG noise and to separate BG levels from the detected signal of immunofluorescently stained biomarkers. Here I demonstrate a custom optical imaging algorithm, written with MATLAB® software, to automate quantification for this type of immunofluorescent staining. The algorithm for fluorescence quantification described in this chapter zeroes pixel intensities at each region of collection (“region-of-interest”, or “ROI”) to the noise level (*e.g.* average pixel intensity plus three standard deviations) of the local BG. Localized noise-subtraction treated collection at each ROI in a field-of-view as unique sampling of biomarkers from the plasma. Localized comparisons were important because BG fluorescence can vary throughout a single field-of-view, across multiple fields-of-view on a single chip, and across multiple fields-of-view for different chips.

To address the challenge of inherently low levels of target biomarkers, it was also necessary to reduce user bias in quantitation. Biomarkers were collected at predictable ROIs, allowing for localized noise subtraction as previously mentioned. The ability to predict the locations of biomarker collection based on the geometry of the planar electrode microarray in the DEP chip also allowed me to automate quantitative calculations, including the removal of optical artifacts. Complete automation of quantitative calculations successfully minimized user bias in fluorescence analysis and reduced analysis time. Automated artifact removal performed as well as a single user following a controlled protocol for manual artifact removal.

I also developed an internal standard, consisting of a known concentration of fluorescent NPs, to correct for the variability in collection efficiency across DEP chips and to further address the challenge of low SNRs. Fluorescent NPs and target biomarkers were collected simultaneously and quantified using the custom algorithm. The internal standard ensured that differences in fluorescence intensity between two or more biological samples reflected physiological differences in endogenous biomarker levels rather than a discrepancy in collection efficiencies across DEP chips.

Many lab-on-a-chip designs focus on the separation and concentration of target particles into predictable locations. Other designs include implementations of DEP where exosomes,^{132, 172} stem cells,^{177, 178} blood cells,^{179, 180} cancer cells,¹⁸¹⁻¹⁸³ bacteria,^{184, 185} viruses,^{186, 187} and synthetic NPs^{159, 188, 189} are all collected in predictable locations. Other collection modes include electrophoretic,¹⁹⁰ magnetophoretic,^{191, 192} and acoustophoretic¹⁹³⁻¹⁹⁵ forces to concentrate targets from surrounding media to known regions on the chip. Several devices utilize immunoaffinity capture of EVs either by functionalized NPs¹⁹⁶ or at patterned locations on the chip.¹⁹⁷ Still others use increased affinity for substrates coated in nanotubes to collect aberrant circulating tumor cells.¹⁹⁸ The automated algorithmic approach described here can be easily adapted for use with other chip geometries by changing the shape of the digital mask to encompass the ROI. Masking makes the algorithm a widely applicable tool to reduce user bias, labor, and time in analyzing large numbers of samples. Masking also reduces BG noise for more accurate quantification of low abundance targets.

2.3 Materials and Methods

2.3.1 *Particle collection using DEP for development of algorithm for optical quantification*

2.3.1.1 Materials

Monoclonal mouse anti-CD9 antibody conjugated to Alexa Fluor 488 was purchased from R&D Systems (Minneapolis, MN; FAB1880G; clone #209306). Monoclonal mouse anti-CD63 antibody conjugated to Alexa Fluor 647 was purchased from Novus Biologicals (Centennial, CO; NB10077913Y; clone MEM-259). FluoSpheres™ Carboxylate-Modified Microspheres (0.1 µm, blue fluorescent 350/440, 2% solids; F8797) and D-(+)-Trehalose Dihydrate (Fisher BioReagents, BP2687100) were purchased from ThermoFisher Scientific (Waltham, MA). Three-chamber DEP chips were purchased from Biological Dynamics (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (P/S) were purchased from Thermo Fisher Scientific (Waltham, MA). Amicon® Ultra-15 Centrifugal Filter Units (25 mL, 100 kDa MWCO; UFC901024) and Ultrafiltration Membrane Filter Discs (10 kDa MWCO, BioMax; PBGC07610; and 300kDa MWCO, BioMax; PBMK07610) were purchased from MilliporeSigma (Burlington, MA). Sterile filtration units (0.1 µm; Nalgene; 5670010) were purchased from Thermo Fisher Scientific (Waltham, MA). BD Difco™ Skim Milk was purchased from Midland Scientific (Omaha, NE; DIFCO 232100).

2.3.1.2 Collection of EVs from HeLa cell culture media

Exosome-depleted cell culture media was prepared to harvest EVs from HeLa cells. EV-depleted FBS was prepared by 300 kDa MWCO ultrafiltration with an Amicon stirred-cell concentrator followed by 0.1 μ M filter sterilization. Filtered FBS was added to DMEM to a final concentration of 10% by-volume. P/S was added to exosome-depleted media at a concentration of 1% by-volume. Non-exosome-depleted cell culture media was prepared with commercial FBS and P/S at final concentrations of 10% and 1% by-volume, respectively. Cells were cultured to approximately 50% confluency in non-exosome-depleted media. Media was discarded, and cells were washed with 1X phosphate-buffered saline (PBS). PBS was discarded, and cells incubated in exosome-depleted media for 48 hours. Exosome-depleted media was collected and cells were removed by centrifugation. D-trehalose was added to media at a final concentration of 25 mM to stabilize EVs during storage. Conditioned media was stored at -80°C until a total of approximately 1.5 L of had been collected.

Conditioned media (1.5 L) was thawed and concentrated to approximately 20 mL using an Amicon stirred-cell concentration (10 kDa filter size). Concentrated media was centrifuged (30 minutes; 10,000 g; 4°C) and protein aggregates were removed. EVs were then concentrated to approximately 5 mL by centrifugal filtration using an Amicon Ultra-15 (10 kDa) spin filter. EV populations were size-separated by fast protein liquid chromatography (FPLC). FPLC fractions corresponding to the size range of exosomes (50 – 150 nm) were collected and suspended into approximately 1 mL of 0.5X PBS. The concentration and size distribution of collected EVs were measured using a qNano Gold

instrument by IZON (Christchurch, New Zealand). Size distribution and concentration data were plotted in **Figure 2.2**. Harvested EVs were aliquoted and stored at -80°C .

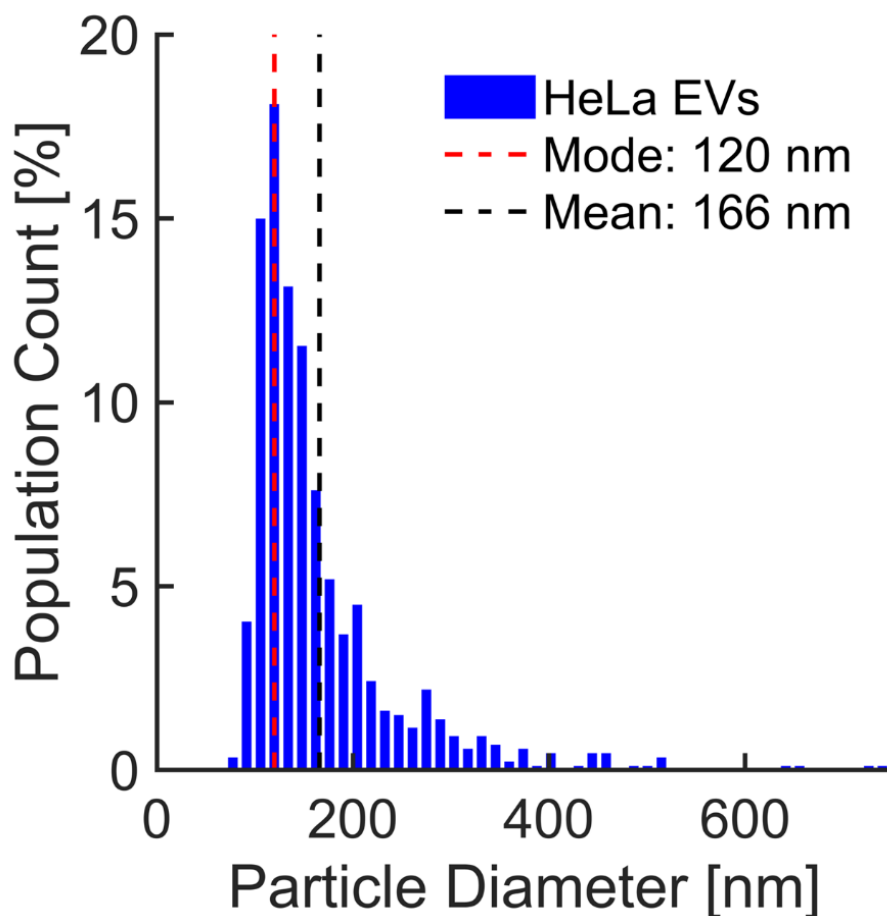


Figure 2.2. Size distribution of EVs collected, purified, and concentrated from HeLa cell culture media. A histogram showing the size distribution of a sample of EVs harvested from cell culture media as the percentage of the measured sample population versus particle diameter (mean particle diameter of 166 nm; $n = 867$ EVs). The mode particle diameter (120 nm) fell within the range of diameters associated with exosomes (50 – 150 nm), suggesting that these EVs were successfully enriched for exosomes. The concentration of EVs was measured to be approximately 4×10^{11} EVs/mL.

2.3.1.3 Positive controls were created by spiking EVs and fluorescent NPs into blood plasma

One whole blood sample from a healthy donor was obtained from the OHSU Knight Cancer Institute Cancer Early Detection Advanced Research (CEDAR) Center Repository (IRB approval #000018048) with use under OHSU IRB approval #00018572. Informed consent was obtained from the subject. All experimental protocols were carried out in accordance with relevant guidelines and regulations outlined by the OHSU Institutional Review Board. Blood plasma from a single healthy human patient was acquired through centrifugation of fresh blood stored in EDTA (1500 g for 15 minutes at 4 °C). The first set of experiments was designed to determine the dynamic range of the system. These experiments involved spiking varying concentrations of fluorescent polystyrene NPs into the plasma and then collecting the particles *via* DEP. Images were acquired in multiple fluorescence channels and quantified using the technique described in this chapter. Fluorescence intensities from the first set of experiments were plotted in **Figure 2.3** (“Exp 1”) and **Figure 2.4a – b**. Plotted fluorescence intensities were calculated from fluorescence images obtained over three experiments.

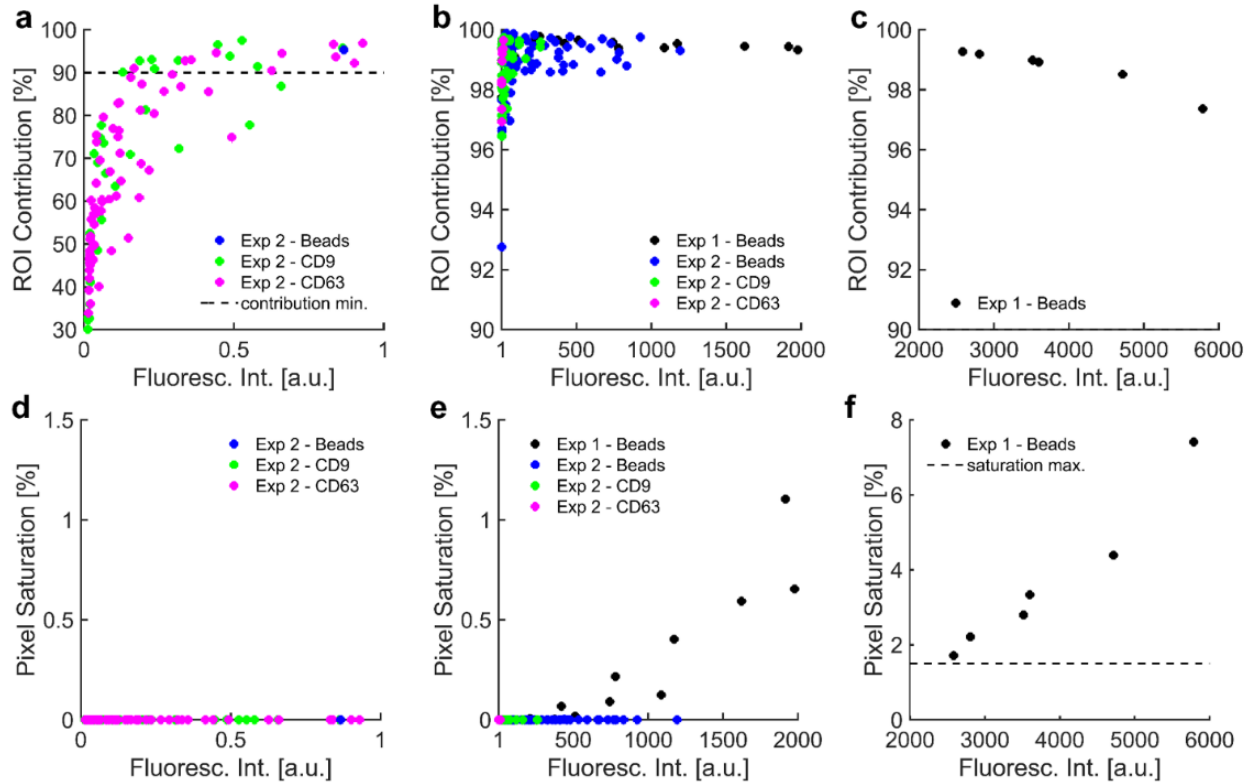


Figure 2.3. Data quality metrics informed a usable dynamic range in fluorescence intensity. Two data quality metrics, contribution from the ROI and pixel saturation, were plotted against three sets of fluorescence intensity values. Contribution from the ROI indicated the percentage of the intensity value that originated from pixels in the ROI, which was the collection region under high-field DEP. Fluorescence intensity values needed a minimum contribution from the ROI of 90% to ensure most of the value was attributed to signal from collected particles. Pixel saturation indicated the percentage of saturated pixels used to calculate each fluorescence intensity value. A maximum allowable pixel saturation of 1.5% ensured that saturation did not adversely affect quantitation. **a)** Fluorescence intensities in the set (0, 1 a.u.] showed a positive correlation ($r = +0.717$) with increasing contribution from ROI. Most intensities were below the 90% cutoff for contribution from the ROI. **b)** All fluorescence intensities in the set (1 a.u., 2000 a.u.) exhibited contributions from the ROI greater than 90%. There was a weak correlation ($r = +0.261$) between increasing fluorescence intensity and contribution from the ROI over this set of intensities. **c)** In the range [2000 a.u., 6000 a.u.), there existed a strong negative correlation ($r = -0.960$) between fluorescence intensities and contribution from the ROI. Increasingly high fluorescence at the ROI could have generated photonic noise, which likely bled into the BG, reduced the contribution from the ROI, and caused the negative correlation between fluorescence intensity and ROI contribution over this set of intensities. Importantly, all intensities between [2000 a.u., 6000 a.u.) exhibited contributions from the ROI above 90%. **d)** Fluorescence intensities in the set (0, 1 a.u.) did not correlate with pixel saturation as no saturation was detected. **e)** All intensities in the set (1 a.u., 2000 a.u.) exhibited minimal pixel saturation (lower than 1.5%), meeting the second quality criterion. Fluorescence intensities correlated ($r = +0.746$) with pixel saturation over this set; however, pixel

saturation was sufficiently low (less than 1.5%) for all values. **f)** There existed a strong positive correlation ($r = +0.976$) between fluorescence intensities in this set [2000 a.u., 6000 a.u.) and pixel saturation. All intensities in this set exhibited pixel saturation rates above 1.5%, indicating that saturation could adversely affect quantitation. Contribution from the ROI and pixel saturation rates suggested that the usable dynamic range of intensities was (1 a.u., 2000 a.u.).

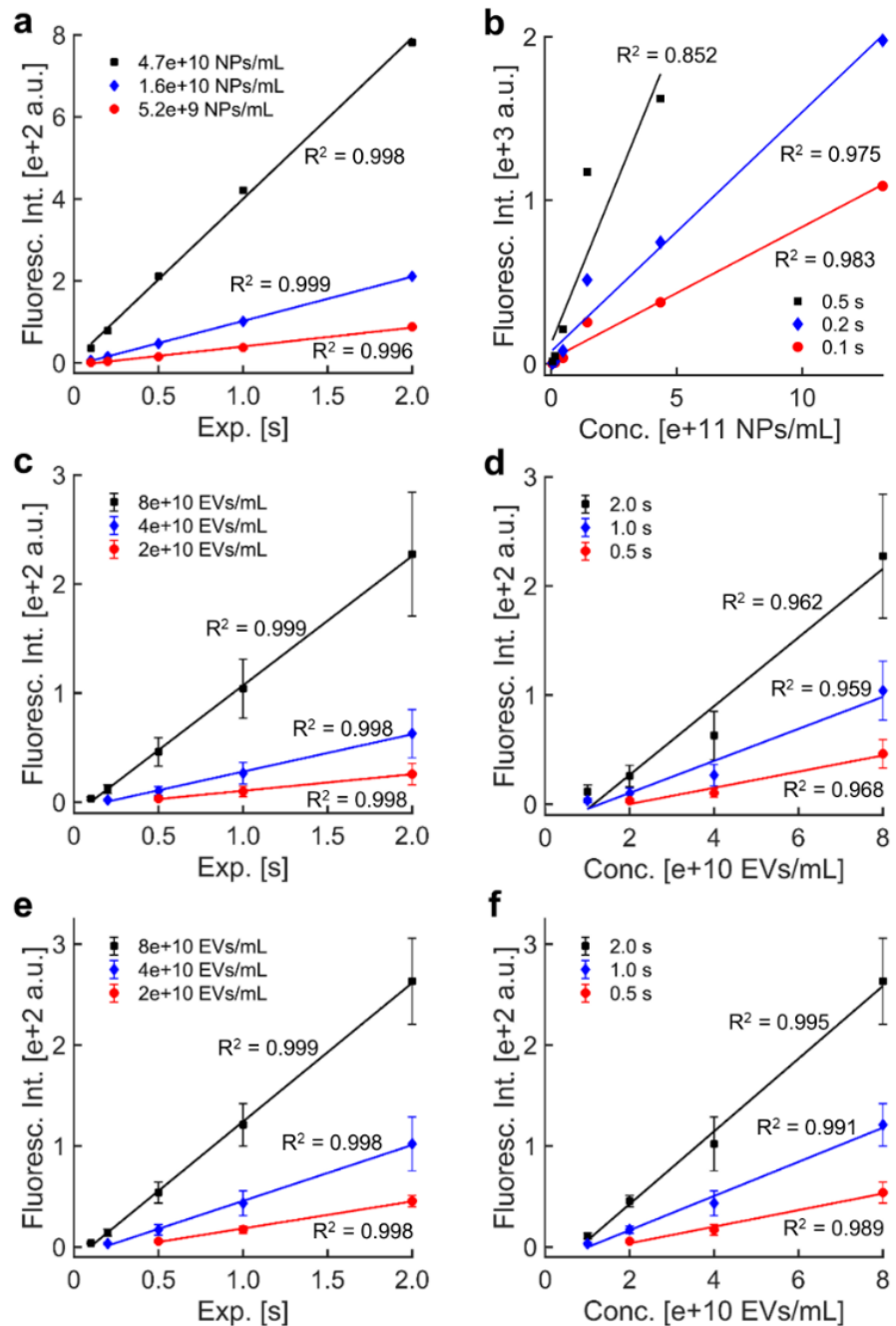
Figure 2.4.
Fluorescence intensity increased linearly with exposure time and with particle concentration.

Trends in exposure time and particle concentration were assessed for fluorescence intensities in panels a through d and for intensities adjusted to the internal standard in panels e through f.

a) Fluorescence intensities of fluorescent polystyrene NPs collected *via* DEP from healthy human plasma were plotted against exposure time at three contours in particle concentration (0.52, 1.6, 4.7 e+10 beads/mL). Best-fit lines through intensities within the dynamic range indicated that the data followed linear trends.

b) Fluorescence intensities from images of fluorescent polystyrene NPs were plotted against particle concentration for three contours in exposure time (0.1, 0.2, 0.5 s). Best-fit lines were plotted through fluorescence intensities within the dynamic range, indicating that fluorescence intensity increases linearly with the concentration of fluorescence particles.

c) Fluorescence intensities of immunofluorescently labeled EVs collected *via* DEP from healthy human plasma were plotted against exposure time at three contours in particle concentration (2, 4, 8 e+10 EVs/mL). Data points represented the mean fluorescence intensity across three technical replicates. Replicates were analyzed in separate microfluidic chambers on DEP chips. Error bars represented one standard deviation above and below the mean



fluorescence intensity. Linear fits were calculated using mean intensities within the dynamic range, confirming that fluorescence intensity increases linearly with exposure time. **d)** Fluorescence intensities of immunofluorescently labeled EVs plotted against EV concentration as contours in exposure time (0.5, 1.0, 2.0 s). The physical presence of the internal standard did not affect the linear relationship between fluorescence intensity and EV concentration. **e)** Chip-to-chip differences in collection efficiency caused variability in fluorescence intensity across technical triplicates in panel c. An internal standard was created to quantify the collection efficiency of each DEP chip. A known concentration of fluorescent polystyrene NPs were spiked into each sample of EVs. EVs and NPs were simultaneously recovered by DEP. Adjusting the EV signal to the internal standard did not change the linear relationship between fluorescence intensity and EV concentration. **f)** Use of the internal standard improved the coefficient of determination for each best-fit line in panel d and reduced variability in fluorescence intensity caused by differences in collection efficiency across DEP chips.

The second experiment was designed to investigate the influence of the internal standard (*i.e.* a fixed concentration of fluorescent polystyrene NPs) on varying concentrations of immunolabeled EVs each run as technical triplicates (“Exp 2” in **Figures 2.3, 2.4c – f, 2.5, 2.6**). HeLa-derived EVs were incubated with mouse monoclonal anti-CD9-Alexa Fluor 488 antibody for one hour at room temperature (1:50 dilution from stock). Immunolabeled EVs were diluted serially into healthy human plasma (2e+10, 4e+10, 8e+10 EVs/mL). Fluorescent polystyrene NPs were added to a final concentration of approximately 5e+9 particles/mL in each plasma dilution of EVs. Technical triplicates of each dilution were loaded into three physically isolated microfluidic chambers on DEP chips. Fluorescent NPs and EVs were simultaneously collected *via* DEP with subsequent immunostaining, fluorescence image acquisition, and image quantification. Fluorescence intensities from the second experiment were also used to determine the dynamic range of the system.

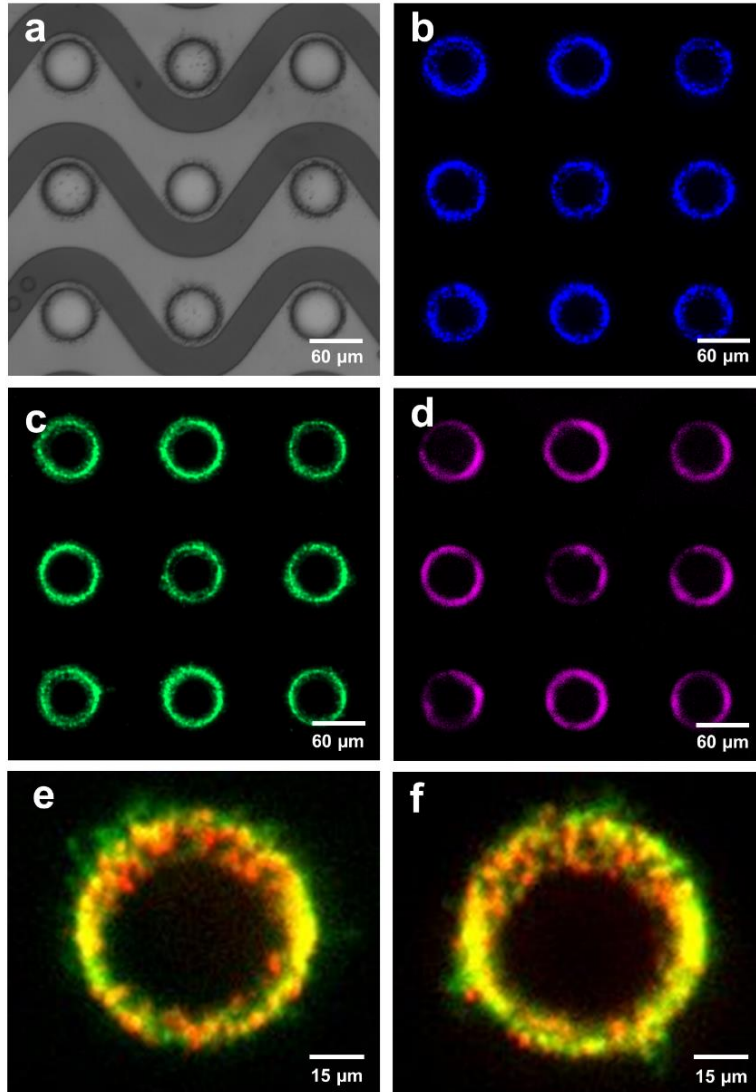


Figure 2.5. Fluorescently labeled NPs were collected at high-field DEP regions. Four images of the same field-of-view were acquired through different optical filters to demonstrate the simultaneous recovery of multiple particle types from healthy human plasma in the high-field regions around the electrode edge *via* DEP. **a)** A bright field image showed nine planar, circular electrodes on the DEP chip. **b)** A DAPI channel image indicated the collection of spiked fluorescent polystyrene NPs (100 nm diameter) collected in high-field regions from healthy human plasma *via* DEP (contrast-enhanced). **c)** An EGFP channel image showed the presence of HeLa-derived EVs, which were immunolabeled with anti-CD9-Alexa Fluor 488 and spiked into the same healthy human plasma prior to collection *via* DEP (contrast-enhanced). **d)** A Cy5 channel image of the same HeLa-derived EVs, which were immunolabeled with anti-CD63-Alexa Fluor 647 after collection (contrast-enhanced). **e – f)** Representative composite images of two electrodes showed the collected polystyrene NPs in red and the collected EVs in green. There was a strong positive spatial correlation between the two, shown in yellow, with a Pearson's R Value of 0.75 ± 0.03 (mean $\pm$ standard deviation, $n = 10$). This correlation indicated that the spatial collection of the 100 nm polystyrene NPs accurately reflected the spatial collection of the EVs.

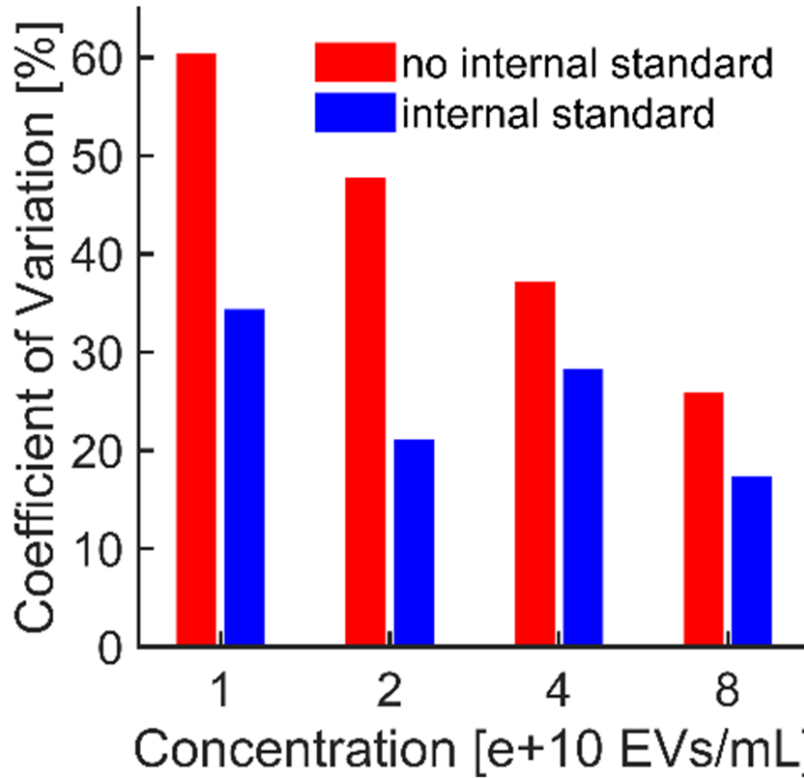


Figure 2.6. Use of the internal standard reduced non-physiological variability in fluorescence intensity. EVs and fluorescent polystyrene NPs (internal standard) were spiked into healthy human plasma, isolated simultaneously *via* DEP in technical triplicates, and imaged at an exposure of 1.0 s. Unadjusted fluorescence intensities of technical triplicates were averaged and standard deviations were calculated at each of four EV concentrations. CVs were calculated at each concentration of EVs (plotted in red). Fluorescence intensities from EVs were also adjusted to corresponding intensities from the internal standard. CVs for adjusted intensities were calculated (plotted in blue). CVs of adjusted intensities were lower than the CVs of unadjusted intensities at all concentrations of EVs, indicating that the internal standard effectively reduced non-physiological (*i.e.* technical replicate) variability in fluorescence intensity. Reductions in variability were most substantial at lower concentrations of EVs, suggesting that use of the internal standard could be critical at the low biomarker concentrations typical of early-stage disease.

2.3.1.4 NP collection from plasma via DEP

Microfluidic chambers on DEP chips were first hydrated with 0.5X PBS to prepare the hydrogel layer prior to loading blood plasma samples. Forty microliters of

plasma were drawn into the chamber prior to applying electrical signal to generate DEP forces at the electrodes in the bottom plane of the chamber (15 kHz; 12 V_{pp}; 15 min). EVs and fluorescent polystyrene NPs were collected simultaneously in experiments involving the internal standard ("Exp 2"); otherwise, only fluorescent polystyrene NPs were collected ("Exp 1"). Samples were washed with 0.5X PBS to remove the bulk plasma. The collected material was blocked using 2% by-weight Difco Skim Milk powder in 0.5X PBS for 30 minutes to reduce nonspecific antibody binding. Samples were washed with 0.5X PBS to remove blocking buffer. For experiments involving the internal standard, the collected EVs and NPs were incubated for one hour at room temperature with 40 microliters of a 1:50 dilution from stock of mouse monoclonal anti-CD63-Alexa Fluor 647 primary antibody added into blocking buffer ("Exp 2"). Samples were washed with 0.5X PBS. Samples spiked with only fluorescent polystyrene NPs were not incubated with antibodies ("Exp 1"). For SEM imaging, the 0.5X PBS in the microfluidic chamber of the chip was exchanged for 1X TE buffer prior to freezing the DEP chip using dry ice and an aluminum block stored at -80 °C.

2.3.1.5 Endogenous NPs were isolated from patient plasma and immunofluorescently labeled

The third experiment was designed to evaluate whether automated artifact removal could reproduce fluorescence intensities obtained *via* a controlled process of manual artifact removal (**Figure 2.7**). Thirty-six clinical plasma samples were obtained from the OHSU Interventional Endoscopy Unit under the Oregon Pancreas Tissue

Registry (IRB approval #00003609) with use under OHSU IRB approval #00018572. Informed consent was obtained from all subjects. All experimental protocols were carried out in accordance with relevant guidelines and regulations outlined by the OHSU Institutional Review Board. Proprietary hydrogel layers in the microfluidic chambers of DEP chips were treated with 0.5X PBS. Thirty microliters of sample were withdrawn into each microfluidic chamber. HeLa-derived EVs and other endogenous NPs were collected *via* DEP (14 kHz; 10 V_{pp}; 15 min) from all samples. Collected material was stained for one target prior to blocking with 2% by-weight milk powder in 0.5X PBS for one hour. Collected material was labeled for the second target using a primary-secondary antibody stain. Material was incubated with the primary antibody solution (1:200 dilution from stock) into blocking buffer for one hour at room temperature prior to washing with 0.5X PBS. Material was incubated with a fluorescently-labeled secondary antibody solution (1:500 dilution from stock into blocking buffer) for one hour at room temperature prior to washing with 0.5X PBS.

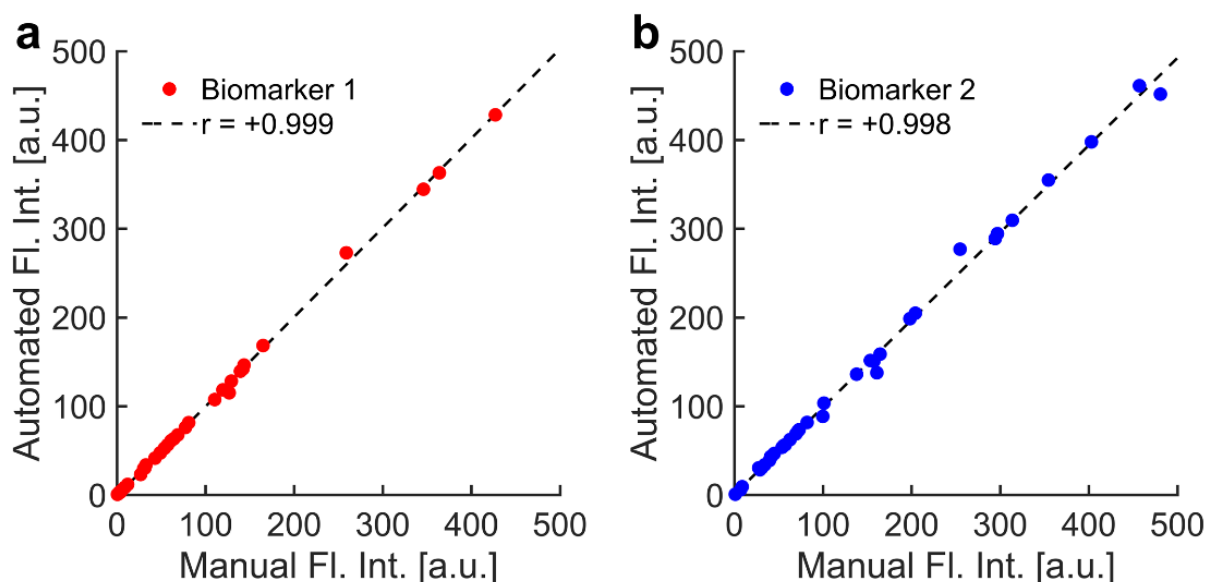


Figure 2.7. Automated artifact removal was faster and as accurate as manual artifact removal. Implementation of automated artifact removal was compared to a standard protocol for manually removing artifacts by assessing correlations in fluorescence intensities resulting from both methods. Plasma samples from thirty-six patients were analyzed for the presence of two biomarkers. Each data point represented a fluorescence image of one immunolabeled biomarker collected from the plasma of a single patient. **a)** One biomarker was fluorescently labeled on endogenous NPs collected from blood plasma of thirty-six patients. Fluorescence images were acquired and analyzed using two methods: a standard procedure for manually removing bright field and fluorescence artifacts (“Manual Fl. Int.”) and a programmatic implementation of that standard procedure for artifact removal (“Automated Fl. Int.”). A strong positive correlation between manual and automated intensities suggested that automatic artifact removal was as accurate as manual removal (Biomarker 1: $r = +0.999$). **b)** A second biomarker was fluorescently labeled on endogenous NPs. Manual and automated intensities for the second biomarker also correlated strongly (Biomarker 2: $r = +0.998$). Automatic artifact removal saved substantial amounts of time for users and minimized user biases, reducing analytical error across experiments.

2.3.1.6 Scanning electron microscopy of DEP chips used to collect EVs and NPs

DEP chips for scanning electron microscopy were flash-frozen by placing ground dry ice on top of the plastic cover of the chip before placing the chip on top of an aluminum block stored at -80°C . After the plastic covers were removed, the chip was

lyophilized and the microelectrode arrays on the surfaces of DEP chips were coated with a conductive layer. Micrographs of DEP chips were acquired with a NanoLab Helios 660 from FEI (Lausanne, Switzerland) at an operating voltage of 2.0 kV.

2.3.1.7 Image Acquisition

Bright field and fluorescence images were acquired at a field-of-view centered in the middle of each microfluidic chamber using DAPI, EGFP, and Cy5 fluorescence filters at five different exposures (0.1, 0.2, 0.5, 1.0, and 2.0 s, respectively). The X-Cite 120LED Boost light source from Excelitas Technologies (Waltham, MA) was used at 100% intensity during fluorescence imaging and at 5 – 7% intensity for bright field imaging. Images were acquired with 14-bit resolution using an AxioCam 506 camera affixed to an Axio Imager A2 fluorescence microscope from ZEISS (Oberkochen, Germany). All images were acquired using a 5x magnification objective lens (Objective EC “Plan-Neofluar” 5x/0.16 Ph1 M27; #420331-9911-000) from ZEISS (Oberkochen, Germany). Catalog Set 39000 (AT375/28x excitation, AT415DC beam splitter, AT460/50m emission), Catalog Set 39002 (AT480/30x excitation, AT505DC beam splitter, AT535/40m emission), and Catalog Set 39007 (AT620/50x, AT655DC beam splitter, AT690/50M emission) filters from Chroma Technology (Bellows Falls, VT) were used for image acquisition in the DAPI, EGFP, and Cy5 fluorescence channels, respectively. Gamma and analog gain were set to unity for image acquisition. Images were acquired with 6.07 Megapixel resolution (2752x2208 pixels). All fluorescence images corresponding to data shown in this chapter were acquired from samples in

0.5X PBS prior to using 1X TE buffer for chip storage at -80°C . Fluorescence images were exported as uncompressed, unedited, greyscale TIFs (16-bit depth) using ZEN 2.3 Blue software from ZEISS. Fluorescence images were quantified with custom image-processing software written in MATLAB®{The MathWorks(TM), 2017 #58} on a Microsoft Windows 10 operating system (Enterprise Version 10.0; Build 15063). Fluorescence quantification for this custom software required pixel values represented in 16-bit depth.

2.3.1.8 Data file preparation for analysis

Image files were exported using ZEN Software and prepared for analysis using the following parameters: [File type: Tagged Image File Format (TIFF); Convert to 8 bit (unselected); Compression (None); Resize (100%); Original Data (selected); Shift Pixel (unselected) Apply Display Curve and Channel Color (unselected); Use channel names (selected); Use Full Set of Dimensions (selected); Export to (...ANALYZE ME); Create folder (selected); Generate xml file (unselected); Generate zip file (unselected)]. Each CZI was exported as an “image folder” of fluorescence channel TIFs and one bright field TIF. Image folders were placed into corresponding “experiment folders” for organizational purposes. Experiment folders were then placed into a folder labeled “ANALYZE ME”. The folder structure was as follows: ANALYZE ME → Experiment Folder(s) → Image Folder(s) → images formatted as uncompressed, unedited, greyscale TIFs with 16-bit depth. Images were acquired using a 14-bit camera. The maximum value for images acquired with a 16-bit resolution camera would be $(2^{16} - 1)$

= 65535. The maximum pixel value of a 14-bit image stored in a 16-bit format was $(2^{14} - 1) \times 2^2 = 65532$, not 65535. Therefore, saturated pixels in across all images had intensity values of 65532.

2.3.1.9 Theoretical simulation of DEP high-field regions in a planar microelectrode array

The gradient distribution of the electric field squared was simulated in COMSOL (Los Angeles, CA) using a model of 60 μm diameter electrodes on a 150 μm spacing. The simulated fluid was modeled with a conductivity of 1.4 S/m and a relative permittivity of 80. The fluid height extended 200 μm from the electrode floor. Boundary conditions were set to assume an infinite array of electrodes. Electrode polarity alternated in a checkerboard pattern of 0 V and 10 V_{rms}, respectively.

2.3.2 *Design of algorithm for optical quantification*

2.3.2.1 Algorithm input consisted of pairs of fluorescence and bright field images

This section references **Figure 2.8a** and **Figure 2.9i – v**. CZI files were exported from Zen 2.3 lite (Blue Edition) with parameters described in Section 2.3.1.8 (**Figure 2.9i**). The executable (.exe) version of the software was opened (**Figure 2.9ii**). The software prompted the user to select the folder (ANALYZE ME) containing image pairs for quantification. The user was not prompted for additional information after this step

(**Figure 2.9iii**). Experiment folders, image folders, and individual images were automatically indexed. Bright field images were paired with each of between one and four fluorescence channel images (**Figure 2.9iv**). The first pair of bright field and fluorescence images were inputted into data structures as two-dimensional sets of 16-bit pixel intensity values (**Figure 2.9v**).

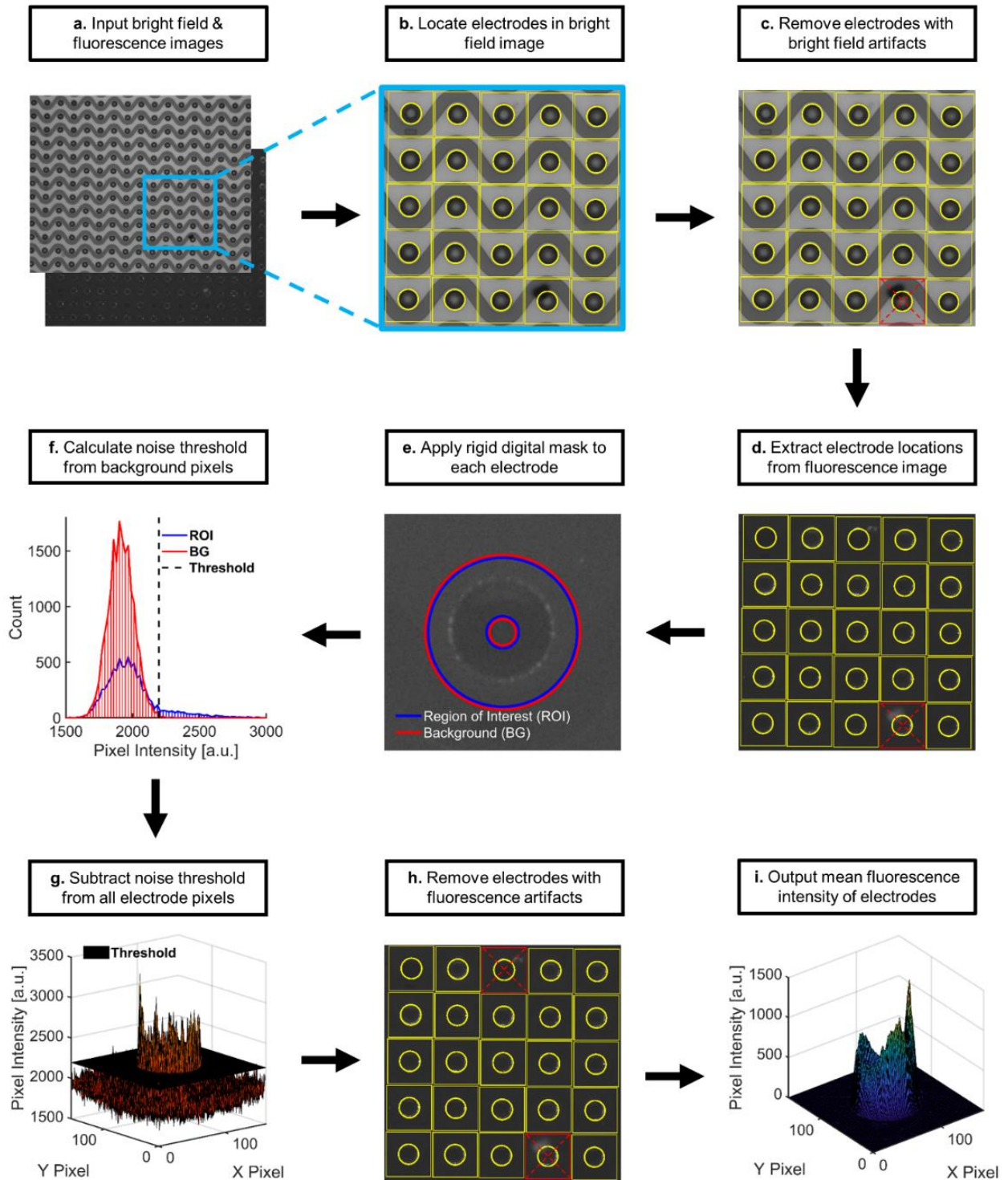


Figure 2.8. Process flow diagram for automated fluorescence quantification. **a)** Pairs of corresponding bright field and fluorescence images were inputted and indexed. **b)** Electrode locations were identified using the bright field image. **c)** Electrode locations were assessed for usability. Usable electrodes were those centered within 160x160 square pixel areas, none of which overlapped. Electrodes with optical artifacts were removed from analysis. **d)** Usable electrode locations were transferred to the paired

fluorescence image. Square regions centered at each electrode were extracted. **e)** A rigid digital mask was applied to each square region, categorizing pixels as either ROI (regions of high-field DEP) or BG (regions of low-field DEP). **f)** Intensity distributions of BG pixels were used to calculate noise thresholds specific to each square region. **g)** The noise threshold value was subtracted from all pixels in respective square regions. The average fluorescence intensity was calculated for each noise-subtracted square region. **h)** Square areas containing apparent fluorescence artifacts were removed from downstream analysis. **i)** The average intensity at each remaining electrode was then used to calculate the sample mean fluorescence intensity, which constituted the data points plotted in all figures for this chapter. All essential calculations and relevant metadata were saved in reports that were automatically generated after each image pair was analyzed. The described processes repeated for remaining image pairs.

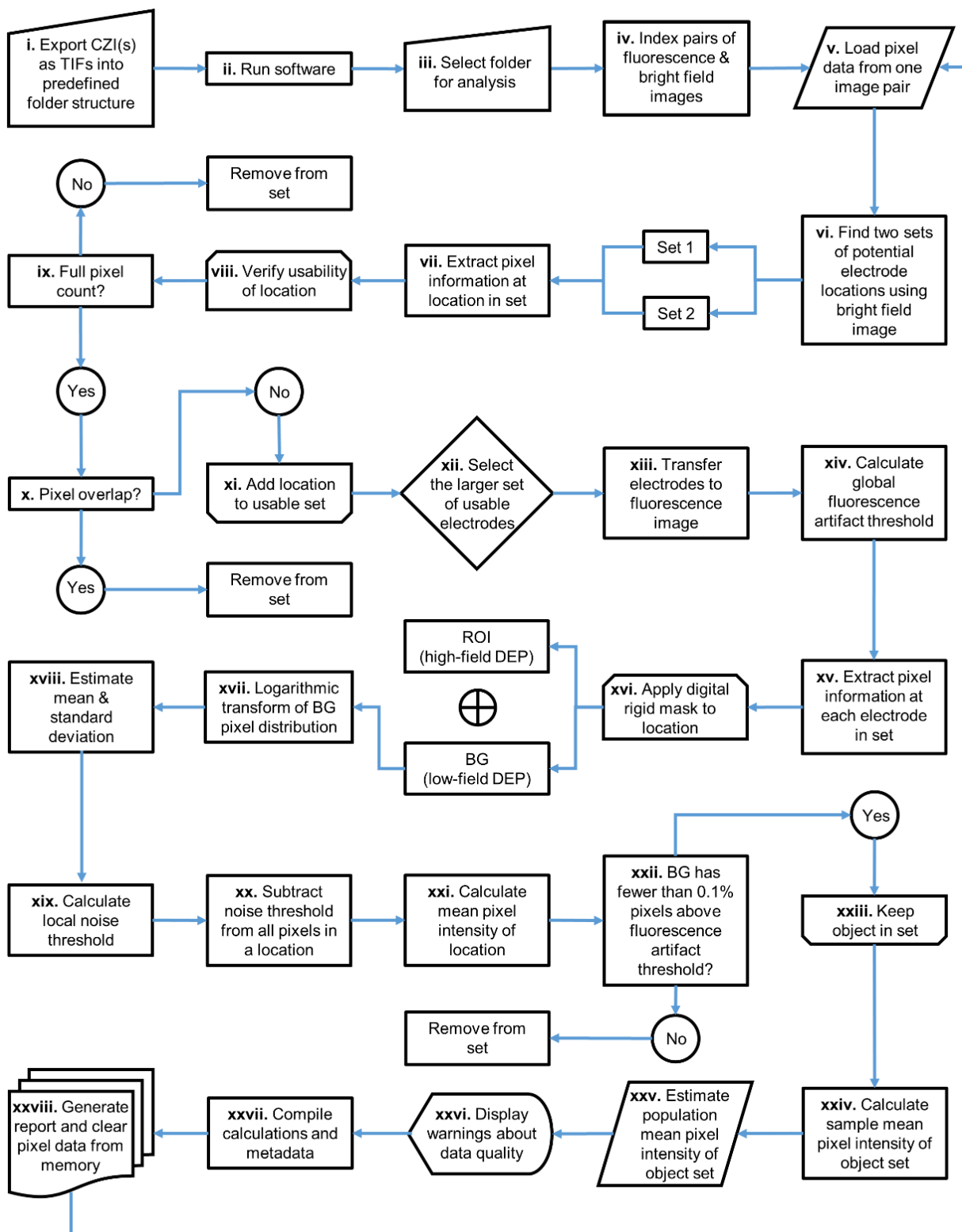


Figure 2.9. Technical flow diagram for automated fluorescence quantification. Flow diagram outlining the computational sequence of the custom algorithm for fluorescence image quantification of NPs isolated *via* DEP. This flow diagram was referenced along with **Figure 2.8**.

2.3.2.2 Electrode locations were identified using bright field images

This section references **Figure 2.8b** and **Figure 2.9vi – vii**. Two Hough Transforms were conducted using the *imfindcircles* function{The MathWorks(TM), 2017 #59} to identify circular objects in the bright field image that resembled electrodes (60 μm diameter) under a 5X objective lens. Each transform yielded a set of potential electrode locations (yellow circles in **Figure 2.8b**; “Set 1” and “Set 2” in **Figure 2.9vi**.) from a single bright field image. An area encompassing the electrode and surrounding BG was defined at the center of each electrode (yellow squares in **Figure 2.8b**). All pixel values were extracted from these square areas (**Figure 2.9vii**).

2.3.2.3 Unusable electrodes with bright field artifacts were removed from analysis

This section refers to **Figure 2.8c** and **Figure 2.9viii – xii**. Electrodes at the perimeter of the field-of-view that lacked sufficient pixel counts were removed from the two sets of potential electrode locations (**Figure 2.9viii – ix**). The DEP chip electrode array was on a regular pattern, allowing me to maximize the extraction area (yellow squares in **Figure 2.8**) while avoiding overlap. Overlap indicated misidentification of optical artifacts as electrodes. All overlapping areas of identification were removed from the set of potential electrodes that contributed to downstream quantitative calculations (**Figure 2.9x – xi**). After all bright-field artifacts and affected electrodes had been removed, the larger of the two sets of potential electrodes was selected for additional processing (**Figure 2.9xii**).

2.3.2.4 A rigid digital mask differentiated the ROI from BG at each electrode

This section refers to **Figure 2.8d – e** and **Figure 2.9xiii, xv – xvi**. The selected set of electrode locations was transferred to the paired fluorescence image (**Figure 2.8d; Figure 2.9xiii**). All pixel values at each electrode location were extracted (yellow squares in **Figure 2.8d; Figure 2.9xv**). A rigid digital mask with fixed geometry was applied to each extracted location, categorizing pixels as either ROI or BG (**Figure 2.9xvi**). Theoretical simulations and empirical observation (**Figure 2.1**) informed the geometry of the mask based on ring-shaped patterns of particle collection at areas of high-field DEP. The ROI entirely encompassed the circumference of the planar electrode, or high-field region, where particles of interest collect under pDEP. The BG encompassed low-field regions surrounding the electrode and at the center of the electrode, away from where particles moved under pDEP. Fluorescence intensity of collected material was determined by comparing pixel intensities in the ROI (high-field region) to pixel intensities in the BG (low-field region).

2.3.2.5 Local noise thresholds were calculated from BG pixels and subtracted from all pixels at each electrode

This section references **Figure 2.8f – g** and **Figure 2.9xvii – xxi**. Pixels from the BG region of each electrode location were used to calculate noise threshold values specific to each electrode. BG pixels exhibited lognormal intensity distributions and were logarithmically transformed (**Figure 2.8f; Figure 2.9xvii**). Transformed mean and

standard deviation values were used to estimate mean and standard deviation values (**Figure 2.9xviii**). Noise thresholds at each electrode location equaled the estimate of the mean plus three times the estimate of the standard deviation (**Figure 2.9xix**). Local noise thresholds were subtracted from all pixels in each respective electrode location (**Figure 2.8g**; **Figure 2.9xx**). The average pixel intensity was calculated using all pixels in each noise-subtracted electrode location (**Figure 2.9xxi**).

2.3.2.6 Unusable electrodes with fluorescence artifacts were removed from analysis

This section refers to **Figure 2.8h** and **Figure 2.9xiv, xxii – xxiii**. A global intensity threshold was calculated as six standard deviations above the average pixel intensity of the entire inputted fluorescence image (**Figure 2.9xiv**). If at least 99.9% of BG pixels around a single electrode had intensities lower than the global intensity threshold, the electrode remained in the set (**Figure 2.9xxii – xxiii**). Otherwise, the electrode likely exhibited at least one fluorescence artifact and was removed from the set (**Figure 2.8h**).

2.3.2.7 Fluorescence intensity was calculated from usable electrodes

This section references **Figure 2.8i** and **Figure 2.9xxiv – xxviii**. The average pixel intensities of each noise-subtracted electrode remaining in the usable set after artifact removal were then averaged to calculate the overall mean fluorescence

intensity, referred to throughout the chapter as “fluorescence intensity” (**Figure 2.9xxiv**). The population mean of the average pixel intensity of all noise-subtracted electrodes in the microfluidic chamber of the DEP chip was estimated within 95% confidence bounds (**Figure 2.9xxv**). Warnings regarding data quality were displayed in the command window for the user when relevant (**Figure 2.9xxvi**). These warnings included: a low contribution to fluorescence intensity from the ROI, a fluorescence intensity below the lower detection limit of 1 a.u., fewer than 100 electrodes used to calculate fluorescence intensity, a high rate of pixel saturation, and a large uncertainty value representing a 95% confidence interval for fluorescence intensity. Calculations, image data, quality control metrics, warnings, and metadata were compiled into a report, which was automatically generated, indexed, and saved to the respective image folder (**Figure 2.9xxvii**). All information extracted and calculated from the first pair of input images was cleared from memory. The next indexed pair of images were inputted automatically. The described process repeated until all images were analyzed. The custom MATLAB® code can be found in Supplemental Section S1.2. Posts on the *MATLAB Answers* online forum{The MathWorks(TM), #60} were accessed during the development of the algorithm.

2.3.3 Statistical analyses

“Fluorescence intensity” referred to the sample mean of the average fluorescent pixel intensity from noise-subtracted areas at each usable electrode in a field-of-view. The “sample” referred to as many as 208 usable electrodes in the field-of-view of the

input image. The quantified sample was a subset of the total population of electrodes in the microfluidic chamber of the chip. The population mean was estimated from the sample of usable electrodes to within 95% confidence (included in automated reports; confidence intervals not plotted). To obtain technical triplicates, three aliquots from one biological sample were obtained. Each aliquot was introduced to a single microfluidic chamber prior to collection of particles *via* DEP, immunofluorescence labeling, washing, and imaging. Variability in fluorescence intensity across technical triplicates represented differences between individual populations of electrodes. Error bars in plotted data represented one standard deviation above and below the average fluorescence intensity of technical triplicates (*i.e.* three quantitative values from three input images, each representing one of three technical replicates).

Pearson correlations between variables on vertical and horizontal axes were calculated for subplots in **Figures 2.3** and **2.7**. Linear fits in **Figure 2.4** were calculated using plotted fluorescence intensities, all of which were within the usable dynamic range (1 – 2000 a.u.). Fluorescence intensities for one midway dilution (1.4×10^{11} NPs/mL) in a serial dilution set ($0.052 - 13.2 \times 10^{11}$ NPs/mL) were excluded from contours in **Figure 2.4b** due to concerns about abnormal collection efficiency. Fluorescence intensities in **Figure 2.4c – d** were adjusted for collection efficiency using the internal standard and plotted in **Figure 2.4e – f**. To adjust for collection efficiency, the fluorescence intensity of the internal standard for one sample (“reference”) was arbitrarily selected. Correction factors were calculated by dividing the reference intensity by the measured intensities of the internal standard in each sample (*e.g.* such that the correction factor for the reference sample was one). The fluorescence intensity of the target biomarker in each

sample was then multiplied by the correction factor. Unique sets of correction factors were calculated for each exposure time. Coefficients of variation (CVs) were calculated for technical triplicates in unadjusted and adjusted fluorescence intensities (**Figure 2.6**).

2.3.4 Automation of software for optical quantification

Previous script versions relied on user input to identify artifacts for removal, which introduced several sources of analytical error. Optical artifacts included debris or bubbles on top of or inside the microfluidic chamber that had abnormally high levels of fluorescence through one or more optical filters. Multiple users could have different interpretations as to whether a given set of pixels qualified as an artifact. These discrepancies were present even amongst users following the same protocol for identifying artifacts. Another source of error could arise within a single user whose interpretation of the protocol for artifact identification changed subconsciously over time. These user biases added a potential source of error in measurements of fluorescence intensity. Manual artifact removal also required substantial amounts of time from users, reducing throughput.

To address these issues, I developed an algorithm that automated the removal of optical artifacts from images. To evaluate the performance of the automated system, fluorescence images of two labeled biomarkers isolated from the plasma of thirty-six patients were acquired (72 images total). This set of images was analyzed using both the manual and automated approaches, the results of which were compared in **Figure 2.7**. The manual approach outlined a protocol for identifying and removing optical

artifacts while minimizing user bias. The protocol required that a single user needed to perform the analysis in less than three days in order to minimize drift in the user's interpretation of the protocol. The automated approach was designed to mimic the user protocol by programmatically encoding artifact removal into the software.

2.3.5 Metrics of data quality

In order to develop a robust, automated method for quantification of collected EV biomarkers, it was necessary to establish metrics for data quality within the optical analysis software. Analysis of these data quality metrics enabled me to define a dynamic range of fluorescence intensity values that exhibited consistent, quantitative trends with both exposure time and particle concentration. The first quality metric was the percentage of the fluorescence intensity that originated from the ROI. This metric was used to ensure that the fluorescence intensity in the ROI was substantially higher than the surrounding BG. I overlaid all the identified electrode areas (yellow boxes in **Figure 2.8**) and averaged respective pixels to generate a representative image of the average electrode. I categorized ROI and BG pixels in the averaged electrode image with the same digital mask I used to calculate noise levels at individual electrodes (**Figure 2.8e**; **Figure 2.9xvi**). I integrated pixel intensities within the ROI as well as intensities within the BG, noting that the sum of the two integrals equaled the fluorescence intensity value. Both fluorescent polystyrene NPs and immunolabeled EVs were collected using DEP, quantified, and presented in **Figure 2.3**. Fluorescence intensities above 1 a.u. consistently exhibited contributions from the ROI of at least 90%

(**Figure 2.3a – c**). Therefore, I set the cutoff for the contribution from the ROI to the overall fluorescence intensity to be greater than 90%.

The second metric for data quality was the percentage of saturated pixels contributing to the fluorescence intensity. Due to inherent limitations of the camera, a saturated pixel indicated that the upper limit of incident photons had been recorded. Photons subsequently incident upon the photodetector were not measured, leading to an underestimation of fluorescence. For this reason, reliable data required negligible numbers of saturated pixels. From the data shown in **Figure 2.3f**, fluorescence intensities above 2000 a.u. showed a strong positive correlation with pixel saturation. Intensities in this set of values exhibited saturation rates above 1.5%. Therefore, the second condition for high-quality data was that the pixel saturation rate must be less than 1.5%.

2.4 Results and Discussion

2.4.1 Automation of software for optical quantification

2.4.1.1 Automatic artifact removal is preferred to manual artifact removal

I evaluated the impact of automated artifact removal on the accuracy of quantifying fluorescence images. Two critical sections of the described algorithm were devoted to the identification and removal of bright field and fluorescence artifacts from analysis of fluorescence intensity around the circumference of the electrodes (**Figure**

2.8c, h; Figure 2.9viii – xi, xxii). Fluorescence intensities from automated artifact removal were plotted against the intensities from manual artifact removal for two fluorescently labeled biomarkers (**Figure 2.7**). Bivariate correlations were performed separately for each biomarker. Automated and manual methods yielded fluorescence intensities that correlated strongly for Biomarker 1 ($r = +0.999$) and Biomarker 2 ($r = +0.998$), confirming that the automated system worked as well as manual artifact removal. I used automated artifact removal for the analysis of the data presented in this chapter.

2.4.2 Characterization of software for optical quantification

2.4.2.1 Metric of data quality for the lower limit of the dynamic range

Data points plotted in **Figure 2.3** were referred to as “fluorescence intensity,” where each value quantitates the average fluorescence signal from up to 208 locally noise-subtracted electrodes in a single field-of-view. The data quality metric for the lower limit of detection was the percentage of the fluorescence intensity originating from the ROI. Both fluorescent polystyrene NPs and immunolabeled EVs were collected using DEP, quantified, and presented in **Figure 2.3**. Fluorescence intensities above 1.5 a.u. consistently exhibited contributions from the ROI of at least 90% (**Figure 2.3a – c**). I noticed empirically during algorithm optimization that fluorescence intensities less than 1 a.u. yielded low signal at the ROI that was usually undiscernible from noise in the BG.

Therefore, I set the cutoff for acceptable contributions from the ROI to be greater than 90%.

Fluorescence from collected particles in the ROI showed a discernible ring-shaped pattern where simulations suggested the accumulation of biomarkers that are responsive to pDEP. I wanted to elucidate the lower limit of fluorescence intensity values that correlated with high contributions from the ROI (*i.e.* above 90%) to yield ring-shaped fluorescence patterns. When there was no difference in the distribution of pixel intensities in the ROI (high-field DEP) and the distribution of pixel intensities in the BG (low-field DEP), there was no discernible pattern of fluorescence after noise-subtraction. Assuming a Gaussian noise distribution of pixel intensities spanning the ROI and BG, the contribution from the ROI to the fluorescence intensity should have been approximately 31% because the BG contained approximately 2.2 times as many pixels as the ROI ($1 / (2.2 + 1) = 0.31$). ROI contributions of 31% therefore indicated pure noise, and fluorescence intensities with contributions from the ROI greater than 31% contained some signal. I empirically decided that contributions from the ROI needed to be at least 90% for patterns of fluorescence to be discernible. The contribution from the ROI could not equal 100% because the process for thresholding noise eliminated approximately 99.7% of pixels in the BG (assuming a normal distribution of pixel intensities). Therefore, approximately 0.3% of pixels in the BG would always contribute to fluorescence intensity values, meaning that there would always be a nonzero BG contribution and a non-unity contribution from the ROI. Therefore, fluorescence intensity values indicative of NP collection should have had contributions from the ROI in the set of percentages [90, 100). Furthermore, fluorescence intensity

values would be greater than zero because there would always be a nonzero level of BG noise, regardless of exposure time during image acquisition and the number of fluorescent particles isolated *via* DEP.

Pearson coefficients were calculated to assess correlations between contribution from the ROI and fluorescence intensity over three sets of intensity values.

Fluorescence intensity values in the set (0, 1 a.u.] correlated strongly with contribution from the ROI ($r = +0.717$; **Figure 2.3a**). Contribution from the ROI tended toward approximately 30% as fluorescence intensities approached zero. Both observations agreed with expectations: contribution from the ROI increased as fluorescence intensity increased, ranging from pure noise (approximately 30%) for intensities approaching zero to fluorescence patterns (at or above 90%) for intensities approaching 1 a.u.

Fluorescence intensities in the set (1 a.u., 2000 a.u.) correlated weakly with contribution from the ROI ($r = +0.261$; **Figure 2.3b**). Fluorescence intensities in this set were also exclusively above the cutoff for detectable fluorescence (contribution from ROI greater than or equal to 90%), meaning that all intensities in the set (1 a.u., 2000 a.u.) exhibited patterns of ring-shaped fluorescence. Fluorescence intensities in the set [2000 a.u., 6000 a.u.) demonstrate a strong negative correlation with contribution from the ROI; however, all intensities in the set [2000 a.u., 6000 a.u.) exhibited discernible patterns of ring-shaped fluorescence through contributions from the ROI above 90% ($r = -0.960$; **Figure 2.3c**). The unanticipated negative correlation was likely caused by increasing amounts of photon noise leaking into the BG region from increasingly strong signal at the ROI (**Figure 2.8e**).

2.4.2.2 Metric of data quality for the upper limit of the dynamic range

The percentage of saturated pixels contributing to the fluorescence intensity was the data quality metric for the upper limit of the dynamic range. From the data shown in **Figure 2.3f**, fluorescence intensities above 2000 a.u. showed a strong positive correlation with pixel saturation. I also observed that fluorescence intensities above 2000 a.u. were usually associated with regions of adjacent pixels that formed saturated intensity plateaus. Most intensities above 2000 a.u. corresponded to pixel saturation rates above 1.5%. Therefore, the second condition for high-quality data was that the pixels saturation rate must be less than 1.5%.

Pearson coefficients were calculated to ascertain correlations between the amount of pixel saturation and fluorescence intensity over three sets of intensity values. I determined that the population of saturated pixels needed to be at or below 1.5% for the fluorescence intensity value to be quantitative. All images were acquired with a 14-bit camera and exported as 16-bit TIFFs. The multiplicative conversion from 14-bit format to 16-bit did not change the resolution of the image as the relative spacing between adjacent intensity values remained the same.

Fluorescence intensities in the set (0, 1 a.u.] did not correlate with pixel saturation because no saturation was detected (**Figure 2.3d**). Fluorescence intensity over the set (1 a.u., 2000 a.u.) positively correlated with pixel saturation, as expected ($r = +0.746$; **Figure 2.3e**). Fluorescence intensities in this set were exclusively below 1.5% pixel saturation. Fluorescence intensities in the set [2000 a.u., 6000 a.u.] demonstrated a strong positive correlation with rates of pixel saturation ($r = +0.976$; **Figure 2.3f**). Pixel

saturation rates for fluorescence intensities in this set exceeded 1.5%, confirming that substantial pixel saturation was prevalent in fluorescence intensities above 2000 a.u.

2.4.2.3 Usable dynamic range

Based on these observations, I concluded that the usable dynamic range over which fluorescence intensities exhibited consistent, quantitative trends with both exposure time and particle concentration would exist between 1 and 2000 a.u. I tested correlations with fluorescence intensities and data quality metrics over three sets of intensity values: (0, 1 a.u.] in **Figure 2.3a,d**; (1 a.u., 2000 a.u.) in **Figure 2.3b,e**; and [2000 a.u., 6000 a.u.) in **Figure 2.3c,f**. All fluorescence intensities in the set (1 a.u., 2000 a.u.) exhibited good data quality, including high contributions from the ROI and low rates of pixel saturation. This dynamic range was further evaluated for robustness with different exposure times and particle concentrations. Understanding these relationships was necessary to make reliable, quantitative comparisons across clinical samples of unknown disease states.

2.4.2.4 Fluorescence intensity increased linearly with exposure time

Fluorescent polystyrene NPs were collected *via* DEP to characterize quantitative trends in fluorescence intensity and to determine the appropriate concentration of NPs to use as an internal standard in later experiments. Fluorescence intensity was plotted against exposure time for three concentrations of fluorescent polystyrene NPs spiked

into healthy human plasma (0.52, 1.6, 4.7 e+10 beads/mL; **Figure 2.4a**). Simple linear regressions were performed on the data in each contour. Coefficients of determination indicated a linear relationship between exposure time and fluorescence intensity over the usable dynamic range

The observed relationship between fluorescence intensity and exposure time was consistent with how exposure time scales distributions of pixel intensities. Both the ROI and BG should scale by the same factor, c , for a c -fold increase in exposure time. The mean and standard deviation of the distribution of BG pixels should also increase by a factor of c . The noise threshold should increase by a factor of c as well (noise threshold = BG mean + 3*BG std). The factor c should propagate through the remainder of the calculation to scale the final fluorescence intensity value, assuming the intensity value is within the dynamic range. Low contributions from the ROI and high pixel saturation outside the usable dynamic range suggested that limitations of the camera caused the ROI and BG pixel distributions to overlap or saturate (**Figure 2.10**), adversely affecting linearity with exposure time.

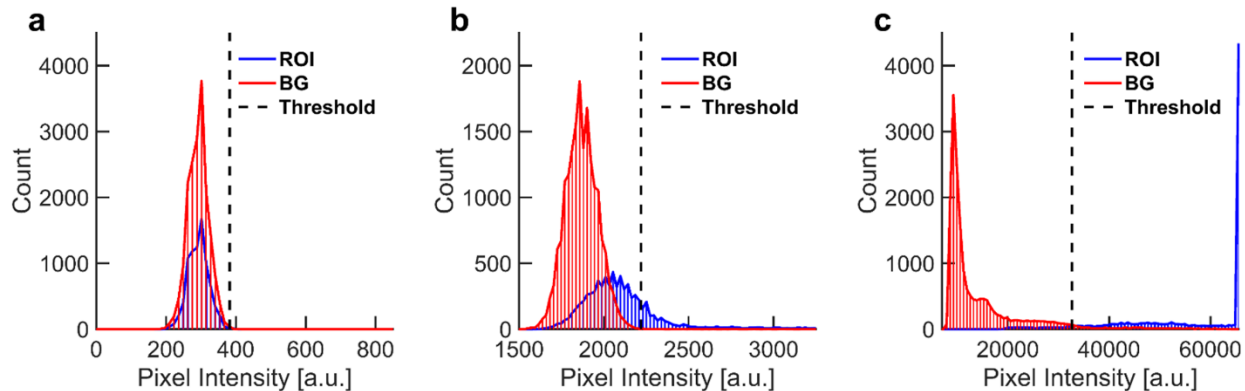


Figure 2.10. Poor data quality can cause overlap and saturation of pixel distributions. **a)** A histogram showing counts versus pixel intensity for a representative electrode in a low-exposure fluorescence image of EVs collected from a low-concentration sample using DEP. The ROI (blue) and BG (red) pixel distributions overlapped completely, indicating that the combination of concentration and exposure time were too low to yield discernible fluorescence patterns after collection from plasma with DEP. This observation was confirmed by a fluorescence intensity value of 0.01 a.u., which was well below the lower limit of the dynamic range (1 a.u.). Accordingly, the contribution from the ROI for the average of electrode in this image was approximately 30%, which was the theoretical minimum value indicating the total absence of fluorescence signal. **b)** A pixel histogram for a single electrode in a representative image of NP collection that yielded discernible fluorescence at the ROI. The ROI and BG pixel distributions overlapped partially with the right-hand tail of the ROI distribution extending beyond the noise threshold. The fluorescence intensity for the corresponding image was 211 a.u., within the bounds of the dynamic range. **c)** The ROI and BG pixel distributions were almost entirely non-overlapping. The ROI pixel distribution saturated at the right end, suggesting substantial pixel saturation. The pixel saturation percentage of the average electrode used to calculate the fluorescence intensity for this image was approximately 7%. The fluorescence intensity calculated for this image was 5787 a.u., which far exceeded the upper limit of the dynamic range (2000 a.u.) for automated analysis.

Similar trends were observed in fluorescence intensity and exposure time for EVs, which were labeled with anti-CD9-AF488 prior to isolation *via* DEP (**Figure 2.4c**). Individual data represent the average of the sample means of fluorescence intensity of usable, noise-subtracted electrodes (one image per replicate; error bars show one standard deviation above and below the mean fluorescence intensity). I wanted to confirm that signal from the internal standard in the DAPI fluorescence channel would

not affect intensity values from labeled EVs in the adjacent EGFP channel. Control experiments confirmed that fluorescence from the internal standard was undetectable in the EGFP channel. Fluorescence levels from immunolabeled EVs through the DAPI channel were less than 1.5% of levels through the EGFP channel (data not shown). Together, these data indicate minimal spectral bleed through in adjacent DAPI and EGFP filter sets for the fluorescent labels used in these experiments.

Linear regressions were performed on fluorescence intensities within the dynamic range for separate contours of EV concentration. Best-fit lines were plotted through data within the dynamic range. All contours exhibited a high degree of linearity, suggesting that the presence of the internal standard had no effect on the relationship between fluorescence intensity and exposure time; however, the size of error bars in technical triplicate data suggested that non-physiological differences in collection efficiency across individual microfluidic chambers might adversely affect linearity. Intensities were adjusted later to account for collection efficiency, reducing variability in biomarker levels.

Fluorescence intensities above 2000 a.u. were not reliably quantitative due to pixel saturation. To obtain quantitative fluorescence values in situations with high pixel saturation, the acquisition exposure time could be reduced by a factor of c to shift the pixel distribution to lower values within limits of the photodetector in the camera. If quantification of the new image yielded a fluorescence intensity within the dynamic range and minimal pixel saturation, multiplying the fluorescence intensity by a factor c would yield a better estimate of the true fluorescence level than the original image

acquired at a higher exposure. Therefore, linearity with exposure time can be exploited to expand the upper limit of the dynamic range.

2.4.2.5 Fluorescence intensity increased linearly with particle concentration

I characterized the relationship between fluorescence intensity and the concentration of fluorescent particles in the plasma samples and found that it was linear over the dynamic range (**Figure 2.4b**). Linear fits were applied to fluorescence intensity values plotted against fluorescent particle concentration as three contours of exposure time (0.1, 0.2, 0.5 s). The physical presence of the internal standard did not prohibit observations of a linear relationship between fluorescence intensity and EV concentration for three contours of exposure time (0.5, 1.0, 2.0 s; **Figure 2.4d**). Linear relationships between fluorescence intensity and particle concentration were anticipated over the usable dynamic range and consistent with conclusions in the literature.

2.4.1 *Characterization of the internal standard*

A critical component of standardized fluorescence quantification is minimizing chip-to-chip variability in collection efficiency. My technique for minimizing variability across technical replicates was to use an internal standard consisting of a known concentration of fluorescent polystyrene NPs, which I spiked into biological samples. DEP forces collected the polystyrene NPs and the biological target particles

simultaneously. The polystyrene NPs were selected to fluoresce at different wavelengths than the reporters used to label biological targets.

Adjusting fluorescence levels of labeled biomarkers to levels of the internal standard accounted for non-physiological differences in intensity. A chip with a low collection efficiency would isolate fewer polystyrene NPs and fewer biomarkers compared to a chip with high collection efficiency. Without the internal standard, I cannot be sure if a difference in biomarker fluorescence is due to a physiological difference in biomarker concentration, a difference in collection efficiencies across chips, or a combined effect. The internal standard enables to adjust biomarker levels to minimize the impact of collection efficiency.

2.4.3.1 Colocalization analysis confirms simultaneous collection of EVs and polystyrene NPs

An analysis was performed using the Image J2 Coloc 2 plugin on individual electrodes within the array to determine the degree to which the polystyrene NPs colocalized with the EVs around the electrode edge. The average Pearson's R Value (no threshold) for the polystyrene NPs (in red) and the CD9-labeled EVs (in green) was 0.75 ± 0.03 (mean $\pm$ standard deviation, $n = 10$). This indicates a strong positive spatial correlation between the polystyrene NPs and the EVs as shown in yellow in **Figure 2.5e – f**. This analysis shows that the DEP force acts similarly on both the polystyrene NPs and the EVs, making the NPs an appropriate internal standard for the EVs.

2.4.3.2 Internal standard: linearity of fluorescence intensity with exposure time

Adjusting intensities of immunofluorescently labeled EVs for collection efficiency using the internal standard did not affect the linear relationship between fluorescence intensity and exposure time. Comparisons of panels c and e in **Figure 2.4** highlighted that coefficients of determination for linear fits did not change after adjusting for collection efficiency.

2.4.3.3 Internal standard: linearity of fluorescence intensity with concentration

Adjusting intensities of immunofluorescently labeled EVs for collection efficiency using the internal standard improved the linear relationship between fluorescence intensity and EV concentration. Comparisons of panels d and f in **Figure 2.4** demonstrated that coefficients of determination for linear fits increased from approximately 0.96 to 0.99 for all contours of exposure time (0.5, 1.0, 2.0 s) after adjusting for collection efficiency.

2.4.3.4 Internal standard reduced variability in technical replicates

To determine how differences in collection efficiency affected biomarker levels, coefficients of variation (CVs) were calculated for technical triplicates of immunofluorescently labeled EVs. CVs were also calculated for intensities that had been adjusted for collection efficiency using the internal standard (**Figure 2.6**). All intensity values used to calculate CVs were within the dynamic range. Adjusted

intensities exhibited lower CVs at all detectable concentrations of EVs, confirming that the internal standard partially corrected for variability in technical replicates.

Taken together with data shown in **Figure 2.4f**, the internal standard effectively reduced the variability in technical replicates while improving the linear relationship between EV concentration and fluorescence intensity. Furthermore, the presence of the internal standard did not affect the linear relationship between fluorescence intensity and EV concentration (**Figure 2.4e**). Therefore, the internal standard was shown to be beneficial for quantifying biomarkers collected with DEP chip technology.

2.4 Conclusion

I designed an algorithm to quantify the fluorescence intensity of labeled NPs collected in predictable locations within a microfluidic device. I demonstrated the utility of this algorithm by using an electrode microarray chip to collect EVs and polystyrene NPs from blood plasma *via* DEP forces. Quantification was achieved by comparing pixel intensity values in regions of high-field DEP, where the particles preferentially accumulated, to values at adjacent regions of low-field DEP, which served as BG. I automated the entire process including removal of optical artifacts, the determination of fluorescence intensity, and the creation of reports. Automation helped standardize analysis by minimizing user bias and improving throughput. I then identified a usable dynamic range in fluorescence intensity values. I established the lower limit of the usable dynamic range by correlating fluorescence intensity with the predicted location of the source of fluorescence. Correlations between fluorescence intensity and the

percentage of saturated pixels defined the upper limit of the usable dynamic range. As anticipated, I observed linear relationships between fluorescence intensity and exposure time at multiple concentrations of particles. I also demonstrated that fluorescence intensity increased linearly with particle concentration at multiple exposure times. By tailoring the digital mask to encompass regions of collection, the algorithm described in this chapter can be translated to other fluorescence-based platforms for liquid biopsy.

I also developed an internal standard to correct for chip-to-chip variability in collection efficiency. Spiking a known concentration of fluorescent polystyrene NPs into biological samples enabled me to partially correct for variability in fluorescence intensity across technical replicates. Adjusting for collection efficiency did not change linear trends between fluorescence intensity and exposure time; however, use of the internal standard improved the linearity of fit for fluorescence intensity and biomarker concentration. Use of the internal standard reduced the measured variability in technical replicates by correcting for differences in collection efficiency across DEP chips. The internal standard will be important for comparing biomarker levels between clinical patient samples of unknown disease states. Controlling for non-physiological variability in biomarker levels is especially critical for the detection of early stage cancer, which often exhibits low circulating concentrations of prognostic biomarkers.

Chapter 3. Cyclic immunofluorescence profiling of nanoparticles collected using DEP

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*In this study, I performed experiments, maintained cell lines, helped design and assemble the quenching device, acquired images, analyzed data, assembled figures, and co-wrote the manuscript.

A portion of this chapter is under review for publication.
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3.1 Abstract

Cancer is a highly heterogeneous disease that requires precise detection tools and active surveillance methods. Liquid biopsy assays provide an agnostic way to follow the complex trajectory of cancer, providing better patient stratification tools for optimized treatment. Here, a cyclic liquid biopsy assay, called cyc-DEP (**cyclic** immunofluorescent imaging on **DEP** chip), was developed to profile biomarkers collected on a DEP microfluidic chip platform. A fluorophore quenching method was optimized to enable sequential rounds of on-chip staining with fluorescently conjugated primary antibodies. Cyc-DEP allows for quantification of a multiplex array of proteins using twenty-five microliters of patient plasma sample. To demonstrate this proof-of-concept technique,

NPs were generated from a prostate adenocarcinoma (LNCaP) cell line and a panel of six biomarkers for prostate cancer detection was developed. Cyc-DEP allowed me to quantify the presence of those biomarkers from the plasma of healthy individuals as well as low-grade and high-grade prostate cancer patients (each $n = 3$). To ensure accurate quantification of signal intensities and to enable comparisons between different samples, I employed the signal intensity normalization method (fluorescent beads, or internal standard) and a custom signal intensity quantification algorithm that accounts for the distribution of signal across hundreds of individual collection regions on the chip (techniques from Chapter 2). These techniques enabled accurate and automated quantification of six biomarkers from patient plasma samples, opening the door to detect a multiplex array of biomarkers from low-volumes of patient samples for cancer early detection and active surveillance.

A second technique was also developed to perform on-chip cycIF using oligonucleotide (oligo) barcoding. This “oligo cyc-DEP” approach condensed antibody incubation into a single step and drastically reduced quenching times to less than fifteen minutes per round. Oligo cyc-DEP achieved four rounds of on-chip cycIF imaging to profile three target proteins in a matter of hours, offering a powerful potential alternative to cyc-DEP that could yield clinically feasible sample-to-answer times.

3.2 Introduction

Tumors become increasingly heterogeneous as they evolve. Cancer cells that harbor different mutations and genomic aberrations can be found within the same tumor, which can lead to resistance to drug therapies and evasion from the immune system¹⁹⁹. Prostate cancer is a highly heterogeneous disease wherein a single prostate gland can contain multiple tumor foci with different histological grades and genomic aberrations²⁰⁰⁻²⁰³. The current method of assessing prostate cancer aggressiveness is through the histological grading of needle biopsy cores taken from the prostate. However, histological grading does not yield information on the molecular heterogeneity of the tumor. Furthermore, needle core biopsies can lead to the under sampling of tumor foci with higher grades, and lead to inaccurate classification of tumors^{204, 205}. This presents a clinical need for an unbiased method to assess prostate cancer aggressiveness, which may be addressed *via* liquid biopsy assays.

Currently there is a lack of liquid biopsy assays with high sensitivity and specificity for detecting clinically significant prostate cancer. The most common liquid biopsy assays to detect prostate cancer quantify PSA levels from plasma²⁰⁶. PSA tests have a high false-positive rate that can lead to unnecessary biopsies, over-detection, and over-treatment of indolent cancers^{33, 207, 208}. About 65-70% of men with high (> 4.0 ng/mL) PSA values have a negative prostate biopsy²⁰⁹. Additionally, 15% of men with prostate cancer have low (< 4.0 ng/mL) PSA values, meaning their disease will be left undetected²⁰⁹. To improve diagnostic accuracy in initial biopsy settings and inform

treatment decisions during active surveillance, novel multi-parametric tests that accurately represent tumor heterogeneity need to be developed²¹⁰.

Recent advances in imaging have enabled the multiplex proteomic profiling of markers from single tissue sections²¹¹⁻²¹⁴. Cyclic immunofluorescence (cycIF) microscopy is one such method. CycIF utilizes oxidation-based chemical quenching of fluorophores to enable up to fifteen rounds of five-channel staining and quenching in tissue sections. CycIF staining can yield spatial information for forty to sixty markers per tissue section²¹⁵. CycIF has so far been utilized on cell lines and tissue sections to characterize the presence of and interactions between molecular markers^{216, 217}. The application of cycIF to liquid biopsy samples can increase the number of biomarkers detected *via* liquid biopsy assays, enhancing the efficiency of these platforms to provide better patient stratification in the clinic.

Challenges in isolating biomarkers consistently from small volumes of patient samples have hindered the development of clinical liquid biopsy assays²¹⁸. Novel microfluidic chip technologies avoid time-consuming and costly sample-processing steps, and some of these technologies successfully distinguish different cancers and cancer subtypes by using multiplex profiling approaches²¹⁹⁻²²¹. My research group has previously used an alternating current electrokinetic microarray chip device (DEP chip), designed by Biological Dynamics in San Diego, CA, to rapidly isolate biomarkers from low volumes of plasma¹³², and I have developed an automated signal intensity quantification system specific to that DEP chip platform (Chapter 2). By applying an alternating current to liquid samples, the microarray chip creates DEP forces that direct biomarkers to predictable locations for collection, on-chip immunolabeling, and

downstream analysis. Through this process, biomarkers can be isolated from less than fifty microliters of undiluted blood plasma within minutes. The DEP chip platform allows for the quantification of proteins associated with EVs or other endogenous NPs by immunofluorescence imaging. Researchers have previously conducted on-chip immunofluorescence imaging of up to two proteins per sample from plasma of patients with various cancers and diseases^{172, 222-225}. Due to a limited number of spectrally non-overlapping fluorophores, higher-multiplicity proteomic profiling of material collected on the DEP chip was previously unattainable.

This study presents a platform called “cyc-DEP,” which enables cyclic immunofluorescent imaging on the DEP chip (**Figure 3.1**) and increases the number of proteins that can be profiled by three-fold. With cyc-DEP, molecules involved in distinct signaling pathways as well as their post-translational modifications can be profiled collectively. This can be used to stratify patients into treatment groups and inform clinicians on precise molecular mechanisms to target. Unlike current multi-marker tests²²⁶⁻²²⁸, this technology can be equally effective in the initial screening of cancer as well as for active surveillance of patients with low-grade localized disease, eliminating repeat tissue biopsies and improving patient quality of life. Cyc-DEP can also be applied to different types of cancer and potentially be used with different sample types (e.g. urine, semen, cerebrospinal fluid, etc.) through modification of the protein panel and DEP conditions. This chapter presents a viable and versatile platform for the multiplex profiling liquid biopsy samples.

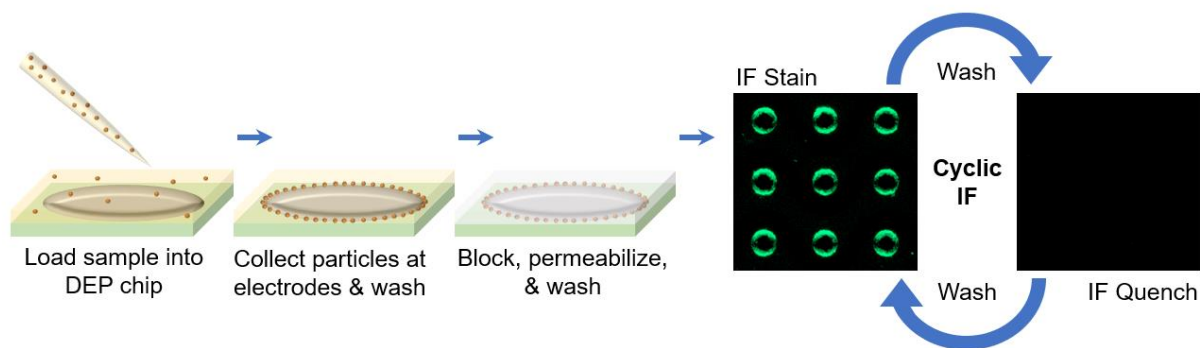


Figure 3.1. Overview of cyc-DEP assay. A biological sample containing protein-associated NPs is loaded into the fluidic chamber of a chip with a planar DEP microarray. Application of an electrical signal to the planar microarray exerts DEP forces on NPs, which collect around the electrode edges. Collected NPs remain at the electrode edges after washing, and the electrical signal is switched off. Blocking and permeabilization prepares the collected material for immunofluorescent labeling by reducing non-specific binding and allowing antibodies to permeate the particles. A solution of Alexa Fluor-conjugated antibodies was added to the fluidic chamber, staining target proteins in the collected material. Washing, imaging, and image quantification revealed fluorescent rings, indicating that target proteins in the collected material had been successfully immunolabeled. A quenching buffer was added to the fluidic chamber, and the sample was exposed to high-intensity full-spectrum light to photo-bleach the fluorophores. Washing, imaging, and image quantification showed effective quenching of fluorophores. Quenching buffer was then removed, and the sample was subsequently stained with a different antibody against another target protein. Repeated rounds of quenching and staining were performed on-chip.

A second platform called “oligo cyc-DEP” was also developed (**Figure 3.2**) to address some of the challenges with cyc-DEP. This second platform used oligo-conjugated antibodies²²⁹ and complementary oligo-conjugated fluorophores to achieve fast labeling and quenching with minimal sample degradation over successive rounds of imaging. The key to this approach was the design of unique oligo barcodes to label specific target proteins. In this approach, primary antibodies were conjugated to unique oligo sequences called docking strands (DSs), and complementary oligo sequences called imaging strands (ISs) were conjugated to fluorophores. After immunolabeling the

collected material with antibody-DSs, specific hybridization of complementary ISs to bound DSs indicated the presence of target proteins.

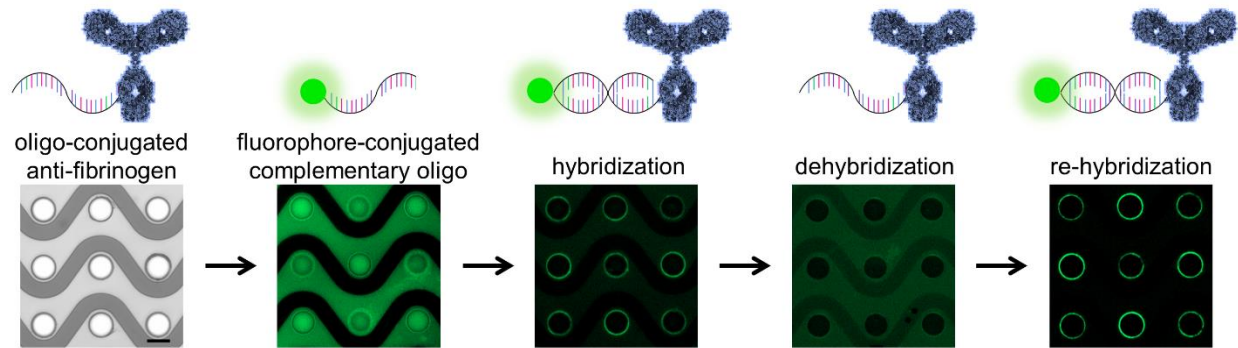


Figure 3.2. Overview of oligo cyc-DEP assay. A plasma sample is loaded into the microfluidic chamber of a DEP chip. An applied electric field exerts DEP forces on endogenous NPs like EVs and cfDNA, which collect at high-field regions around the circumferences of each planar microelectrode. The collected material is blocked, permeabilized, and washed. NPs are immunolabeled with an oligo-conjugated antibody and washed. Complementary oligo-fluorophore conjugates are introduced into the microfluidic chamber, hybridize with the bound antibody-oligo conjugates within minutes. Excess oligo-fluorophore conjugates are washed away before the sample is imaged and fluorescence levels are quantified. A dehybridization agent is added to the sample, removing oligo-fluorophore conjugates from the bound antibody-oligo conjugates within minutes. The sample is washed again, imaged, and quantified to confirm the removal of fluorophores. Once more, oligo-fluorophore conjugates are withdrawn into the chamber, hybridizing with the bound antibody-oligo conjugates. Re-hybridization confirmed that collected material remained at the high-field regions after exposure to the dehybridization agent. Dehybridization and rehybridization can be repeated, demonstrating the feasibility of oligo-based cycIF for multiplex proteomic profiling of liquid biopsies. Scale bar is 60 μm .

Oligo cyc-DEP utilized ethylene carbonate (EC) buffer to quickly dehybridize DS-IS pairs. It is believed that EC interferes with the hydrophobic stacking of base-pairs, effectively destabilizing DNA helices. Civitci *et al.* recently utilized EC to alter the localization kinetics of DS-IS pairs in DNA-PAINT experiments²³⁰. Here, EC-induced dehybridization of DS-IS pairs was applied to cycIF imaging, enabling fast multiplex

profiling across successive rounds of staining. Importantly, EC-mediated dehybridization exhibited high quenching efficiency and had no noticeable negative effect on sample integrity.

Oligo cyc-DEP offers a fast, high-multiplicity alternative to cyc-DEP for profiling the protein content of liquid biopsy biomarkers. The presented studies highlight the design of three unique DS-IS pairs, the EC-mediated dehybridization quenching of those DS-IS pairs, and the binding validation of the antibody-oligo library against protein targets in cells. Four rounds of oligo-based cycIF were successfully conducted on cell-derived NPs collected from approximately twenty-five microliters using DEP. Furthermore, oligo cyc-DEP offered a sample-to-answer time on the scale of hours, which is appropriate for clinical use. Oligo cyc-DEP is also adaptable to profile a variety of diseases through conjugation of the DS library to different disease-specific antibody panels.

3.3 Materials and Methods

3.3.1 Antibodies

Antibodies against CD9, phospho-histone H2A.X (γ H2AX), phosphoglycerate kinase 1 (PGK1), Claudin 3 (CLDN3), fatty acid synthase (FASN) and fibrinogen were obtained. The following antibodies were conjugated to AlexaFluor 488 or AlexaFluor 647 by the manufacturers: CD9-AF488 (R&D Systems, FAB1880, 209306; Minneapolis, MN), γ H2AX-AF647 (Cell Signaling Technology, 9720, 20E3; Danvers, MA), PGK1-

AF488 (Abcam, ab222117, EPR19057; Cambridge, UK). Information regarding unconjugated antibodies are as follows: CLDN3 (Thermo Fisher Scientific, 34-1700, polyclonal rabbit IgG; Waltham, MA), FASN (Abcam, ab22759, polyclonal rabbit IgG; Cambridge, UK), Fibrinogen β -chain (Abcam, ab193932, EPR19765; Cambridge, UK) Fibrinogen γ -chain (Abcam, ab119948, 5A6; Cambridge, UK).

3.3.2 Conjugation of fluorophores to antibodies for cyc-DEP experiments

IgG antibodies were conjugated to Alexa Fluor 488 and Alexa Fluor 647 dyes via labeling of primary amine residues on antibodies with NHS ester functionalized fluorophore dyes. For antibodies that contain preservatives (e.g. sodium azide, glycerol, etc.), a buffer exchange step into 1X DPBS(-) was performed using 10 kDa Amicon Ultra spin columns (UFC5010) from Sigma-Aldrich (St. Louis, MO). Alexa Fluor NHS ester reagents (A20006 and A20000) from Thermo Fisher Scientific (Waltham, MA) were dissolved in DMSO to a final concentration of 10 mM. Antibodies were reacted with fluorophore dyes at a 1:10 antibody to dye molar ratio in 0.1 M sodium bicarbonate buffer of pH 8.3. Conjugation reactions were carried out on a rocker for two hours at room temperature in the dark. A buffer exchange step using 10 kDa Amicon Ultra spin columns was performed to remove the excess Alexa Fluor dye from conjugation reactions.

3.3.3 *Site-specific modification of antibodies for oligo cyc-DEP experiments*

Unconjugated antibodies against the tetraspanin protein CD9 were purchased from R&D Systems (Minneapolis, MN). The following unconjugated antibodies were purchased from Abcam (Cambridge, UK): phosphoglycerate kinase 1 (PGK1) and fatty acid synthase (FASN). Buffer exchanges were performed on all antibodies that were stored with carriers like sodium azide and bovine serum albumin into preparation buffer from the SiteClick™ Antibody Azido Modification Kit (Cat # S20026), which was purchased from Thermo Fisher Scientific (Waltham, MA). These kits were used for the site-specific azide modification of carbohydrate residues on primary antibodies, enabling conjugation of DS oligo sequences to antibodies. A NanoDrop™ spectrophotometer from Thermo Fisher Scientific (Waltham, MA) was used to determine the concentrations of DSs and modified antibodies prior to antibody-DS conjugation. DSs were incubated overnight with modified antibodies in a 4:1 molar ratio at 25 °C. To purify the antibody-DS conjugates, excess DS was removed with six rounds of spin column filtration using 30 kDa Amicon Ultra Centrifugal Filter Units (UFC501024) from Fisher Scientific (Hampton, NH).

3.3.4 *Design of oligonucleotide DSs and ISs for oligo cyc-DEP*

DSs consisted of oligo sequences that were either 28 or 32 nucleotides long. The 5' ends of DSs were capped with dibenzylcyclooctyl-triethylene glycol (DBCO-TEG) spacers to which modified antibodies could be conjugated. Experimental optimization

necessitated that all ISs used in this study exhibit molar guanine-cytosine (GC) percentages less than 40% and melting temperatures lower than 40 °C. Optimal ISs were approximately 13 nucleotides long and were confirmed through the open-source Basic Local Alignment Search Tool (BLAST) to be orthogonal to the human genome. All ISs used in this study were conjugated with two AF488 fluorophores, one on the 3' end and another on the 5' end. Fluorophore-conjugated ISs and DBCO-TEG-modified DSs were purchased from Integrated DNA Technologies (Coralville, IA).

3.3.5 *Cell culture*

LNCaP (FGC CRL-1740) human prostate epithelial cells from ATCC (Manassas, VA) were maintained in DMEM with 10% FBS at 37 °C and 5% CO₂ at recommended densities. Adherent cells were detached using Trypsin and collected at mid-log phase for all experiments. Cell viability and concentration were measured with Trypan blue on the Countess II FL Automated Cell Counter from Life Technologies (Carlsbad, CA). Cell viability was at least 90% for all samples.

3.3.6 *Immunofluorescent labeling of LNCaP cells for cyc-DEP*

For the validation of antibody-antigen binding, staining patterns of each antibody on LNCaP cells were visualized using immunofluorescent microscopy. LNCaP cells were plated at 4e+4 cells/well concentration on 96 well plates with #1.5 polymer coverslip bottoms (P96-1.5P) from Cellvis (Mountain View, CA). At approximately 80%

confluence, cells were fixed with 4% paraformaldehyde (PFA) for fifteen minutes at room temperature. PFA was diluted from a freshly opened 16% PFA ampoule (15710) from Electron Microscopy Sciences (Hatfield, PA). Fixed cells were permeabilized in ice cold 100% methanol for ten minutes at 4 °C, followed by a blocking step in 3% BSA (A7906) from Sigma-Aldrich (St. Louis, MO) that was dissolved into PBS for thirty minutes at room temperature. Primary antibodies were diluted 1:100 in 3% BSA solution and incubated with cells for either two hours at room temperature or overnight at 4 °C. Cells were stained with 0.01 mg/mL DAPI nuclear dye (32670-5MG-F) from Sigma-Aldrich (St. Louis, MO) for ten minutes at room temperature. After each step of the protocol, cells were washed three times in 1X DPBS(-). Cells were imaged at 20x magnification with either a CSU-X1 spinning disk confocal microscope from Yokogawa (Tokyo, Japan) on an Axio Observer from ZEISS Microscopy (Jena, Germany), or an ImageXpress Micro Confocal microscope from Molecular Devices (San Jose, CA).

3.3.7 Immunofluorescent labeling of LNCaP cells for oligo cyc-DEP

For cell line validation of DS-IS hybridization, LNCaP cells were plated and fixed as previously described. Sheared salmon sperm DNA (AM9690) from Fisher Scientific was added at 0.5 mg/mL to the 3% BSA blocking solution described previously, and LNCaP cells were incubated with this modified blocking solution for thirty minutes at room temperature. Primary antibody-DS conjugates were diluted 1:100 by-volume from stock into 3% BSA solution. Block LNCaP cells were incubated overnight at 4°C with antibody-DSs. Prior to addition of ISSs, LNCaP cells were washed with Buffer C (equal

parts by-volume of 1M NaCl and DPBS-) three times. All ISs were reconstituted into TE buffer at a concentration of 100 μ M. ISs were then diluted 1:1000 into a hybridization buffer consisting of 0.5 mg/mL sheared salmon sperm DNA in Buffer C. ISs incubated with the LNCaP cells for ten minutes at room temperature, hybridizing bound antibody-DS conjugates. LNCaP cells were washed three times with Buffer C to remove excess ISs. Nuclei were stained with DAPI nuclear dye as previously described. LNCaP cells were imaged as previously described using 100% intensity and 1500 ms exposure. All images were exported as 8-bit TIFFs.

3.3.8 *Formation of LNCaP-derived NPs*

LNCaP cells were trypsinized and centrifuged at 300 g for five minutes and the supernatant was discarded. Cells were resuspended in DMEM. The concentration of cells in suspension was measured as previously described. Cells were centrifuged again at 300 g for five minutes, and the supernatant was discarded. LNCaP cells were resuspended in 0.5X PBS at a concentration of 1×10^6 cells/mL. Cell suspension volumes between one and three milliliters were necessary for further sample preparation. The suspension of LNCaP cells was immediately placed in a bath of ice water and probe sonicated for a total of six minutes (four minutes of active sonication with two minutes of interspersed rest) at a pulse ratio of two seconds on to one second off and an amplitude of 50% using the Q125 probe sonicator (#Q125) affixed to a probe (#4422), both supplied by Qsonica (Newtown, CT). These sonication parameters were sufficient to create DEP-responsive NPs from cellular material (**Figure 3.3**). The size distribution,

Zeta potential, and concentration of the LNCaP-derived NPs were characterized using the ZetaView® QUATT nanoparticle tracking system from Particle Metrix (Munich, Germany).

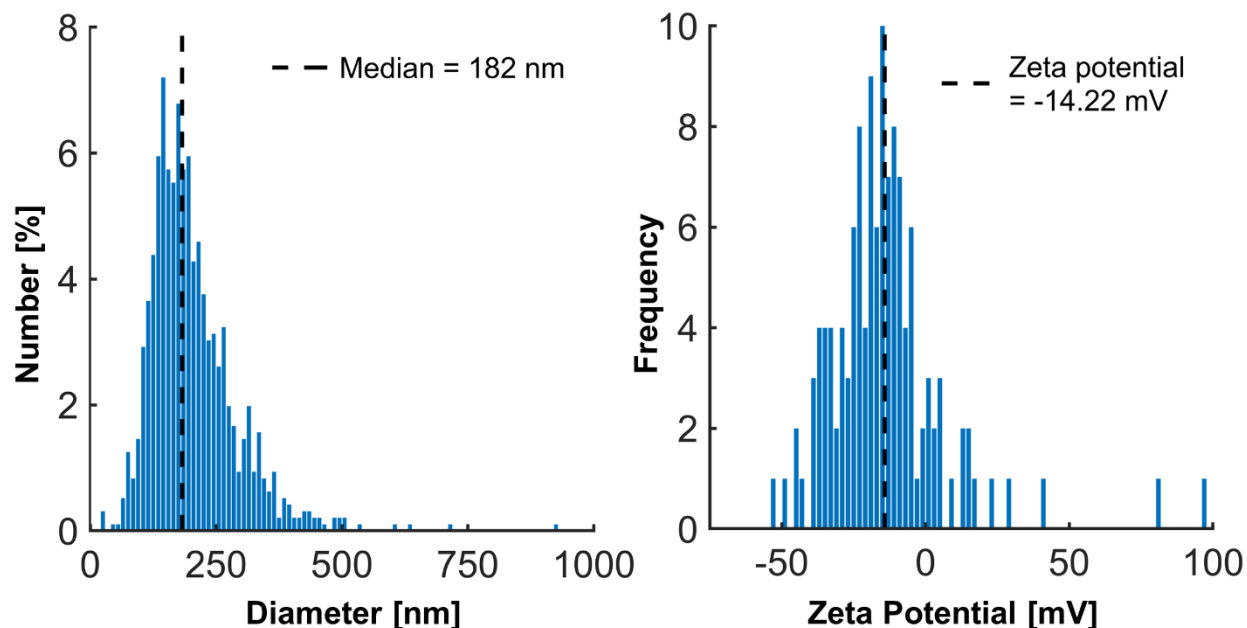


Figure 3.3. Characterization of LNCaP-derived NPs. Size and charge distribution of $n = 958$ NPs formed from probe sonication of LNCaP cells were measured at 25 °C in 0.1X PBS. **(a)** Plot showing the size distribution of $n = 958$ particles that formed from probe sonicating a buffer suspension of LNCaP cells. The median diameter of particles was 182 nm. **(b)** NPs exhibited a mean Zeta potential of -14.22 ± 0.14 mV.

3.3.9 Processing of clinical plasma samples

Blood plasma and FFPE tissue sections were collected from prostate cancer patients (IRB #00020159). Normal samples were also acquired with informed consent from healthy volunteers and stored at -80 °C. One whole blood sample from a healthy donor was acquired from the OHSU Knight Cancer Institute Cancer Early Detection

Advanced Research (CEDAR) Center Biorepository (IRB approval #00022404). The whole blood sample was collected in an EDTA tube and centrifuged (at 4 °C for fifteen minutes at 1500g) once to obtain plasma. Two blood plasma samples from healthy donors were obtained from the Oregon Clinical & Translational Research Institute (OCTRI) and the OCTRI Research Volunteer Repository (IRB approval #00022404). All experimental protocols were performed in adherence with state and federal regulations and with relevant policies as outlined by OHSU and the OHSU IRB. The OHSU IRB determined that the activities of the study associated with IRB #00022404 and IRB #00020159 were not research involving human subjects.

3.3.10 *Preparation of the internal standard for DEP chip collection*

FluoSpheres™ fluorescent microparticles (0.1 µm diameter, 2% solids; F8797) from Thermo Fisher Scientific (Waltham, MA) were diluted 1:2000 into 0.5X PBS to create a working solution. The working solution was diluted 1:25 into LNCaP-derived NPs and thawed plasma samples to a final dilution of 1:50,000 from stock. Samples were stored on ice during experimentation. All samples were vortex mixed for two minutes prior to loading onto the DEP chip to ensure homogeneous distribution of the internal standard.

3.3.11 *DEP collection of LNCaP-derived NPs and endogenous NPs*

All multi-chamber microfluidic DEP chips (ExoCell™ EVF1P flow cell, 3-channel; EF-CRT-00002) were acquired from Biological Dynamics (San Diego, CA). DEP chips were hydrated with 0.5X PBS prior to loading samples in order to remove byproducts of microfabrication from the fluidic chambers. Twenty-five microliters of each sample were loaded into separate fluidic chambers each atop a DEP microarray at a flow rate of 7 $\mu\text{L}/\text{min}$. An electrical signal (14 V_{pp} , 15 kHz) was applied to DEP chips for twenty minutes to collect NPs from each sample. For the first ten minutes, collection was enriched by flowing an additional 5 μL of sample over the array at a rate of 0.5 $\mu\text{L}/\text{min}$. No enrichment occurred over the remaining ten minutes. The electrodes were washed with 0.5X PBS to remove excess sample or bulk plasma (140 μL at a flow rate of 7 $\mu\text{L}/\text{min}$). The applied electrical signal was turned off after washing. The collected material was blocked for at least twenty minutes at room temperature with a filtered solution of 2 wt% BD Difco™ Skim Milk powder (232100-500g) from Midland Scientific (Omaha, NE) and 0.1 v% TWEEN®20 were dissolved into 0.5X PBS and filtered with a 0.45 μm syringe filter. Blocking buffer was removed with additional washing.

3.3.12 *Immunofluorescent labeling of proteins from LNCaP-derived NPs and clinical plasma using cyc-DEP*

Collected NPs were immunolabeled with the first round of antibodies. Fluorophore-conjugated primary antibodies were diluted 1:100 from stock into blocking

buffer without TWEEN®20. Thirty microliters of antibody solution were introduced into the fluidic chamber at a flow rate of 7 μ L/min. The collected NPs incubated with antibodies for one hour in the dark at room temperature. The sample was then washed with 0.5X PBS. To photo-bleach the fluorophores, thirty microliters of quenching buffer (0.1 M NaOH) was drawn into the microfluidic chamber and subjected to high-intensity light from a full-spectrum LED panel for a minimum of four hours at 4 °C. Quenching buffer was removed from the fluidic chamber with additional washing with 0.5X PBS. Additional rounds of cycIF staining and quenching were performed as described.

3.3.13 *Immunofluorescent labeling of proteins from LNCaP-derived NPs using oligo cyc-DEP*

For oligo cyc-DEP assays, LNCaP cell-derived NPs were collected with DEP, blocked, and permeabilized using the method previously described. Antibody-DS stock solution was diluted 1:100 by-volume into blocking buffer (2 wt% skim milk powder into filtered 0.5X PBS). Collected NPs were incubated with antibody-DS dilutions for one hour at room temperature and washed. Four rounds of cycIF imaging were then conducted. ISs were reconstituted from stock into TE buffer as previously described. Reconstituted ISs were added to Buffer C with added sheared salmon sperm DNA (respective final dilutions of 1:10 by-volume IS and 1:5 by-volume salmon DNA). ISs were incubated with the collected material for ten minutes at room temperature and washed with 70 μ L filtered 0.5X PBS at 7 μ L/min. Fluorescent images were acquired using the equipment and settings outlined in Chapter 2.

3.3.14 *Fluorophore photo-bleaching for cyc-DEP*

A custom fluorescence-quenching device was built to photo-bleach fluorophores used in immunofluorescent labeling of target proteins. A microcontroller-based circuit was designed and assembled to tailor the amount of light delivered by the quenching device to the surface of the DEP chip *via* pulse-width modulation (**Figure 3.4**). The central component of the quenching device was the full-spectrum, high-intensity LED panel, capable of exhausting fluorophores of different excitation wavelengths spanning UV and visible spectrums. A fan and a heatsink were affixed to the back of the LED panel to dissipate heat. A focusing lens was inserted into a lens holder, which was attached to the front of the LED panel. Protective housing encased the fan, heatsink, lens, and lens holder. Lead wires connected the fan and LED panel to two different pairs of nodes in the circuit. The circuit consisted of: the LED panel, a transistor, a resistor, a cooling fan (motor), a microcontroller, and an AC power supply. Custom software was uploaded to the microcontroller, dictating the amount of light incident upon the DEP chip. Electrical power was supplied to the microcontroller and LED panel, which emitted light necessary to photo-bleach fluorophores conjugated to bound antibodies. Alternatively, a full spectrum LED light source can be commercially obtained and used for the photo-bleaching of fluorophores.

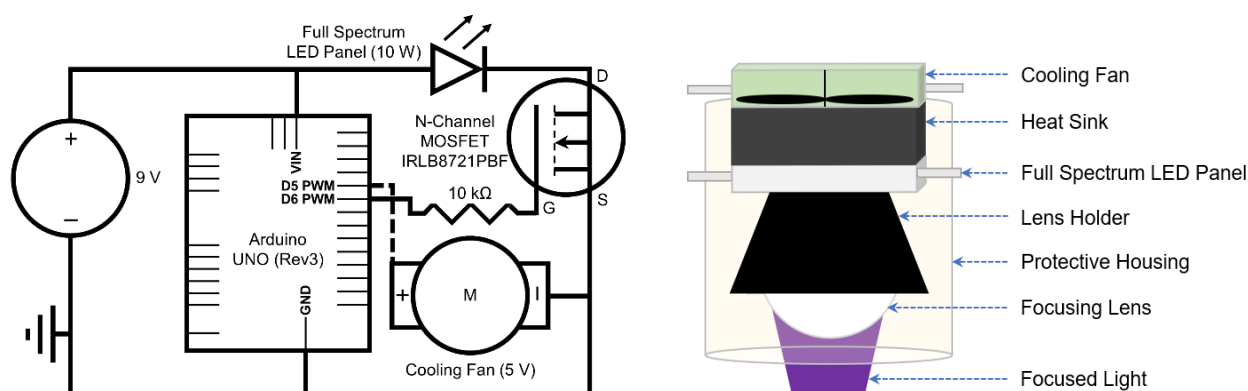


Figure 3.4. Custom optical device for photo-bleaching fluorophores. A circuit diagram (left) consisting of a power source, LED panel, MOSFET, resistor, cooling fan, and Arduino controlled the amount of light incident upon the immunofluorescently labeled sample. Custom software used pulse-width modulation to control LED intensity. The LED panel and cooling fan were incorporated into a quenching device (right) that was affixed to the chip holder. Light from the LED panel exited the quenching device and interacted with the sample through the optical window of the chip.

3.3.15 *Ethylene carbonate-mediated dehybridization for oligo cyc-DEP*

Ethylene carbonate (676802) was purchased from Sigma-Aldrich (St. Louis, MO), liquified at 70 °C, and diluted to 25 v% in distilled water. Incubation of the collected material with diluted EC for at least five minutes at room temperature dehybridized DS-IS pairs, and unbound ISs were removed with washing.

3.3.16 *Image acquisition, exportation, and analysis*

Images were acquired and exported as previously described in Chapter 2. The microscope, camera, filters, and light source were also unchanged from previous work. Biomarker levels were quantified from fluorescence images using an automated

algorithm written in MATLAB and presented in Chapter 2. The collection efficiency of each DEP microarray was measured using the internal standard. The internal standard was quantified using an image taken after DEP collection but prior to conducting the on-chip cyc-DEP assay. Fluorescence levels of the internal standard were used to calculate correction factors, which were multiplied with biomarker levels in order to adjust for differences in collection efficiency across DEP microarrays. All plotted biomarker levels in this chapter were adjusted for collection efficiency. All heatmaps contain biomarker levels plotted as the base-two logarithm of fluorescence intensities quantified from images taken over the course of the experiment. Fluorescence intensities greater than or equal to the lower limit of detection (*i.e.* 1 a.u. multiplied by an appropriate adjustment factor determined by use of the internal standard) were detectable (increasing color scale of yellow to red) and within the established dynamic range of the algorithm. Fluorescence intensities less than the adjusted lower limit of detection were undetectable and shown in white. Undetectable fluorescence levels were categorized as “not a number,” or “NaN”. Other figures showed biomarker levels for which there was no logarithmic transformation of the set of fluorescence intensities. All fluorescence images shown in this chapter were contrast-enhanced. The methods of contrast-enhancement were consistent for images acquired in a given fluorescence channel within each figure.

3.4 Results and Discussion

3.4.1 Overview of cyc-DEP strategy: multiplexing and quenching

Cyclic immunofluorescent imaging on **DEP** chip assay (cyc-DEP) is an on-chip cycIF imaging and quantification platform for the multiplex proteomic analysis of liquid biopsy samples. Cyc-DEP utilizes DEP-mediated microfluidic technology for collecting endogenous NPs directly from undiluted liquid biopsies like whole blood and plasma samples. This enables the combined proteomic profiling of EVs and other cellular NPs, protein aggregates, and nucleic acids like high-molecular-weight circulating cfDNA²²⁴. The proteomic multiplicity of previous studies that utilized DEP-mediated microfluidic technology was limited by the number of available fluorescence filters/channels and spectral overlap of fluorophores used in on-chip immunoassays. Cyc-DEP expands the number of proteomic targets that can be profiled from a given sample volume by a factor of three or more.

Figure 3.1 summarizes cyc-DEP. Twenty-five microliters of blood plasma is withdrawn into a microfluidic chamber atop a planar array of microelectrodes (one electrode shown). An electrical signal is applied to the array of microelectrodes to create DEP forces on endogenous NPs, which collect at the circumferences of the electrodes. The bulk plasma is washed away while the NPs are held in place. The collected material is blocked, permeabilized, immunolabeled with primary antibody-fluorophore conjugates, and washed. Immunolabeled proteins appear as patterns of ring-shaped fluorescence, which are detected and quantified *via* fluorescence imaging and custom

software. Fluorophores are quenched using a combination of a chemical buffer and photo-bleaching using an LED panel (**Figure 3.4**). The sample is then washed to remove the chemical buffer, and fluorescence quenching is confirmed using fluorescence imaging and quantification. The sample is incubated with additional primary antibody-fluorophore conjugates. Successive rounds of immunolabeling and quenching are performed. A known concentration of fluorescent NPs, called the internal standard, was added to every sample prior to DEP in order to measure the relative strength, or collection efficiency, of each microelectrode array. Measured fluorescence levels were adjusted for differences in collection efficiency using the quantitative methods from Chapter 2.

3.4.2 Selection and validation of protein panel for cyc-DEP method development

A panel of six target proteins was selected based on the protein expression profile of the prostate adenocarcinoma LNCaP cell line as well as their association with EVs and/or prostate cancer (**Table 3.1**). PGK1, CLDN3 and FASN are known markers associated with EVs that are secreted by prostate cancer cell lines into the serum or plasma of prostate cancer patients²³¹⁻²³⁴. CD9 is a common tetraspanin marker for EVs²³¹. γ H2AX is a histone phosphorylation marker for replication stress that indicates high rates of proliferation, which may indicate tumor growth²³⁵. Fibrinogen is present in the blood and is often upregulated in cancer patients²³⁶. Five proteins (PGK1, FASN, CLDN3, CD9, and γ H2AX) are positively expressed in LNCaP cells; one protein (Fibrinogen) is not expressed in LNCaP cells. Antibodies were conjugated to either AlexaFluor 488 (AF488) or AlexaFluor 647 (AF647) dyes to minimize the impact of

spectral overlap on quantification. Cell and prostate tissue staining experiments validated the selection of antibodies against each target protein (**Figure 3.5, Figure 3.6**), which was confirmed using images published by the Human Protein Atlas.

Table 3.1. Panel of target proteins for cyc-DEP assay of LNCaP-derived NPs. Six target proteins were selected to demonstrate three rounds of on-chip cycIF: phosphoglycerate kinase 1 (PGK1), γ H2A histone family member X (γ H2AX), the tetraspanin protein CD9, fatty acid synthase (FASN), fibrinogen, and Claudin 3 (CLDN3). Antibodies against each target protein were conjugated to either Alexa Fluor (AF) 488 or AF647 dyes. Each row (P1-P3) shows an AF488 and AF647 tagged antibody pair.

Target Pair (P)	Alexa Fluor 488	Alexa Fluor 647
P1	PGK1	γ H2AX
P2	CD9	FASN
P3	Fibrinogen	CLDN3

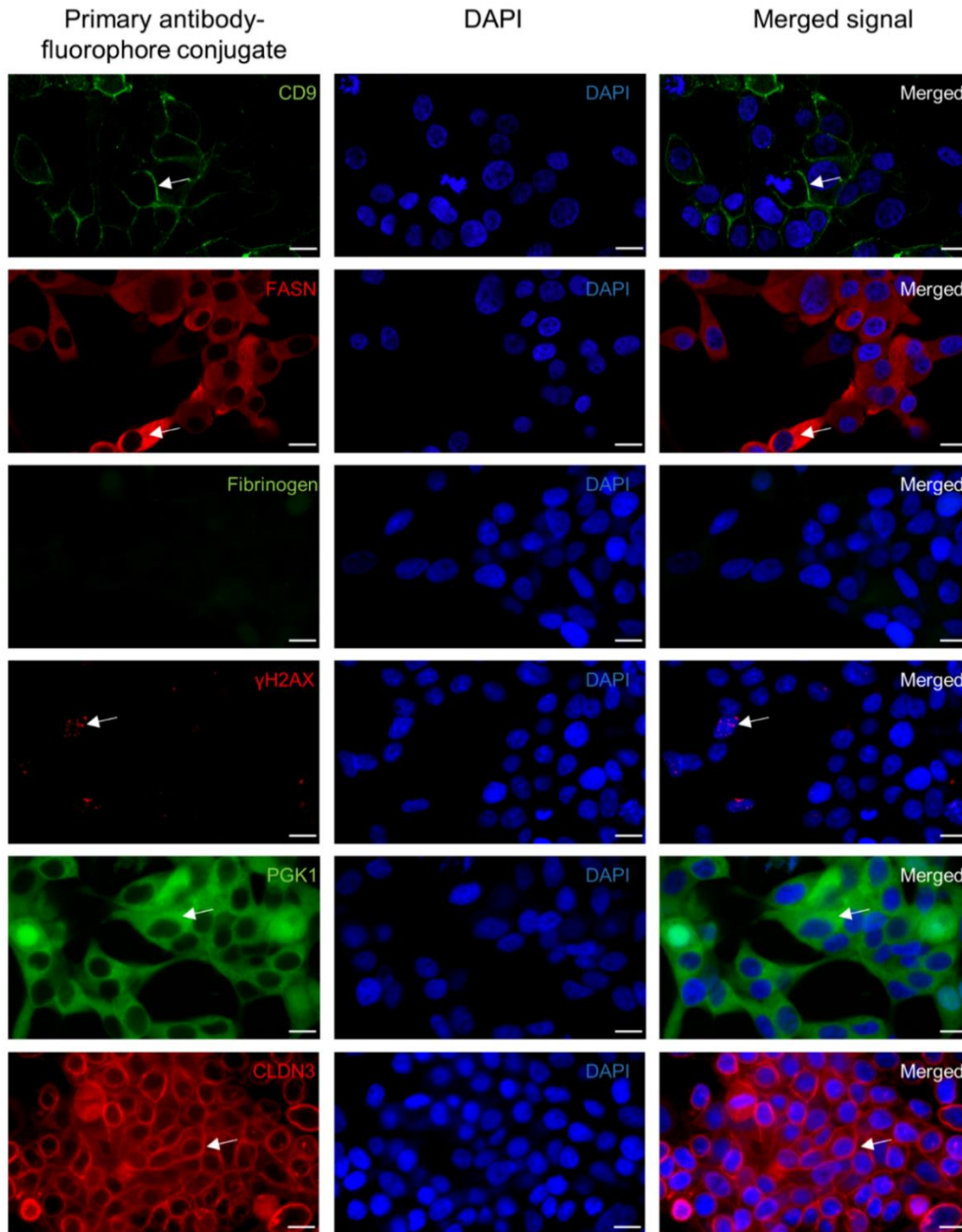


Figure 3.5. Validation of fluorophore conjugated primary antibodies on LNCaP cells. LNCaP cells were fixed, permeabilized, incubated with 1:100 of each antibody, and imaged with a confocal microscope. Fluorescent signal from primary antibody is shown in first column, nuclear DAPI stain in second column and merged image in third column. Arrows point to accurate cellular locations of specific fluorescent signal acquired from each antibody staining. Antibodies conjugated to AF488 and AF647 are shown in green and red respectively, DAPI signal is shown in blue. CD9 stains cell membrane, FASN is found in cytoplasm, fibrinogen is not expressed by LNCaP cells, γH2AX is visualized as nuclear puncta, PGK1 stains the cytoplasm, and CLDN3 stain is localized to the membrane (scale bars are 20 μm).

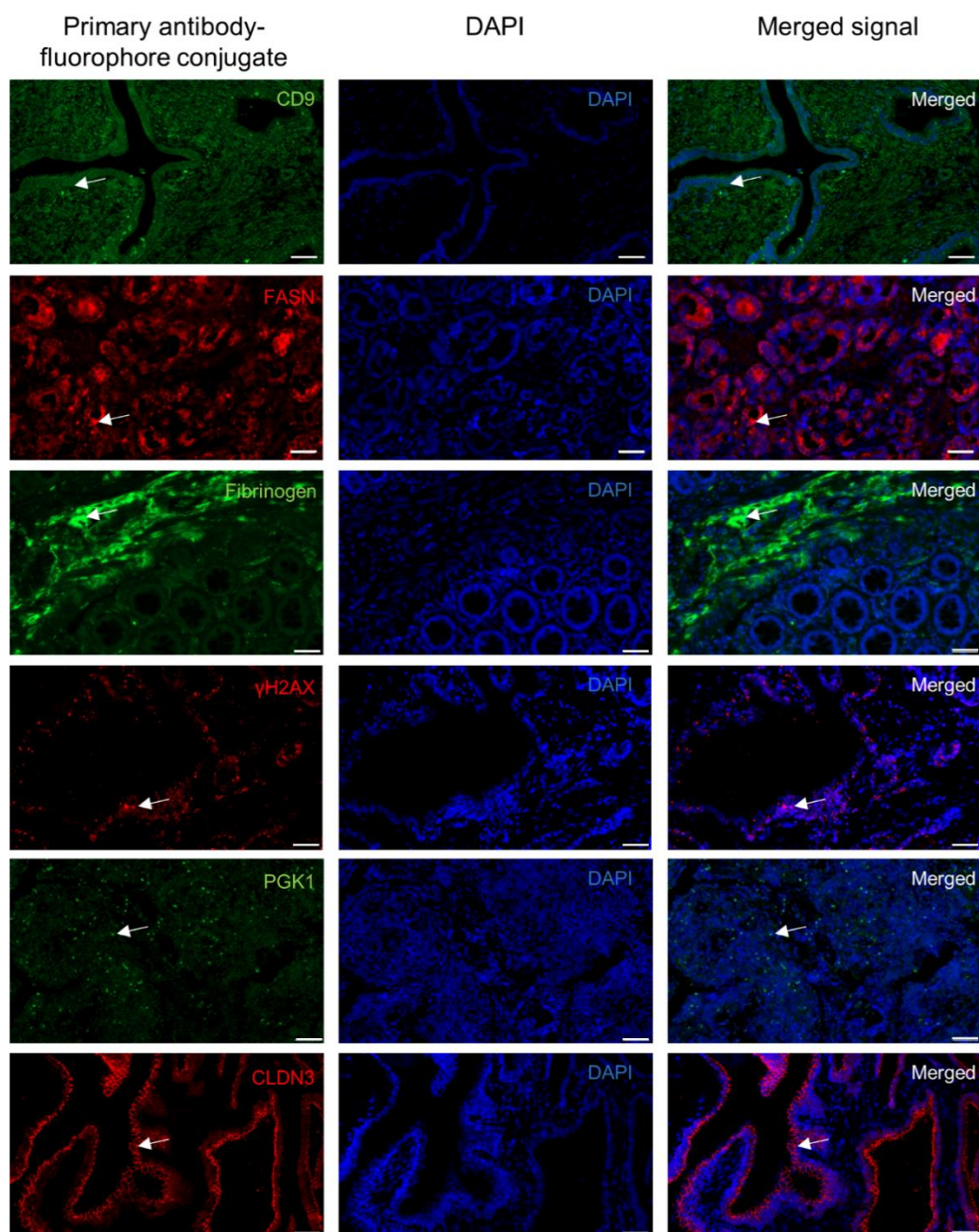


Figure 3.6. Validation of fluorophore-conjugated primary antibodies on FFPE tissues. FFPE tissue sections from tissues with known presence of each antibody target were stained and imaged. Fluorescent signal from primary antibody is shown in first column, nuclear DAPI stain in second column and merged image in third column. Antibodies conjugated to AlexaFluor488 and AlexaFluor647 are shown in green and red respectively, DAPI signal is shown in blue. Arrows point to specific fluorescent signal acquired from each antibody staining accurate cellular locations, as follows; cell membrane signal on prostate gland epithelial cells for CD9, prostate gland epithelial cell cytoplasm for FASN, colon tissue extracellular matrix for fibrinogen, prostate gland nuclei for γ H2AX, prostate tissue cell nuclei and cytoplasm for PGK1 and prostate gland epithelial cell membrane for CLDN3 stain (scale bars 50 μ m).

3.4.3 *Synthesis and structural characterization of NPs derived from LNCaP cells*

NPs were synthesized *via* probe sonication of LNCaP cells suspended in buffer (**Figure 3.3**). Approximately 5×10^{10} NPs/mL were formed from roughly 1×10^6 cells/mL (suspension volume of 1.5 mL). LNCaP-derived NPs exhibited two characteristics that were important to the development of cyc-DEP. Any target in the panel that was detected in LNCaP cells should be detectable in NPs derived exclusively from LNCaP cells; the inverse is also true for negative targets. Therefore, cell staining (**Figure 3.5**) cross-validated the fluorescent patterns of LNCaP-derived NPs that were collected and immunolabeled on the DEP chip through cyc-DEP (**Figure 3.7**). By including both positive and negative targets in the protein panel, specific identification of target proteins using cyc-DEP was confirmed. LNCaP-derived NPs respond to DEP under the same electrical parameters typically used to isolate EVs and other endogenous NPs from plasma. Therefore, it follows that a successful method for cyc-DEP of NPs is directly translatable to liquid biopsy samples.

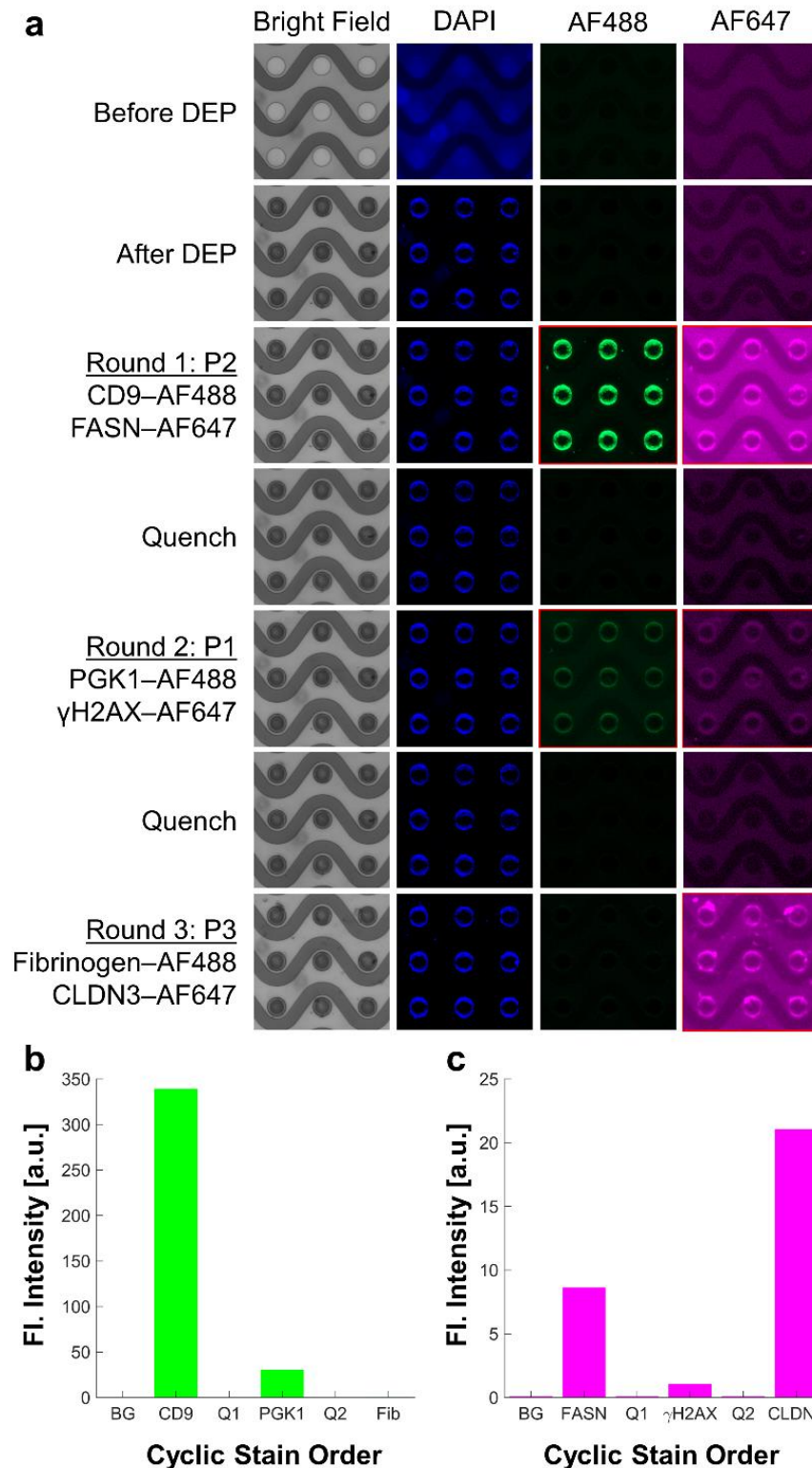


Figure 3.7. Cyc-DEP of target proteins in LNCaP-derived NPs. (a) NPs formed from LNCaP cells and the internal standard were collected simultaneously *via* DEP. Fluorescent polystyrene NPs were used as the internal standard and were visualized through the DAPI fluorescence filter. Three rounds of cycIF staining and two rounds of fluorescence quenching were performed on the collected material. Bright field images showed nine electrodes (each 60 μ m diameter) in the planar DEP microarray; all images showed the same field-of-view. DAPI channel images revealed collection of the internal standard. AF488 and AF647 channel images showed alternating rounds of successful immunofluorescent labeling and quenching. Five target proteins (CD9, FASN, PGK1, γ H2AX, and CLDN3) were detected over three rounds of cycIF. All images acquired through a given filter (e.g. DAPI, AF488, AF647) were contrast-enhanced using the same parameters. Fluorescence

quantification was performed on fluorescence images without contrast-enhancement. (b) Fluorescence levels of labeled proteins (CD9, PGK1, and Fibrinogen) associated with LNCaP-derived NPs were quantified from images in the AF488 channel.

Fluorescence levels of BG before the first round of immunolabeling were below the lower limit of detection. Fibrinogen was undetectable, CD9 and PGK1 were detected after respective rounds of immunolabeling. Fluorescence levels of CD9 in Round 1 and PGK1 in Round 2 were quenched to below the lower limit of detection. (c) Fluorescence levels of labeled proteins (FASN, γ H2AX, and CLDN3) associated with LNCaP-derived NPs were quantified from images in the AF647 channel. Fluorescence levels of BG before the first round of immunolabeling were below the lower limit of detection. Each target was detected after respective rounds of immunolabeling. Fluorescence levels of FASN in Round 1 and γ H2AX in Round 2 were quenched to below the lower limit of detection.

Figure 3.7 shows fluorescence images of the cyc-DEP method performed on LNCaP-derived NPs using the protein panel in **Table 3.1**. Prior to the application of DEP, no fluorescence was observed. After DEP, fluorescence in the DAPI channel indicated collection of the internal standard, confirming that the DEP chip was functional. CD9 and FASN were immunolabeled and detected in the first round of staining; fluorescence signal was subsequently quenched. PGK1 and γ H2AX were immunolabeled and detected in the second round prior to quenching. While fibrinogen and CLDN3 were immunolabeled in the third round, only CLDN3 was detected. All five proteins that were expressed in LNCaP cells (**Figure 3.5**) were also detected on LNCaP-derived NPs (**Figure 3.7**); one negative target was also undetectable on-chip. Quantification of image data indicated that quenching reduced fluorescence levels by more than 90% in all events (**Figure 3.7**). Furthermore, quenching reduced fluorescence to below the limit of detection of the quantification software as described in Chapter 2. This quenching method was effective for both AF488 and AF647.

3.4.4 Optimization of round order based on fluorescence levels

Based on findings from previous cycIF studies, it was expected that fluorescence levels of some targets would vary based on the order in which those targets were immunolabeled²¹⁵. To test whether round order would affect on-chip fluorescence levels, six unique cyc-DEP assays were performed on LNCaP-derived NPs in which the stain order of antibody pairs were altered. The image data shown in **Figure 3.7** represented one of six possible permutations of antibody pairs across three rounds of cycIF: P1-P2-P3, P1-P3-P2, **P2-P1-P3**, P2-P3-P1, P3-P1-P2, and P3-P2-P1. Fluorescence data from all six cyc-DEP assays were represented as a heatmap in **Figure 3.8**. All fluorescence levels were adjusted for the collection efficiencies of respective DEP microarrays using the internal standard. The concentration of LNCaP-derived NPs was constant across all experiments.

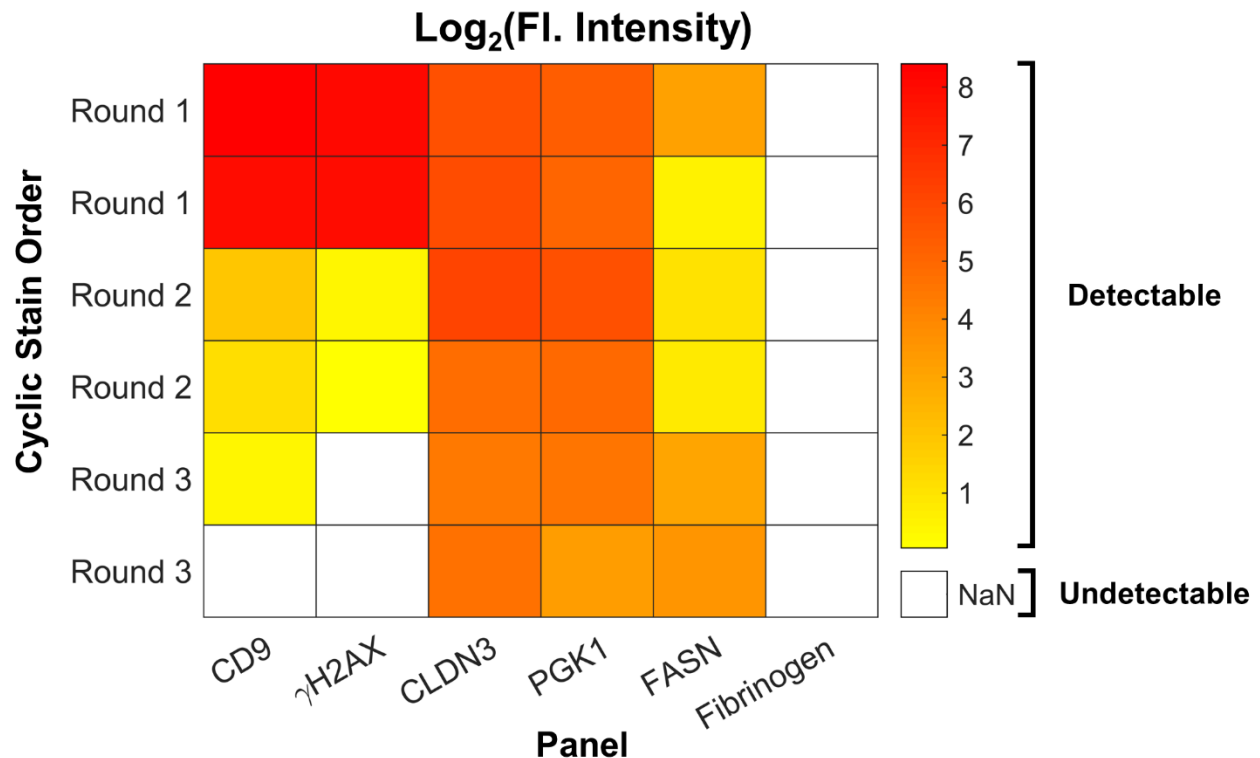


Figure 3.8. Stain order affected signal intensity levels in cyc-DEP. Heatmap of the base-2 logarithm of fluorescence intensity of each protein in the panel for each different round in which they were used. Two replicates were used for each protein – round order pairing. Heatmap shows that the order in which proteins were immunolabeled affected their measured values. Values ranging from yellow to red were above the lower limit of detection; values in white were undetectable. Three pairs of target proteins were ordered into six permutations (e.g. P1-P2-P3, P3-P1-P2, etc.). Three separate cyc-DEP experiments were performed to test each permutation. The data inform optimal pairing and ordering of targets for on-chip cyclIF staining of LNCaP-derived NPs.

Data indicated that CD9 and γH2AX were most impacted by round order. CD9 and γH2AX stained brightest when labeled in the first round, showed substantial reductions in intensity in the second round, and were either undetectable or barely detectable by the third round. CLDN3 and PGK1 were detectable in all rounds and predominantly unaffected by stain order; however, slight overall decreases in fluorescence intensity were observed in later rounds. Interestingly, FASN levels

appeared to be highest in the third round. Fibrinogen was undetectable regardless of stain order, as anticipated.

Based on the information in **Figure 3.8**, antibody pairs and the order in which those pairs stained the sample were restructured to optimize the detection of target proteins (**Table 3.2**). CD9 and γ H2AX were paired in the first round of cyc-DEP. FASN, which yielded the highest signal in the third round, and fibrinogen, which was undetectable in all rounds, were paired and immunolabeled in the third round. The second round consisted of CLDN3 and PGK1, both of which were only mildly impacted by round order. Similar to what is done with tissue cycIF experiments, it is recommended that research groups identify the antibodies in their panels that are most impacted by round order and optimize cyc-DEP assays accordingly.

Table 3.2. Optimized antibody-round order pairings for cyc-DEP. Signal intensity values measured by the cyc-DEP assay were partially dependent on the order in which each target was immunolabeled. Antibody pairings and round orders were optimized according to the data obtained from round order shuffling experiment shown in **Figure 3.8**. This optimized panel was used in further cyc-DEP experiments.

Round Order (R) / Target Pair (P)	Alexa Fluor 488	Alexa Fluor 647
R1, P1	CD9	γ H2AX
R2, P2	PGK1	CLDN3
R3, P3	Fibrinogen	FASN

Averaged across all permutations of round order shown in the heatmap, fluorescence quenching was 98 $\pm$ 3 % effective. Quenching in the AF488 channel was 99 $\pm$ 2 % effective (n = 8 quenching events). Quenching in the AF647 channel was 98

+/- 3 % effective (n = 11 quenching events). There was no significant difference between quenching in the AF488 channel and the AF647 channel (p = 0.31; two-tailed t-test assuming equal variance). Fluorescence intensities that were below the limit of detection prior to quenching were not included in calculations of quenching efficiency. All fluorescence intensities after quenching were below the limit of detection and more than 90% effective.

3.4.5 Cyc-DEP as a multiplex imaging platform for patient plasma samples

To demonstrate the clinical application of cyc-DEP, I analyzed plasma samples from three healthy individuals and six prostate cancer patients (n = 3 of Gleason Pattern 3+3; n = 3 of Gleason Pattern 4+4). I used the optimized antibody panel in **Table 3.2** to profile the presence of six biomarkers from twenty-five microliters of patient plasma. The heatmap shown in **Figure 3.9** summarizes the protein profiles of each plasma sample. CD9, which is a common EV marker, was detected in all samples as expected. Fibrinogen, which is generally present in blood plasma, was detected in the majority of patients regardless of disease state. Fibrinogen is involved in angiogenesis and tumor metastasis²³⁶, which might account for its elevated presence in certain subsets of Gleason 3+3 and Gleason 4+4 patients. CLDN3 showed high signal intensity in some prostate cancer patients relative to healthy individuals, which was in accordance with previous findings^{231, 232}. γ H2AX, a histone marker, and FASN, an enzyme involved in fatty acid metabolism²³⁷, were both undetected in all but one patient. An enzyme known to be involved in aerobic glycolysis²³⁸ and present in EVs from some prostate cancer

cell lines²³⁹, PGK1, was undetectable across all patients. All fluorescence intensities reported in **Figure 3.9** were adjusted for the collection efficiencies of respective DEP microarrays using the internal standard.

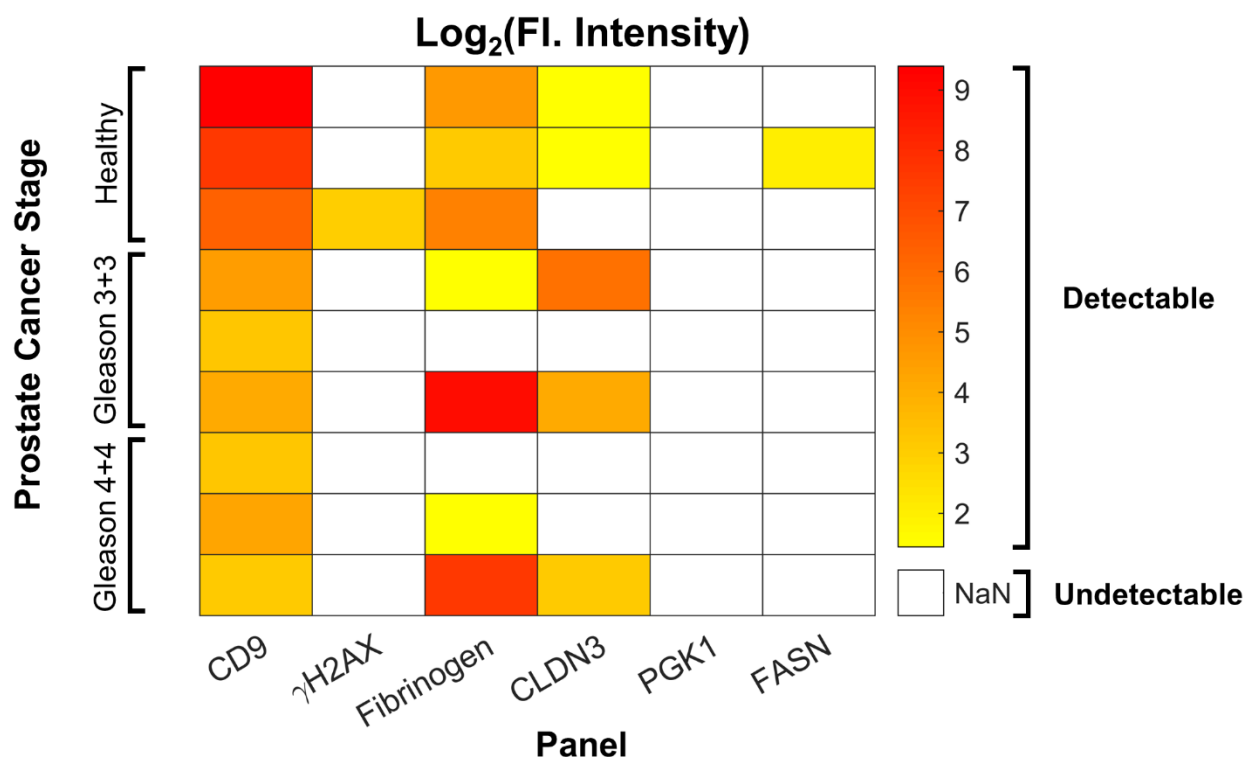


Figure 3.9. Cyc-DEP with plasma samples. A three round cyc-DEP assay using antibody pairings and round orders from **Table 3.2** was conducted on twenty-five microliters of plasma from healthy individuals ($n = 3$), patients with Gleason 3+3 scores ($n = 3$), and patients with Gleason 4+4 scores ($n = 3$). Signal intensity values from each target are quantified and base-2 logarithm transformed. Detectable fluorescent levels are depicted in the yellow-red color scale. Non-detectable (NaN) values are shown in white. PGK1 was undetectable across all patient cohorts.

It is important to note that this panel was selected based on the proteomic profile of LNCaP cells. It was not known whether the selected panel would be relevant for liquid biopsy analysis using DEP because plasma-derived, DEP-responsive NPs had not been profiled for those markers. This was partially due to an incomplete

characterization of endogenous NPs that respond to the particular AC signal used to generate electrokinetic forces like DEP. While previous work demonstrated collection of high MW cfDNA²²⁴, EVs¹³², and a variety of synthetic NPs¹⁵⁹ (e.g. polymeric, liposomal, etc.), the full scope of DEP-responsive endogenous NPs present in blood plasma has yet to be characterized. Furthermore, it is not currently known which circulating NPs, if any, contain detectable amounts of proteins from the panel. While some of these markers are known to be present in circulation (e.g. CD9 and Fibrinogen), none are clinically accepted as prognostic indicators of prostate cancer. Furthermore, there is an insufficient number of patients in this study to conclude meaningful differences in proteomic profiles within and across cohorts. As such, sufficient information was lacking to indicate that the panel selected for method development with LNCaP-derived NPs would provide clinically meaningful proteomic information from patient samples. Therefore, the data in **Figure 3.9** must be contextualized in terms of technological assay development.

Cyc-DEP enabled screening of patient plasma samples for the presence of six proteins. Across all patient samples, five of the six proteins were detected, and four proteins were detected in a single patient. Cyc-DEP increased the screening capacity of DEP assays threefold from two targets to six. Cyc-DEP also doubled the maximum number of targets identified per patient sample from two to four. Notably, cyc-DEP was directly translatable from LNCaP-derived NPs suspended in buffer to endogenous NPs isolated directly from blood plasma without altering experimental parameters. Modifications to the current cyc-DEP platform could further increase the multiplicity of on-chip detection. Future work will focus on generating disease-specific panels by

surveying clinically relevant proteins using cyc-DEP. Further characterization of DEP-responsive, endogenous NPs present in circulation will help inform future panel selections. Additional fluorophores and fluorescence filters will also be utilized to increase the number of targets per round.

While the data in **Figure 3.9** cannot indicate statistically significant differences in biomarker levels across clinical subgroups, the data serve as an important proof-of-concept for the potential clinical relevance of cyc-DEP for the highly multiplexed proteomic profiling of liquid biopsy samples. DEP has previously been shown as a successful diagnostic tool for several diseases, including cancer and traumatic brain injury^{172, 222-225}. Cyc-DEP improved the efficiency of on-chip immunofluorescence detection by increasing the number of biomarkers that can be detected on the DEP chip by at least three-fold. Cancer is a complex disease that follows a unique trajectory for each patient. Any liquid biopsy assay that has the potential to be successfully implemented to the clinic must measure activity along several biological pathways in order to best characterize disease state. High parameter capability is also a crucial feature for any liquid biopsy assay that can provide diagnostic or prognostic information despite patient-to-patient variation. This chapter summarizes the development of cyc-DEP, a low-volume, multiplexed assay for liquid biopsy that is geared towards early cancer detection. Furthermore, cyc-DEP has tremendous potential in research settings as a cost-effective sample-to-answer platform that is based on open-source software designed to standardize signal intensity measurements.

3.4.6 Overview of oligo cyc-DEP

Despite its versatility, the cyc-DEP platform exhibited three characteristics that would limit its clinical translation. The first limitation was the amount of time required to quench fluorescence levels. A second setback was the need for successive antibody incubations with each round of imaging. The third restraint was the degree of variability in fluorescence levels depending on when antibodies were introduced to the sample throughout the experiment, potentially due to limited epitope access, sample degradation, and sample loss. Here, oligo cyc-DEP is presented for the multiplex proteomic profiling of liquid biopsy biomarkers using oligo barcodes. Oligo cyc-DEP eliminates the need for multiple antibody incubation periods and avoids long quenching protocols that can degrade samples. The primary benefit of oligo cyc-DEP is that it enables multiple rounds of imaging in a matter of hours rather than days.

Figure 3.2 demonstrates the concept of oligo cyc-DEP using a single target protein. Endogenous NPs were collected from twenty-five microliters of plasma using DEP and immunolabeled with oligo-conjugated anti-fibrinogen antibodies (antibody-DS conjugates). Complementary IS-fluorophore conjugates were introduced into the fluidic chamber, binding the DSs within minutes. Excess ISs were washed away, and the sample was imaged. DS-IS pairs dehybridized within minutes after adding EC to the sample, leaving the sample and its bound antibody-DS conjugates intact. Excess EC was washed away before imaging. Once more, complementary IS-fluorophores were added to the sample. Fluorescent signal was recovered after washing, demonstrating that antibody-DS conjugates remained bound after selective DS-IS dehybridization.

Oligo cyc-DEP enables repeated hybridization and dehybridization of complementary DS-IS pairs through EC-mediated quenching.

3.4.7 Validation of DS-IS hybridization by immunofluorescent staining of LNCaP cells

Three DS-IS pairs were designed (DS6-IS6, DS7-IS7, DS8-IS8). Each DS was conjugated to a primary antibody against one of three target proteins (FASN, CD9, PGK1), yielding anti-FASN-DS6, anti-CD9-DS7, and anti-PGK1-DS8. Antibody clones used in oligo cyc-DEP were the same as those used in the cyc-DEP platform presented in this chapter. To validate hybridization of DS-IS pairs, LNCaP cells were first fixed, blocked, and immunolabeled with each DS-conjugated antibody. Complementary ISs were then added to respective DSs, and excess ISs were removed with washing. CD9 was observed at the cellular membrane, while FASN and PGK1 stained the cytoplasm. Cell staining patterns in **Figure 3.10** exactly matched patterns in **Figure 3.5** that corresponded to FASN, CD9, and PGK1, respectively. Cell staining in **Figure 3.10** therefore confirmed hybridization of DS-IS pairs.

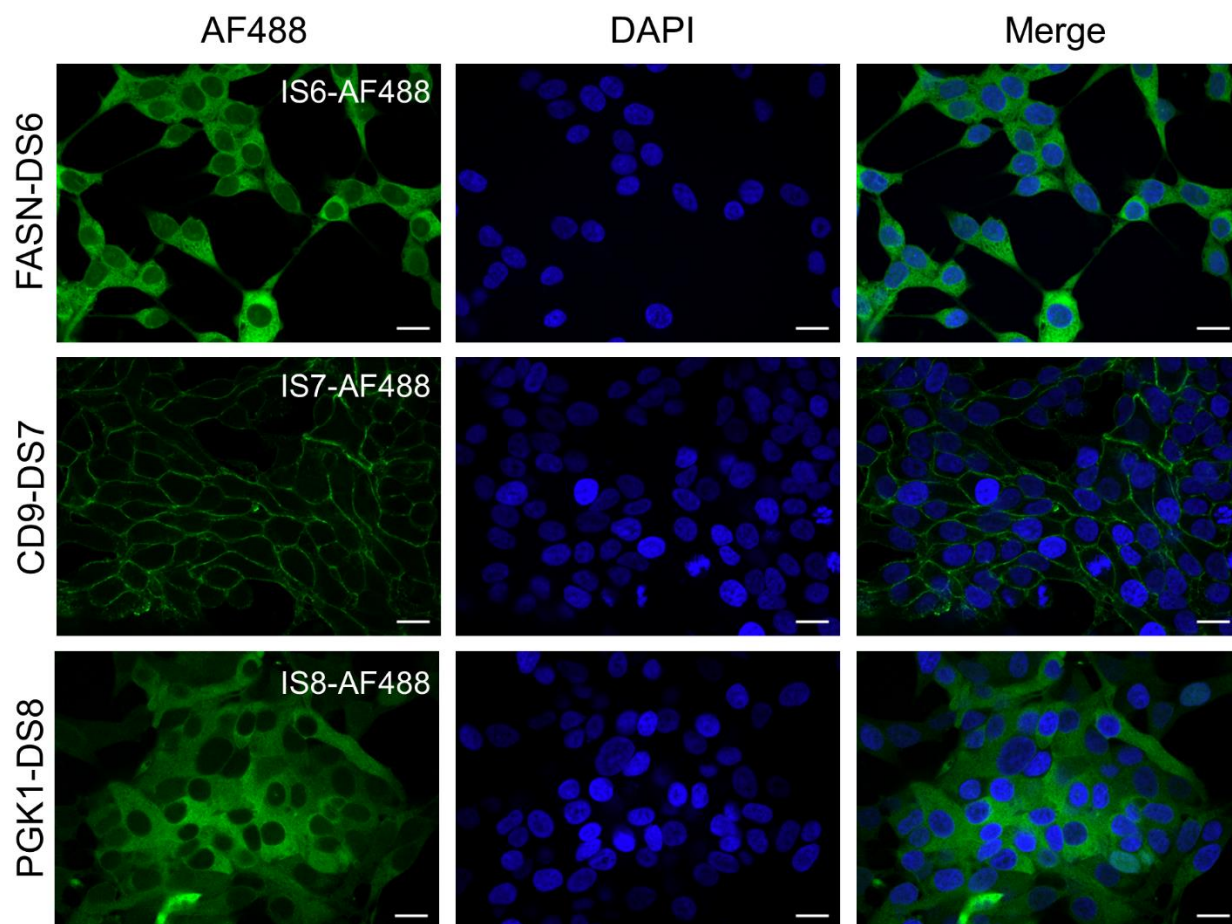


Figure 3.10. Hybridization of oligo-fluorophores with bound complementary antibody-oligos. LNCaP cells were fixed, permeabilized, and incubated with primary antibody-oligo (DS) conjugates in blocking buffer. Complementary oligo-fluorophores (ISs) were hybridized for ten minutes and washed prior to confocal imaging. Staining patterns from DS-IS hybridization matched patterns from primary-fluorophore antibodies. All images were acquired in AF488 and DAPI channels. All scale bars are 20 μm .

3.4.8 Four rounds of oligo cyc-DEP were performed on cell-derived NPs

Since the antibody staining patterns for oligo cyc-DEP matched those for cyc-DEP and each target (FASN, CD9, and PGK1) was detectable in LNCaP cell-derived NPs using cyc-DEP, each target protein should be detectable in cell-derived NPs using

oligo cyc-DEP. Cell-derived NPs were collected using DEP, labeled simultaneously with all three antibody-DS conjugates, and fluorescently stained across four rounds of on-chip cycIF using complementary IS-fluorophores (**Figure 3.11**). In the first round, IS8-AF488 hybridized DS8 to stain PGK1. EC-mediated removal of IS8-AF488 was 99.3% effective. The second round of staining involved hybridization of IS6-AF488 to anti-FASN-DS6, and the subsequent dehybridization of DS6-IS6 was 99.7% effective. IS7-AF488 was introduced in the third round of staining to stain CD9, and downstream EC-mediated removal was 91.7% effective. In the fourth and final round of staining, all three ISs were added simultaneously and removed with 99.5% quenching efficiency. After the first three rounds of staining, EC-mediated dehybridization quenched fluorescence below the lower limit of detection as established in Chapter 2.

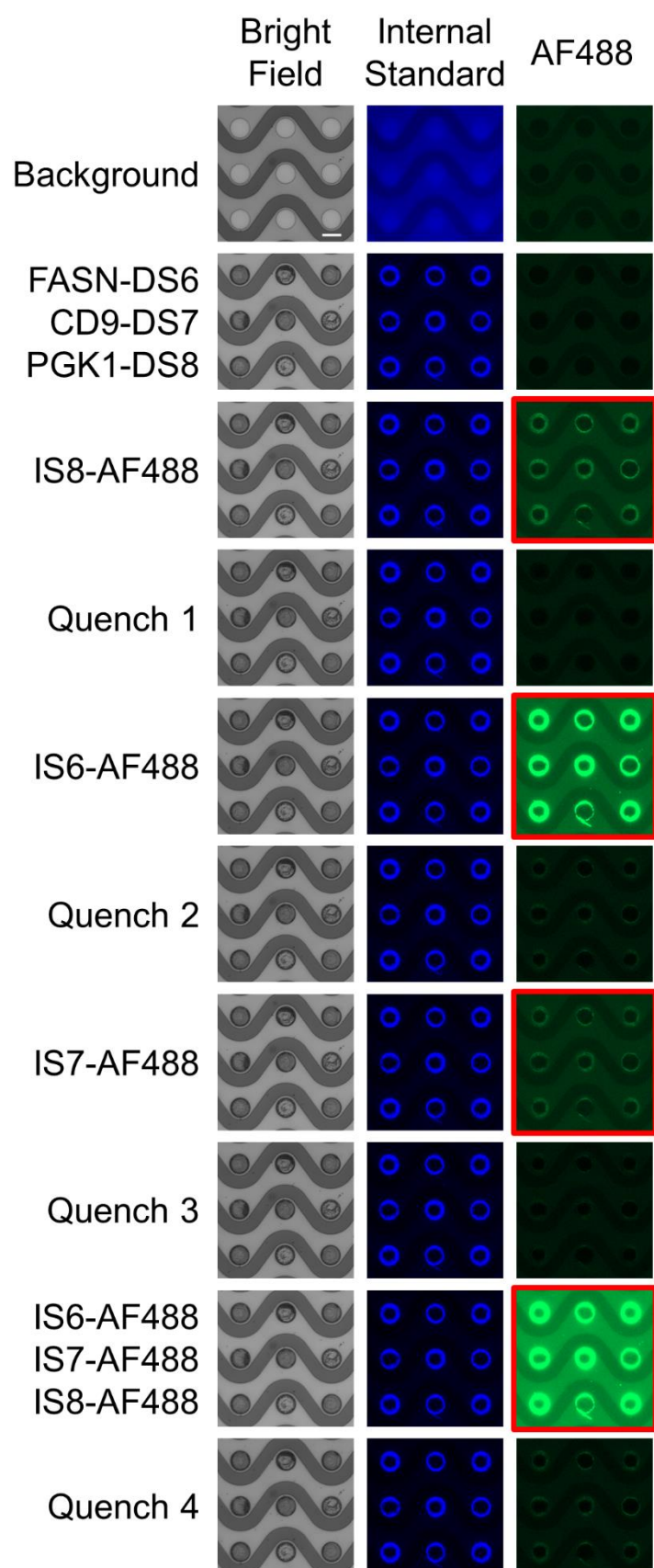


Figure 3.11. Four rounds of oligo cyc-DEP against FASN, CD9, and PGK1. LNCap cell-derived NPs and the internal standard NPs were collected simultaneously from buffer using DEP. Collected material was immunolabeled with anti-FASN-DS6, anti-CD9-DS7, and anti-PGK1-DS8. Imager strands (IS8-, IS6-, IS7-AF488) were introduced and removed one at a time in the first three rounds of hybridization and dehybridization. Fluorescence signal was detected after hybridization with all three imager strands. The sample was then incubated with all three imager strands simultaneously, and fluorescence was detected. All fluorescent images were quantified using the algorithm presented in Chapter 2. Fluorescence values in the AF488 channel were adjusted for the collection efficiency of the particular DEP microarray. All dehybridization quenching events were at least 91% effective (97.6% $\pm$ 3.9%; $n = 4$). Scale bar is 60 μm .

Similar experiments were performed with slight variations in **Figure 3.12** and **Figure 3.13**. In the experiment corresponding to **Figure 3.12**, cell-derived NPs were labeled with anti-FASN-DS6 and anti-CD9-DS7 prior to four rounds of staining. Fluorescence was detected after staining with IS6, IS7, and the combination of IS6, IS7, and IS8. Importantly, no fluorescence was detected after staining with IS8, which indicated no nonspecific interactions with DS6, DS7, unlabeled regions of the collected material, or the chip. Furthermore, IS8 did not interfere with either IS6-DS6 or IS7-DS7 hybridization. EC-mediated dehybridization yielded quenching efficiencies of 99.2% (Quench 2), 95.3% (Quench 3), and 98.6% (Quench 4); however, only Quench 3 reduced fluorescence below the lower limit of detection.

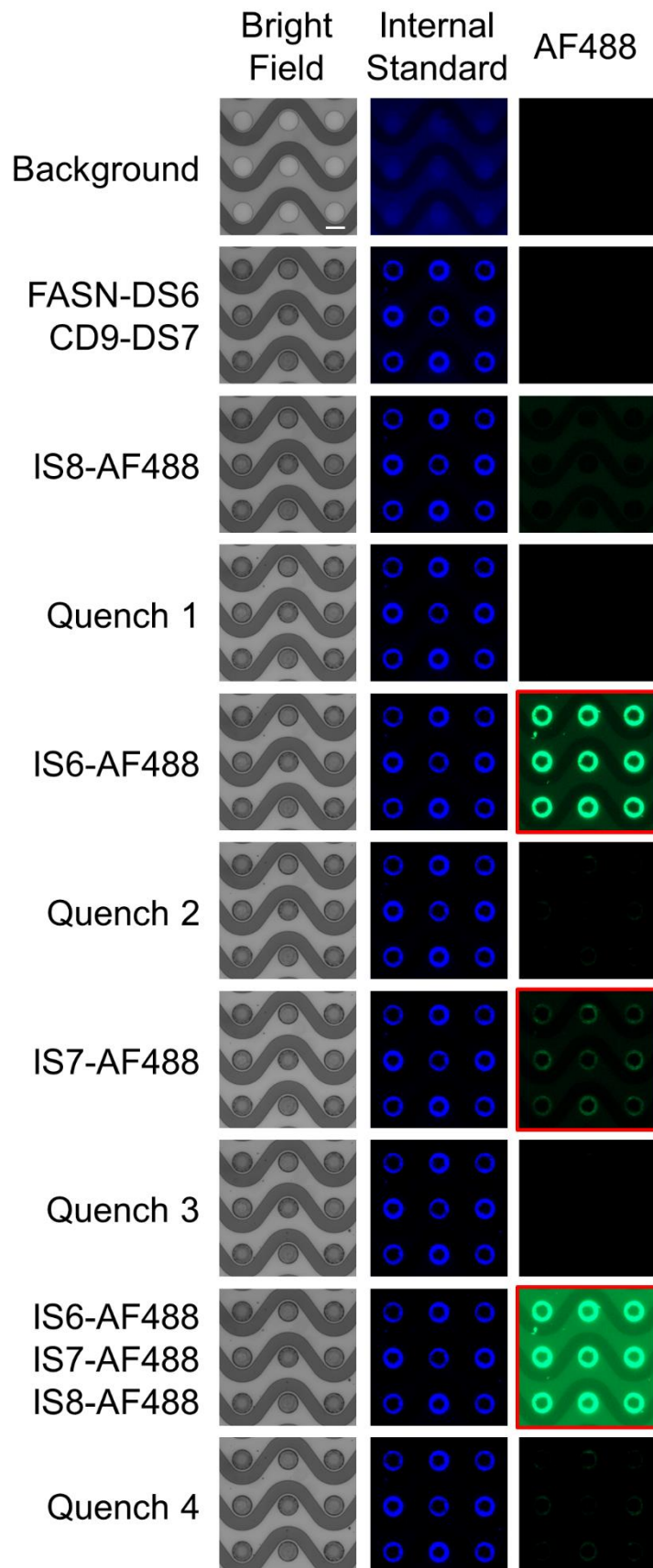


Figure 3.12. Four rounds of oligo cyc-DEP against FASN and CD9. LNCap cell-derived NPs and the internal standard NPs were collected simultaneously from buffer using DEP. Collected material was immunolabeled with anti-FASN-DS6 and anti-CD9-DS7. Imager strands (IS8-, IS6-, IS7-AF488) were introduced and removed one at a time in the first three rounds of hybridization and dehybridization. Fluorescence signal was detected after hybridization with IS6-AF488 and IS7-AF488. No signal was detected after incubation with IS8-AF488. The sample was then incubated with all three imager strands, and fluorescence was detected. All fluorescent images were quantified using the algorithm presented in Chapter 2. Fluorescence values in the AF488 channel were adjusted for the collection efficiency of the particular DEP microarray. All dehybridization quenching events were at least 95% effective (97.7% $\pm$ 2.1%; $n = 3$). Scale bar is 60 μ m.

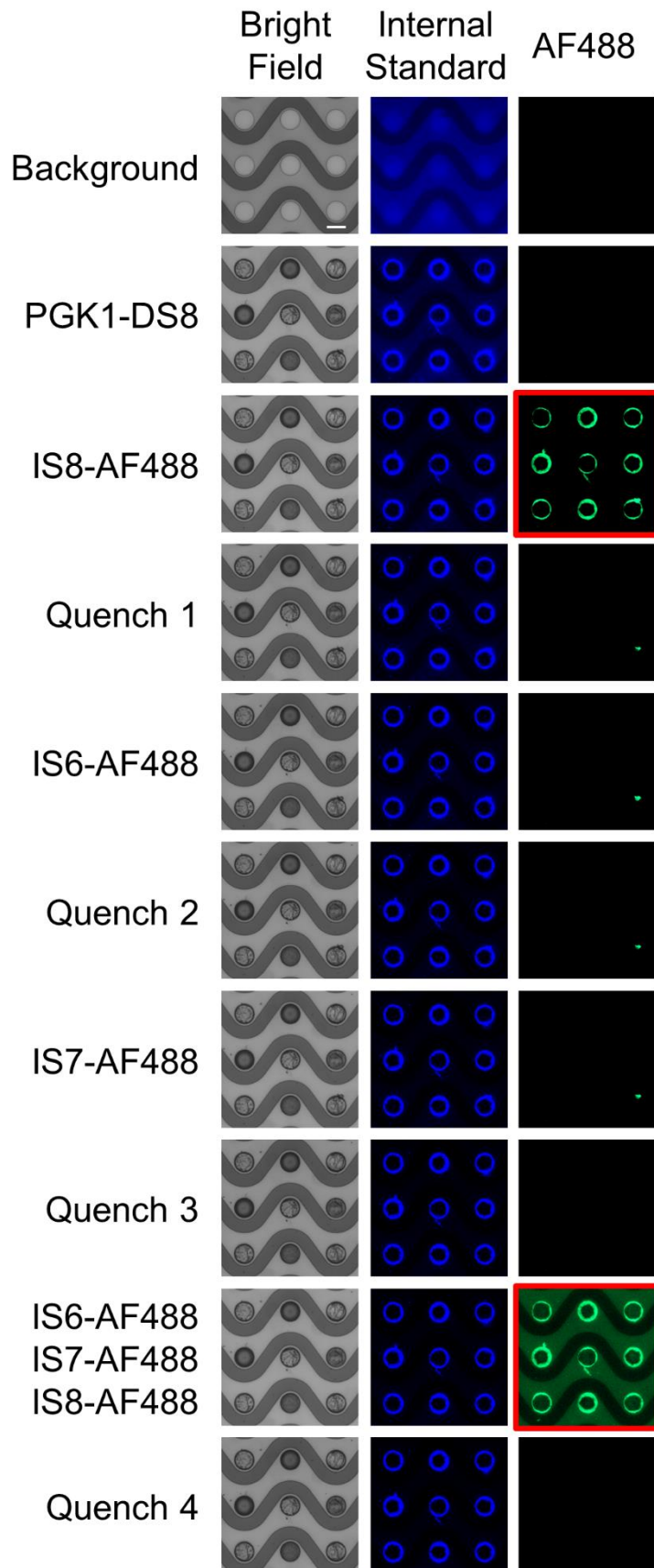


Figure 3.13. Four rounds of oligo cys-DEP against PGK1. LNCap cell-derived NPs and the internal standard NPs were collected simultaneously from buffer using DEP. Collected material was immunolabeled with anti-PGK1-DS8. Imager strands (IS8-, IS6-, IS7-AF488) were introduced and removed one at a time in the first three rounds of hybridization and dehybridization. Fluorescence signal was detected after hybridization with IS8-AF488. No signal was detected after incubation with IS6-AF488 and IS7-AF488. The sample was then incubated with all three imager strands, and fluorescence was detected. All fluorescent images were quantified using the algorithm presented in Chapter 2. Fluorescence values in the AF488 channel were adjusted for the collection efficiency of the particular DEP microarray. All dehybridization quenching events were at least 99% effective (99.8% $\pm$ 0.1%; $n = 2$). Scale bar is 60 μ m.

In **Figure 3.13**, cell-derived NPs were labeled with anti-PGK1-DS8, and fluorescence was detected only after staining with IS8 and with the combination of IS6, IS7, and IS8. Importantly, IS6 and IS7 did not interact with DS8, nor did IS6 or IS7 appear to interfere with DS8-IS8 hybridization. Additionally, IS6 and IS7 did not nonspecifically bind to material inside the microfluidic chamber, and DS8-IS8 binding appeared to be specific. Dehybridization led to quenching efficiencies of 99.8% (Quench 1) and 99.7% (Quench 4). Both instances of dehybridization reduced fluorescence below the lower limit of detection.

3.4.9 EC-mediated dehybridization of DS-IS pairs for complete fluorescence quenching

Quantitative values for all images in the AF488 columns of **Figures 3.11 – 3.13** are included in the heatmap shown in **Figure 3.14**. All fluorescence intensities were quantified using the custom image processing algorithm and adjusted for collection efficiency using the internal standard. **Figure 3.14** shows the base-two logarithm of fluorescence intensity values above the lower limit of detection (“Detectable”). Values below the lower limit of detection were not shown in the heatmap (“Undetectable”); however, “undetectable” values were nonzero and therefore used to calculate quenching efficiency of EC-mediated dehybridization. Across all dehybridization events shown in the heatmap, the mean quenching efficiency was 98.1% +/- 2.8% (n = 9).

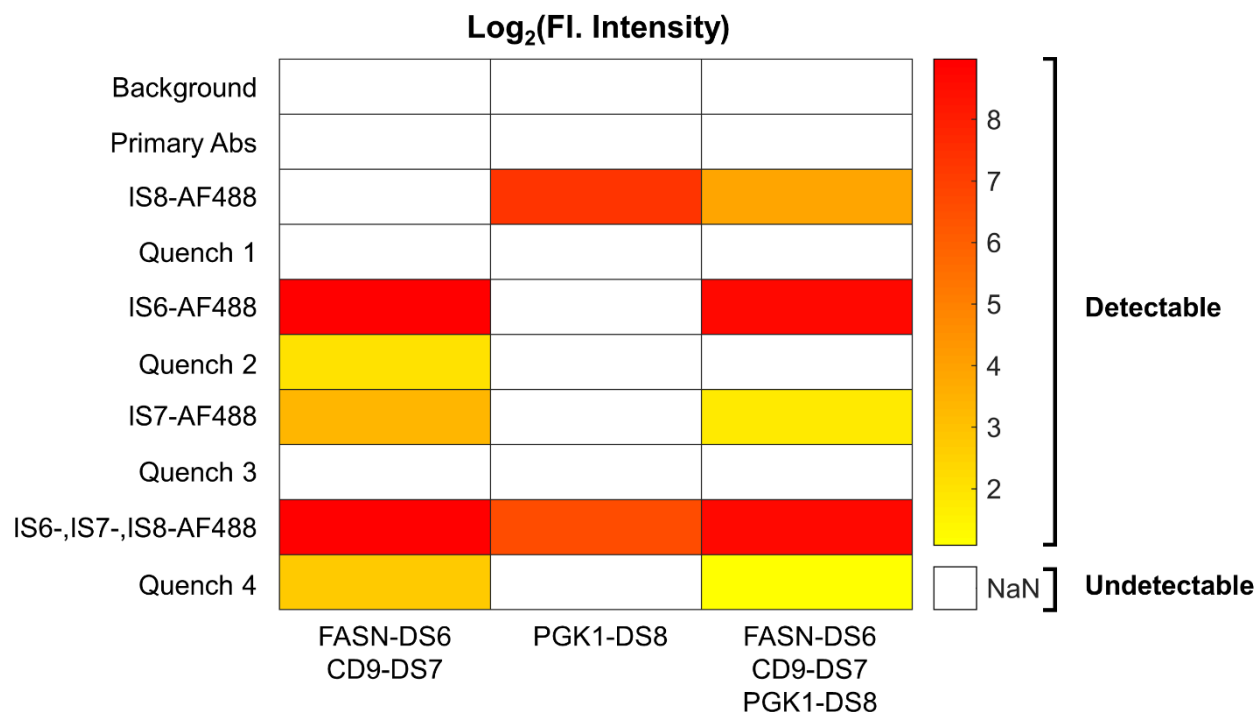


Figure 3.14. Heatmap showing four rounds of oligo cyc-DEP on cell-derived NPs. LNCaP cell-derived NPs were collected in each of three microfluidic chambers on a DEP chip. NPs in the first chamber (left column) were immunolabeled with FASN-DS6 and CD9-DS7. In the second chamber (middle column), NPs were stained with PGK1-DS8, and NPs in the third chamber (right column) were immunolabeled with all three antibody-oligos (FASN-DS6, CD9-DS7, and PGK1-DS8). Fluorescence intensity values were quantified using the custom image-processing algorithm presented in Chapter 2. All values were adjusted for differences in collection efficiency as measured by the internal standard (Chapter 2) and were plotted using a base-two logarithmic scale. Rectangles in white represent fluorescence levels below the lower limit of detection. Dehybridization quenching was more than 90% effective in all instances (98.1% +/- 2.8%; n = 9).

Data presented in this chapter suggest that proteins associated with LNCaP cell-derived NPs can be labeled using oligo barcodes in a multiplex manner through complementary hybridization and EC-mediated dehybridization. While additional cross-talk validation is necessary, the specific hybridization of at least three complementary DS-IS pairs is achievable per round of staining, and at least four rounds of oligo-based

on-chip cycIF is feasible. Furthermore, oligo cyc-DEP requires less than one hour per cycle, whereas cyc-DEP requires more than six hours per cycle.

3.4.10 *Additional considerations for oligo cyc-DEP*

In oligo cyc-DEP, the number of unique oligo barcodes, rather than the number of available fluorescence channels, determined the number of interrogatable target proteins. The primary benefits of oligo barcoding are fast hybridization and dehybridization processes that require approximately ten minutes, expediting cycIF staining. Another advantage is that EC buffer did not appear to damage the sample nor affect the internal standard, perhaps because short incubation periods limited overall exposure. Successful dehybridization was achieved at room temperature exclusively through the use of EC. Importantly, dehybridization quenching for oligo cyc-DEP was exactly as effective as the combined chemical and photobleaching technique for cyc-DEP (98% +/- 3% versus 98% +/- 3%).

An important observation was made across the three experiments involving DEP collection of cell-derived NPs. The fluorescence intensity of a given target protein (e.g. PGK1-AF488) was highest when labeled exclusively with its complementary IS (e.g. IS8-AF488) rather than a combination of multiple ISs (e.g. IS6-, IS7-, and IS8-AF488) that included the complementary IS. This discrepancy could be caused by one or more factors. It is possible that the combination of ISs prompts heterodimer formation, which limits complementary DS-IS hybridization. Another possibility is tripling the total concentration of ISs increased the BG noise, which effectively reduced the fluorescence

level determined by the custom image-processing algorithm. These particular increases in BG noise can be observed in **Figures 3.11 – 3.13**. It is also possible that sample loss or degradation over the course of the experiment caused the reduction in fluorescence. Slight reductions (approximately 5% or less) in the fluorescence level of the internal standard were observed between the second row and the last row in **Figures 3.11 – 3.13**; however, these reductions might be due to imaging-induced photobleaching of the internal standard rather than sample loss and/or degradation.

In an effort to understand why levels of the three target proteins decreased when labeled simultaneously (as in **Figure 3.11**) rather than individually (as in **Figure 3.13**) or in pairs (as in **Figure 3.12**), the concentration of each antibody stock was measured. Anti-FASN-DS6 stock was approximately fifty times more concentrated than anti-FASN-DS6 and anti-CD9-DS7, suggesting that anti-FASN-DS6 could have possibly affected the binding kinetics of the other antibodies through steric hindrance. This observation highlighted the need to use antibody stocks of equal concentrations in future experimentation.

The main challenge of oligo barcoding is the need for comprehensive cross-validation studies to identify nonspecific oligo binding. Prohibitive nonspecific binding could include ISs hybridizing with non-complementary DSs, ISs hybridizing with each other, or ISs nonspecifically binding to collected material on the DEP chip or the chip itself. Unpaired DS and IS sequences with partial sequence complementarity will need to be identified through experimentation. It will also be necessary to investigate potential heterodimer formation between unique ISs that are introduced simultaneously during a

given round of staining. Future work will involve additional cross-validation experiments and expanding the oligo barcode library to include all target proteins used in cyc-DEP.

By using the same sample types, antibodies, fluorophores, and quantification techniques, direct comparisons will be made between oligo cyc-DEP and cyc-DEP. Specifically, it will be important to assess the impact of round order on fluorescence levels using oligo cyc-DEP. Considering that sample degradation is less likely to occur through short incubations with EC rather than long incubations with NaOH, round order might affect fluorescence levels to a lesser extent in oligo cyc-DEP than in cyc-DEP. Furthermore, the introduction of all antibodies in a single incubation period should reduce the likelihood of altering epitope availability during sequential rounds of immunolabeling throughout the course of the experiment. Overall, oligo cyc-DEP offers several critical advantages over cyc-DEP, most notably the reduction in sample-to-answer times. In order to further demonstrate its clinical utility, oligo cyc-DEP will be performed on biomarkers from patient plasma samples.

3.5 Conclusions

3.5.1 Utility of cyc-DEP for multiplex proteomic profiling of liquid biopsy biomarkers

This work demonstrates a unique method of multiplex profiling of biomarkers from low-volume, minimally processed liquid biopsy samples *via* on-chip cycIF imaging. The cyc-DEP method utilizes the cyclic staining of biomarkers concentrated on the DEP chip with sets of fluorescently tagged antibodies followed by a photo-bleaching step to

quench fluorescence signal and obtain multiplex information from 25 microliters of minimally processed patient sample. A panel of six target proteins was selected. Antibodies against each target were validated in LNCaP cells and prostate tissues. Staining patterns were confirmed with images published by the Human Protein Atlas²⁴⁰. NPs were synthesized *via* probe sonication of LNCaP cells suspended in buffer. Cell-derived NPs exhibited proteomic profiles that were consistent with precursor LNCaP cells, as confirmed using DEP capture and on-chip immunofluorescence staining. Measured fluorescence levels were affected by the order in which certain target proteins on NPs were immunofluorescently labeled, similar to observations in traditional cycIF. Quantitative comparisons of six cyc-DEP assays enabled optimization of round order and antibody pairing based on the selected panel and sample type. The optimized cyc-DEP assay was conducted on patient plasma samples for which four of the six target proteins were collectively detected. Cyc-DEP provides a method for increasing the number of targets that can be measured from twenty-five microliters of undiluted liquid biopsy sample by a factor of at least three.

To the best of my knowledge, the use of this particular DEP chip technology for the rapid isolation of biomarkers, including exosomal proteins, from low-volumes of plasma has previously been demonstrated for up to two proteins per assay²⁴¹. DEP chips have been successfully applied to diagnose patients with glioblastoma²²², post-concussive syndrome²²³, pancreatic and colon cancer¹⁷², and chronic lymphocytic leukemia^{224, 225}. The efficiency of on-chip biomarker profiling increased three-fold through cyc-DEP, opening the door to detect several biomarkers from small amounts of patient plasma samples. This enabled the development of a panel of biomarkers that

measured epigenetic and transcriptional markers, as well as EV proteins, in a single assay. By increasing the number of fluorophores per round and the number of rounds per assay, cyc-DEP has the potential to screen for large protein panels for improved proteomic characterization of liquid biopsies.

3.5.2 Clinical feasibility of oligo cyc-DEP for disease screening

Cancer is a highly heterogeneous disease that follows a unique trajectory for each patient. The development of multiplex multi-omic technologies such as cyc-DEP and oligo cyc-DEP is necessary for accurate small-volume diagnostics of early disease, precise disease stratification, targeted treatment options, and active surveillance of patients (**Figure 3.15**). In the context of prostate cancer, cyc-DEP has the potential to help patients to avoid unnecessary tissue biopsies, inform therapy, and improve quality of life. Cyc-DEP can be conducted with commercially-available antibodies and a conventional widefield microscope. When coupled with automated quantification software, cyc-DEP can be easily adapted by clinicians as a cost-effective pipeline for patient screening, diagnosis, prognostication, and therapeutic monitoring at multiple timepoints throughout disease progression; however, cyc-DEP quenching requires substantial amounts of time that could hinder its clinical translation.

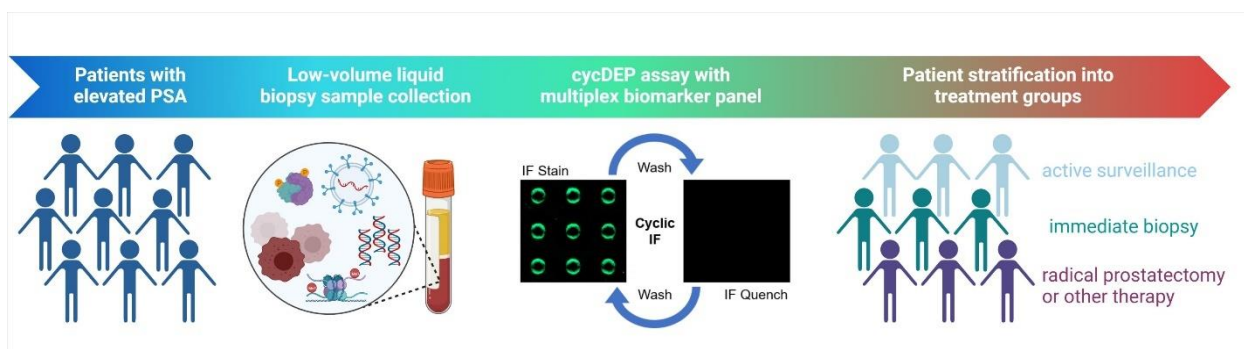


Figure 3.15. Patients with elevated PSA levels can be further stratified into risk groups using cyc-DEP. Low-volume, unprocessed patient plasma samples would be surveyed using biomarkers for circulating tumor cells, protein aggregates, EVs, cfDNA and DNA bound histones, simultaneously collected and profiled on the DEP chip. Automated biomarker quantification with open-source software produces a unique vector of protein levels for each patient, which can be used to group patients with similar profiles into specific treatment groups, eliminating unnecessary biopsies. Cyc-DEP can also be used for active surveillance of patients across time to track disease progression in a minimally invasive way. This figure was created using Biorender.com (BioRender 2021).

Oligo cyc-DEP offers a faster alternative for on-chip cycIF proteomic profiling of liquid biopsy biomarkers that could be well-suited for clinical use. Four rounds of oligo cyc-DEP were performed in approximately four hours as demonstrated with a panel of three target proteins. EC-mediated dehybridization in oligo cyc-DEP was equally as effective as NaOH quenching and photobleaching in cyc-DEP. Future work will expand the library of usable oligo barcodes and cross-validate nonspecific oligo interactions to ensure complementary hybridization of DS-IS pairs. All antibodies against the panel of target proteins in **Table 3.1** will be conjugated to unique DSs from the expanded oligo library. Oligo cyc-DEP and cyc DEP will be performed on the same sample types using analogous parameters to directly compare the two methods.

Chapter 4. Hybrid silica-coated PLGA nanoparticles for enhanced enzyme-based therapeutics

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*In this study, I optimized NP synthesis, prepared samples, conducted experiments, collected data, analyzed results, and assembled the manuscript.

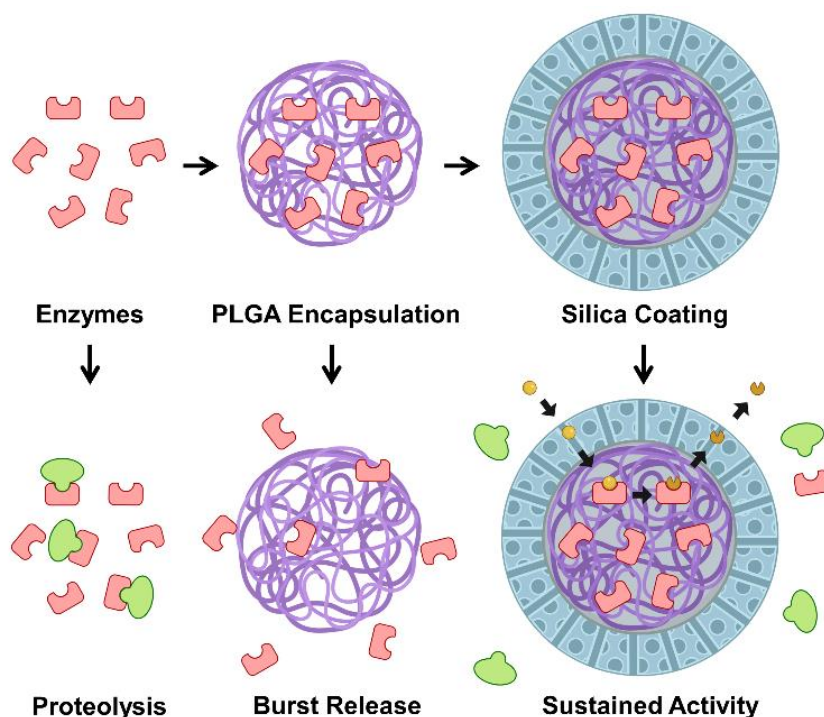
A portion of this chapter is in submission for publication.

4.1 Abstract

Enzyme-based cancer treatments eliminate non-essential biomolecules that tumors rely on for survival, growth, and proliferation, thereby specifically and safely killing neoplastic cells; however, enzyme therapeutics are susceptible to immune clearance, exhibit short half-lives, and require frequent administration. Encapsulation of therapeutic cargo within biocompatible and biodegradable poly(lactic-co-glycolic acid) (PLGA) NPs is a common strategy for controlled release. Unfortunately, PLGA NPs typically release the bulk of their therapeutic cargo shortly after delivery, which negates the purpose of encapsulation. This work demonstrates that coating enzyme-loaded PLGA NPs with silica reduces the initial burst release of cargo and protects against proteolytic inactivation, thereby enhancing enzyme activity. Biocompatible silica-coated PLGA (SiLGA) NPs are viable drug delivery vehicles exhibiting sub-200 nm diameters,

a metastable Zeta potential, and high loading efficiency (86%) and content (8.9%). Compared to uncoated PLGA NPs, SiLGA NPs offer greater retention of enzymatic activity *in vitro* after proteinase treatment. SiLGA NPs also slow the burst release of protein cargo after intramuscular (IM) injection in mice. By sustaining enzymatic activity after delivery, SiLGA encapsulation of the FDA-approved therapeutic enzyme, asparaginase, could reduce frequency of administration, increase half-life, and improve efficacy for patients with acute lymphoblastic leukemia (ALL). Furthermore, the ability to isolate SiLGA NPs from plasma and high-conductance buffer using DEP offers great potential for further characterization of pharmacokinetics as well as therapeutic monitoring after intravenous (IV) administration.

4.2 Graphical abstract



4.3 Introduction

Active enzymes offer attractive therapeutic strategies for many diseases²⁴²⁻²⁴⁶, including cancer²⁴⁷⁻²⁶¹ (**Table 4.1**). Amino acid depletion therapy (AADT) is a promising strategy that often employs active enzymes to target metabolic processes that tumor cells depend upon for survival, growth, and proliferation²⁵⁸. Active enzymes for AADT (e.g. asparaginase, arginine deiminase, arginase, methioninase, cysteinase, etc.) deprive cancer cells of amino acids necessary for tumor growth²⁵⁸. Unfortunately, the immune response to foreign enzymes often prompts short elimination half-lives, which can limit clinical implementation of these therapies.

Table 4.1. Active enzymes used to treat diseases.

Enzyme	Formulation(s)	FDA Status	Disease(s)
asparaginase	colaspase; crisantaspase; pegaspargase; calaspargase pegol-mknl	approved	acute lymphoblastic leukemia; lymphoblastic lymphoma
phenylalanine ammonia lyase	pegvaliase	approved	phenylketonuria
arginine deiminase	pegargiminase; ADI-PEG 20	phase 2	metastatic melanoma; hepatocellular carcinoma; mesothelioma
arginase	pegzilarginase	phase 3; phase 1	Arginase 1 deficiency; melanoma, leukemia, lymphoma
methioninase		phase 1	lung carcinoma, breast cancer, renal cancer, lymphoma
kynureninase	PEG-KYNase	preclinical research	melanoma, breast carcinoma, colon carcinoma
cysteinase		preclinical research	prostate cancer, breast cancer

The linkage of polyethylene glycol (PEG) to an active drug or drug delivery vehicle (PEGylation) is one strategy developed to improve the half-life of therapeutic molecules including enzymes²⁶²⁻²⁶⁶. **Table 4.1** includes several formulations of PEGylated enzymes (e.g. pegaspargase, calaspargase pegol-mknl, pegvaliase, pegargiminase, pegzilarginase, and PEG-KYNase) that are FDA-approved, undergoing clinical trials, or currently being researched. PEGylation shields enzymes from antibodies and proteases *in vivo*; however, the immune system can develop antibodies against PEG with repeated doses, reducing the long-term efficacy of PEGylated drugs²⁶⁷⁻²⁷¹. Patient sensitization to PEGylated formulations can complicate treatment regimens²⁷², highlighting an urgent clinical need for novel approaches to enzyme delivery.

NPs offer high surface-area-to-volume ratios and loading efficiencies to improve the half-life of enzymes after delivery. PLGA NPs are of particular interest as they decompose into natural byproducts of human metabolism (lactic acid and glycolic acid)²⁷³. Several commercially available therapies utilize PLGA particles for drug delivery²⁷⁴⁻²⁷⁹, some of which are listed in **Table 4.2**. PLGA hydrolysis prompts the release of enzymatic cargo^{280, 281}, which can begin hours after injection. While the rate of hydrolysis degradation can be tailored to an extent by modifying the molecular weight and monomer ratio of PLGA, dose dumping related to the characteristic burst release of enzymatic cargo from PLGA NPs remains a challenge for controlled release²⁷³. Surface modifications have been shown to help reduce the burst release of cargo from PLGA NPs²⁸²⁻²⁸⁴; however, burst release remains a major limitation for the controlled delivery of cargo over extended periods.

Table 4.2. Commercial formulations using PLGA particles for drug delivery.

Commercial name(s)	Active ingredient	Disease(s)
Decapeptyl®; Decapeptyl™ SR	triptorelin	prostate cancer, endometriosis
Bydureon®; Bydureon Bcise®	exenatide	type 2 diabetes
Sandostatin LAR®; Sandostatin LAR® Depot	octreotide	acromegaly
Suprecur® MP	buserelin	prostate cancer, endometriosis
Somatuline® LA	lanreotide	acromegaly
Trelstar™ Depot	triptorelin	prostate cancer
Lupron Depot®	leuprolide	prostate cancer
Nutropin Depot®	somatropin	pediatric growth hormone deficiency

Here a strategy is reported to slow the burst release of protein cargo by modifying the outer surface of PLGA NPs with porous silica. This hybrid drug delivery platform offers high enzyme loading efficiency, protection against proteolytic cleavage, and prolonged in-tissue stability. I outline the synthesis of enzyme-loaded core-shell PLGA NPs using a modified water-oil-water double emulsion technique²⁸⁰ and the subsequent sol-gel condensation of an outer nanoporous silica layer²⁸⁵⁻²⁸⁸. The resultant SiLGA NPs permit diffusion of small molecule substrates into the NP core while protecting the enzymatic cargo from deactivation *via* proteinases and slowing burst release kinetics. Silica also offers unique surface modification strategies for targeted delivery. Both PLGA and silica are biodegradable and biocompatible, meaning that SiLGA NPs could have important clinical benefits as delivery vehicles for FDA-approved therapeutic enzymes like asparaginase. Furthermore, the DEP-responsiveness of these hybrid NPs could enable future characterization of NP

biodistribution, circulation half-life, pharmacokinetics, and patient response to therapy after IV delivery, highlighting a novel application for DEP chip technology.

4.4 Materials and Methods

4.4.1 Preparation of reagents

PLGA NPs were synthesized using a water-oil-water double emulsion technique modified from Singhal *et al*²⁸⁰. Fourteen milligrams of penicillinase (Penicillinase from *Bacillus cereus*, lyophilized powder, 1,500-3,000 units/mg protein using benzylpenicillin; P0389-25KU) from Sigma-Aldrich (St. Louis, MO) were added to 450 μ L of 1X PBS and vortex mixed (water phase 1). For *in vivo* experiments involving IM injections, 2 mg of BSA-Cy7 (BS1-S7-1) from Nanocs, Inc. (New York, NY) were added to 450 μ L of 1X PBS and vortex mixed (water phase 1). To form the oil phase, 81 mg of PLGA (Resomer® RG 504 H, Poly(D,L-lactide-co-glycolide), acid terminated, lactide:glycolide 50:50, Mw 38,000-54,000; 719900-1G) and 9 mg of (+)-Dimethyl L-tartrate (DMT, 99%; 163457-25G) were added to 3 mL of DCM (dichloromethane, contains 40-150 ppm amylene as stabilizer, ACS reagent, $\geq$ 99.5%; D65100-500ML) and vortex mixed until solids visibly dissolved; all reagents were obtained from Sigma-Aldrich (St. Louis, MO). The second water phase consisted of 5% by weight PVA (polyvinyl alcohol; 87-90% hydrolyzed, average Mw 30-70 kDa; P8136-1KG) from Sigma-Aldrich (St. Louis, MO) in milliQ water. Water was heated to 80 °C on a hotplate, and PVA was stirred into solution slowly. Once the PVA visibly dissolved at elevated temperature, the solution was

vacuum filtered using the Stericup® and Steritop® Vacuum Driven Disposable Filtration System from MilliporeSigma (Burlington, MA) and stored at 4 °C for at least one week prior to use.

4.4.2 Synthesis of PLGA NPs

PVA solution (water phase 2) was warmed to room temperature. Three hundred microliters of water phase 1 was added dropwise to the oil phase while vortex mixing on low. The resultant solution was vortex mixed on high for one minute to create the first emulsion (emulsion 1). The tube containing emulsion 1 was placed in ice water prior to probe sonication for two minutes at 30% amplitude (pulsed 2 s on and 1 s off) using the Q500 Ultrasonic Processor (#Q500) from QSonica (Newtown, CT) at an output of 500 W and 20 kHz. A total energy of 1,432 J was transferred to emulsion 1, which consisted of aqueous nanodroplets (water phase 1; encapsulated penicillinase) suspended in oil (oil phase). Emulsion 1 was then immediately added dropwise to 18 mL of water phase 2 while stirring at 300 rpm to create the second emulsion (emulsion 2). The stir rate was increased to 600 rpm for 40 s. Emulsion 2 was transferred to a Falcon tube and vortex mixed for one minute on high. Emulsion 2 was then placed in ice water prior to probe sonication using the settings previously described. A total energy of 2,314 J was transferred to emulsion 2, which consisted of oil nanodroplets (oil phase) with an aqueous core (water phase 1; encapsulated penicillinase) that were suspended in an aqueous buffer (water phase 2). Emulsion 2 was capped with Parafilm™, vented with a syringe tip, and stirred at room temperature overnight at 200 rpm, allowing the DCM to

evaporate out of the oil phase to yield solid PLGA NPs. PLGA NPs were placed in a vacuum desiccator for one hour. Approximately 16 mL of the initial 21.3 mL of emulsion 2 were recovered, suggesting that DCM evaporated out of solution.

4.4.3 Washing PLGA NPs

PLGA NPs were added to an empty ultracentrifuge tube (OPTI SEAL BELL TBE/56PK; 361625) from Beckman Coulter (Brea, CA), which was then filled with milliQ water and mixed thoroughly. PLGA NPs were recovered *via* ultracentrifugation using the Optima Max-XP Table Top Ultracentrifuge from Beckman Coulter (Brea, CA) at 32,000 rpm for thirty minutes at 4 °C. The supernatant was collected for downstream analysis, and the pellet was resuspended in 5 mL of milliQ water by sonication. This process was repeated three times. After the final wash, PLGA NPs were resuspended in 3 mL of milliQ water. PLGA NPs were then centrifuged at 1,500 rpm for ten minutes at 4 °C. The supernatant was collected and sonicated. This step was repeated once. PLGA NPs were centrifuged a third time at 1,500 rpm for thirty minutes at 4 °C. The supernatant was collected and sonicated.

4.4.4 Coating PLGA NPs with silica

Half of the PLGA NPs (1.5 mL) were added to 30 mL of 0.1X PBS and vortex mixed thoroughly. The remainder of the PLGA NPs were stored at 4 °C. To form silicic acid, 74 μ L of tetramethyl orthosilicate (TMOS, purum, $\geq$ 98% (GC); 87682-250ML) from

Sigma-Aldrich (St. Louis, MO) was added to 500 μL of hydrochloric acid (HCl; 0.1 mM). The silicic acid was vortex mixed thoroughly on high. While vortex mixing the suspension of PLGA NPs on low, 450 μL of silicic acid was added dropwise. The suspension was vortex mixed thoroughly on high and shaken overnight at 4 °C to allow silicic acid to condense to silica on the surface of the PLGA NPs.

4.4.5 Washing SiLGA NPs

Approximately 13 mL of milliQ water was added to the SiLGA NPs. The resultant suspension was vortex mixed thoroughly and then centrifuged at 3,900 rpm for fifteen minutes at 4 °C. The supernatant was collected for downstream analysis. The pellet was resuspended in one milliliter of milliQ water by pipetting and bath sonication for two minutes. Approximately 44 mL of milliQ water was added to the suspension of SiLGA NPs, which was then vortex mixed thoroughly. The SiLGA NPs were washed three more times using this technique. On the final wash, the pellet was resuspended in 1.5 mL of milliQ water and then stored at 4 °C.

4.4.6 Dynamic light scattering measurements of PLGA and SiLGA NPs

PLGA and SiLGA NPs were sonicated to resuspend any particles that may have aggregated. One microliter of NPs was added to one milliliter of milliQ water. The resultant suspension was inverted ten times and loaded into a cuvette (folded capillary Zeta cell; DTS1070) from Malvern Panalytical (Malvern, UK). The cuvette was inserted

into the Zetasizer Nano ZSP from Malvern Panalytical (Malvern, UK). Appropriate material properties were imported into the Malvern Zetasizer Software prior to measuring the hydrodynamic particle diameter, polydispersity index (PDI), and Zeta potential of the NPs. Three replicate measurements were obtained.

4.4.7 Nanoparticle tracking analysis of PLGA and SiLGA NPs

Samples were analyzed using the ZetaView QUATT Nanoparticle Tracking Video Microscope PMX-420 from Particle Metrix (Munich, Germany). The machine was calibrated with alignment beads (100 nm diameter) supplied by Particle Metrix. PLGA and SiLGA NPs were sonicated to resuspend aggregates that may have formed. NPs were diluted by a factor of at least 10^5 into filtered (0.1 μm) milliQ water and 0.1X PBS to obtain particle counts within the linear range of the instrument. Samples were inverted ten times prior to being analyzed. Particle Metrix recommended the following settings, which were used consistently across samples: sizing and enumerating particles (70%); frame rate (30%).

4.4.8 Transmission electron microscopy of PLGA and SiLGA NPs

Transmission electron microscopy samples were prepared by spotting 10 μL of a 1:20 dilution of stock NPs in milliQ water on top of 200 mesh carbon film copper TEM grids (NC1513260) from Fisher Scientific (Waltham, MA). Samples dried overnight.

Micrographs were acquired using a Tecnai F20 TEM by FEI (Lausanne, Switzerland) at an accelerating voltage of 120 kV.

4.4.9 Nitrocefin assays determine penicillinase activity

Five milligrams of nitrocefin (Nitrocein β -lactamase substrate; 484400-5MG) from EMD Millipore (Burlington, MA) were thoroughly dissolved into 500 μ L of DMSO. The dissolved nitrocefin was then added to 9.5 mL of 1X PBS to create a working solution, which was aliquoted, protected from light, and stored at -20 °C for up to two weeks. Aliquots of nitrocefin working solution were thawed at room temperature for at least thirty minutes prior to use in activity assays. Penicillinase-containing samples were diluted into 1X PBS at a volume of 100 μ L per well in an assay plate (96-Well Clear Bottom Plates with Lids, Tissue Culture Treated, White, Well Volume: 360 μ L, Sterile; 3610) from Corning (Corning, NY) and mixed thoroughly. The Spark® 20M Te-cool™ from Tecan (Männedorf, Switzerland) was utilized to measure absorbance at 486 nm in each well on the assay plate (at least four measurements per well per timepoint). The TECAN chamber was set to 37 °C prior to conducting assays.

Twenty-five microliters of nitrocefin working solution was added to each well at a total reaction volume of 125 μ L per well. The assay plate was immediately withdrawn into the TECAN assay chamber, which was set to shake the assay plate in-between timepoints. Activity assays ran for at least one hour. Multiple measurements per well were averaged at each timepoint. Means of these average measurements were calculated across wells containing technical replicates. Mean measurements of

absorbance at 486 nm were plotted against time. Penicillinase activity was calculated as the slope of the best-fit line through the linear region of those plots. Proteinase K (PK) was suspended in 1X PBS and added to penicillinase-containing samples at a final concentration of 0.1 mg/mL. Samples were incubated with PK for 4.5 hours at 37 °C while shaking before adding nitrocefin. Control samples were incubated with an equivalent volume of 1X PBS.

4.4.10 IM injection of PLGA and SiLGA NPs in mice

Nod scid gamma (NSG™) (005557) and albino C57Bl/6 (B6a) (000058) mice were purchased from the Jackson Laboratories. Mice were housed in high-efficiency particulate air (HEPA) cages in a specific-pathogen free (SPF) facility at OHSU. Mice were fed a diet of PicoLab Mouse Diet 20 (LabDiet, 5058) ad libitum beginning one week before imaging. Male and female mice were given a 50 µL IM injection into each hind limb of Cy7-Bovine Serum Albumin (Nanocs) as free protein, PLGA encapsulated, or SiLGA encapsulated (N=3 mice for each treatment and mouse strain). Mice were imaged for fluorescence (excitation wavelength = 740 nm, emission wavelength = 780 nm, exposure = 1 s) after correcting for BG fluorescence using the MIS Lumina XRMS Series III (PerkinElmer). All experiments performed were approved by the Institutional Animal Care and Use Committee (IACUC) at OHSU.

4.4.11 *DEP isolation of SiLGA and PLGA NPs from plasma and buffer*

BSA conjugated to AF594 and AF647 was encapsulated with PLGA and SiLGA NPs using the described synthesis and coating techniques. After the final wash step, PLGA and SiLGA NPs were resuspended in 1X PBS buffer to form stock solutions. PLGA and SiLGA NPs loaded with BSA-AF647 were spiked into plasma at a dilution of 1:5 by-volume from stock. PLGA and SiLGA NPs loaded with BSA-AF594 were undiluted from stock.

Three-channel DEP chips were purchased from Biological Dynamics (San Diego, CA). Filtered 0.5X PBS was withdrawn into microfluidic chambers on DEP chips, and DEP microelectrode arrays were imaged. Each of the four NP samples were then withdrawn into isolated microfluidic chambers on DEP chips. Alternating current was applied to the planar microelectrode arrays in the microfluidic chambers to generate DEP forces. A voltage of 15 V_{pp} at a frequency of 15 kHz was applied for at least 15 minutes. During the first ten minutes of applied voltage, the samples were enriched by 5 µL at 0.5 µL/min followed by at least five minutes of collection under static fluid. DEP forces were then applied while washing with 140 µL of filtered 0.5X PBS at 7 µL/min. Samples were then imaged using previously described methods. NPs loaded with BSA-AF594 were then imaged through a DsRed optical filter. NPs encapsulating BSA-AF647 were imaged through a Cy5 optical filter.

4.5 Results and Discussion

Silica-coated PLGA (SiLGA) NPs are biocompatible drug delivery vehicles designed to enhance the efficacy of therapeutic enzymes by shielding cargo from proteolysis and reducing the rate of initial cargo release. SiLGA NPs permit the diffusion of small-molecule substrates into the particle core for catalysis while protecting enzymatic cargo against proteolysis from proteinases, which are abundant in the blood. SiLGA NPs also retain a high percentage of protein cargo one day after IM injection, suggesting that silica slows the initial rate of PLGA hydrolysis *in vivo*. Compared to PLGA NPs, SiLGA NPs improve the retention of enzymatic activity by approximately 40% in the presence of proteinases and reduce dose dumping by approximately 20% after one day. SiLGA NPs exhibited high loading efficiency (86%) and content (8.9%) relative to chitosan-coated PLGA NPs reported in the literature. Formation of the silica layer followed a straightforward sol-gel polycondensation reaction of silicic acid at the NP surfaces that can be readily applied to existing FDA-approved PLGA formulations (**Table 4.2**). Silica also offers a wide variety of surface modifications for immune cloaking and targeted delivery, suggesting future applications of SiLGA NPs might further enhance enzyme-based delivery.

4.5.1 Structural characterization of SiLGA NPs

Transmission electron microscopy (TEM), dynamic light scattering (DLS), and nanoparticle tracking analysis (NTA) characterized the formation of complete and

uniform silica layers around individual PLGA NPs. I utilized a water-oil-water double emulsion technique for obtaining protein-loaded PLGA NPs²⁸⁰ followed by silica coating to produce SiLGA NPs (**Figure 4.1**). Silica layers were formed by a polycondensation reaction of silicic acid, generated by adding TMOS to HCl²⁸⁵. **Figure 4.2** shows TEMs of PLGA NPs, which exhibited diameters of approximately 125 nm to 175 nm. To obtain TEMs of PLGA NPs, the electron beam was focused outside the field-of-view and moved into the frame immediately prior to micrograph acquisition, as depicted in **Figure 4.3**. SiLGA NPs were slightly larger (135 nm to 195 nm in diameter) due to the addition of uniform and complete silica layers, approximately 10 nm to 20 nm thick. Silica at the surface of PLGA NPs can be observed as the change in contrast at particle edges²⁸⁸ in panels f and g of **Figure 4.2**. Under DLS, an increase in Cumulant particle diameter from 223 nm $\pm$ 2 nm (PDI: 0.099 $\pm$ 0.016) to 394 nm $\pm$ 26 nm (PDI: 0.252 $\pm$ 0.015) also suggested formation of silica layers. Under NTA, NPs exhibited an average diameter of 162.7 nm $\pm$ 61.4 nm before silica coating and 187.7 nm $\pm$ 73.6 nm afterward. DLS measured a shift in Zeta potential from -13.4 mV $\pm$ 0.1 mV prior to silica coating to -20.5 $\pm$ 1.4 mV afterward. Zeta potential measurements using NTA were consistent with DLS, indicating a negative shift from -8.91 mV $\pm$ 0.26 mV to -17.10 mV $\pm$ 1.32 mV upon silica coating (data not shown). Results showed that complete silica layers with uniform thickness formed around PLGA NPs.

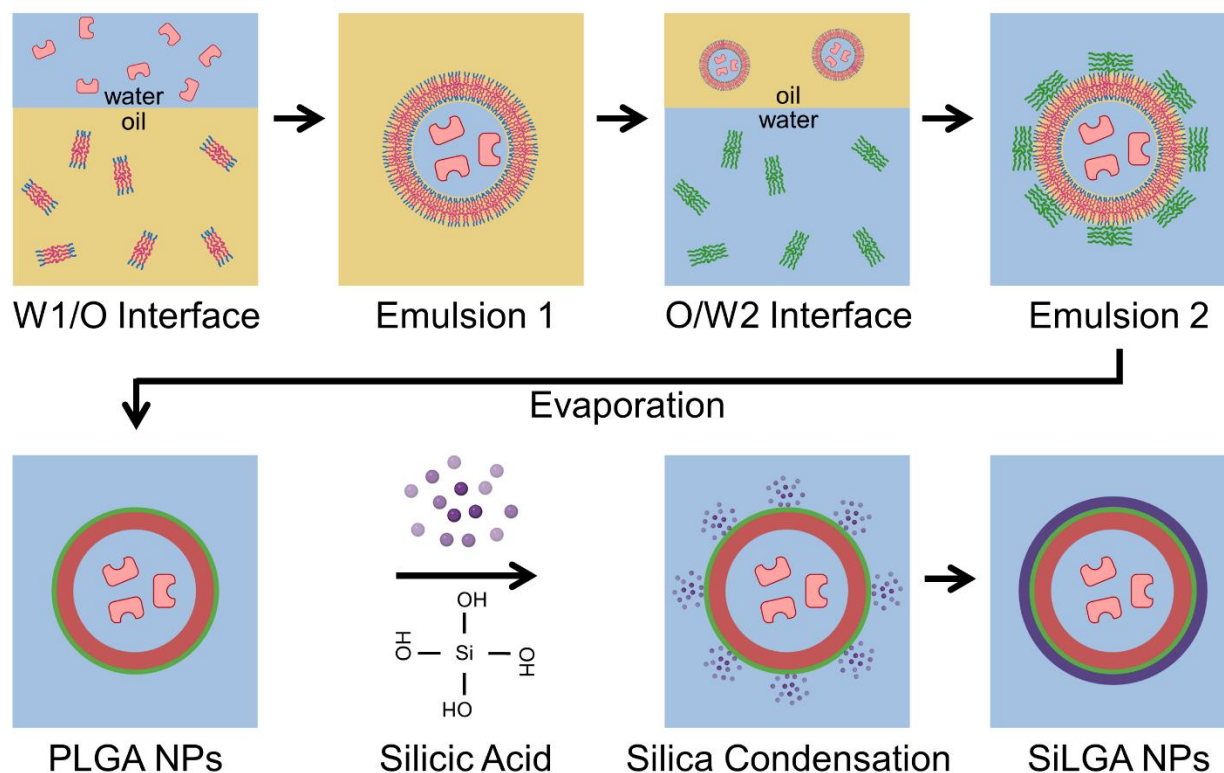


Figure 4.1. Synthesis of PLGA and SiLGA NPs. PLGA NPs are synthesized using a water-oil-water (W1/O/W2) double emulsion process. Active enzymes suspended in aqueous solution (W1) are interfaced with PLGA dissolved in dichloromethane (O). Emulsification at the W1/O interface creates aqueous nanodroplets in dichloromethane (Emulsion 1). Emulsion 1 is interfaced with a second aqueous solution containing polyvinyl alcohol (W2). Emulsification at the O/W2 interface yields oil-phase NPs containing enzymes in an aqueous core (Emulsion 2). Evaporation of dichloromethane forms an aqueous suspension of PLGA NPs with active enzymatic cargo. Addition of silicic acid prompts the formation of silica at the surface of PLGA NPs *via* sol-gel polycondensation, resulting in SiLGA NPs. This figure was created using Biorender.com.

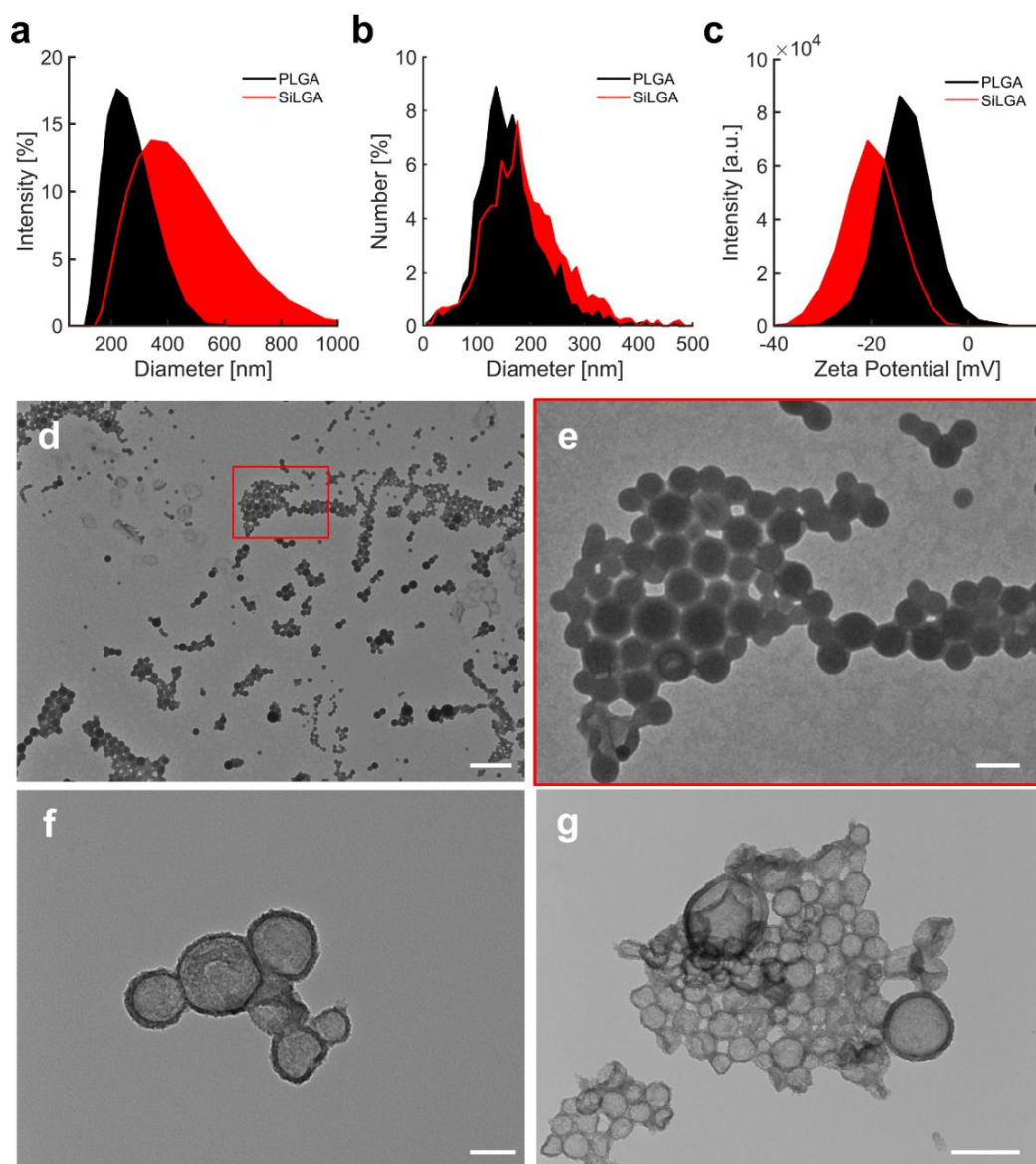


Figure 4.2. DLS, NTA, and TEM show silica coating. a) Dynamic light scattering measured the Cumulant hydrodynamic diameter of PLGA NPs to be 223 nm $\pm$ 2 nm (black), which increased to 394 nm $\pm$ 26 nm (red) after addition of the silica layer. PLGA NPs exhibited a PDI of 0.099 $\pm$ 0.016. SiLGA NPs showed a PDI of 0.252 $\pm$ 0.015. b) PLGA and SiLGA NPs exhibited diameters of 162.7 nm $\pm$ 61.4 nm and 187.7 nm $\pm$ 73.6 nm, respectively, using nanoparticle tracking analysis. c) The Zeta potential of the PLGA NPs was measured to be -13.4 mV $\pm$ 0.1 mV. After addition of the silica layer, the Zeta potential decreased to -20.5 $\pm$ 1.4 mV. Panels a and c show the average of technical triplicate measurements. Panel b shows size distribution for 1687 PLGA NPs and 1501 SiLGA NPs. d) Electron micrograph shows PLGA NPs formed from a water-oil-water double emulsion synthesis (scale bar is 1 μ m). e) Zoomed in area from panel a (highlighted in red) shows PLGA NPs with radii approximately between 125 nm and 175 nm (scale bar is 200 nm). f-g) Electron micrographs show complete coating of PLGA NPs with silica to form SiLGA. Silica layers are approximately 10 nm to 20 nm thick (scale bars for panel c and d, respectively: 100 nm; 200 nm).

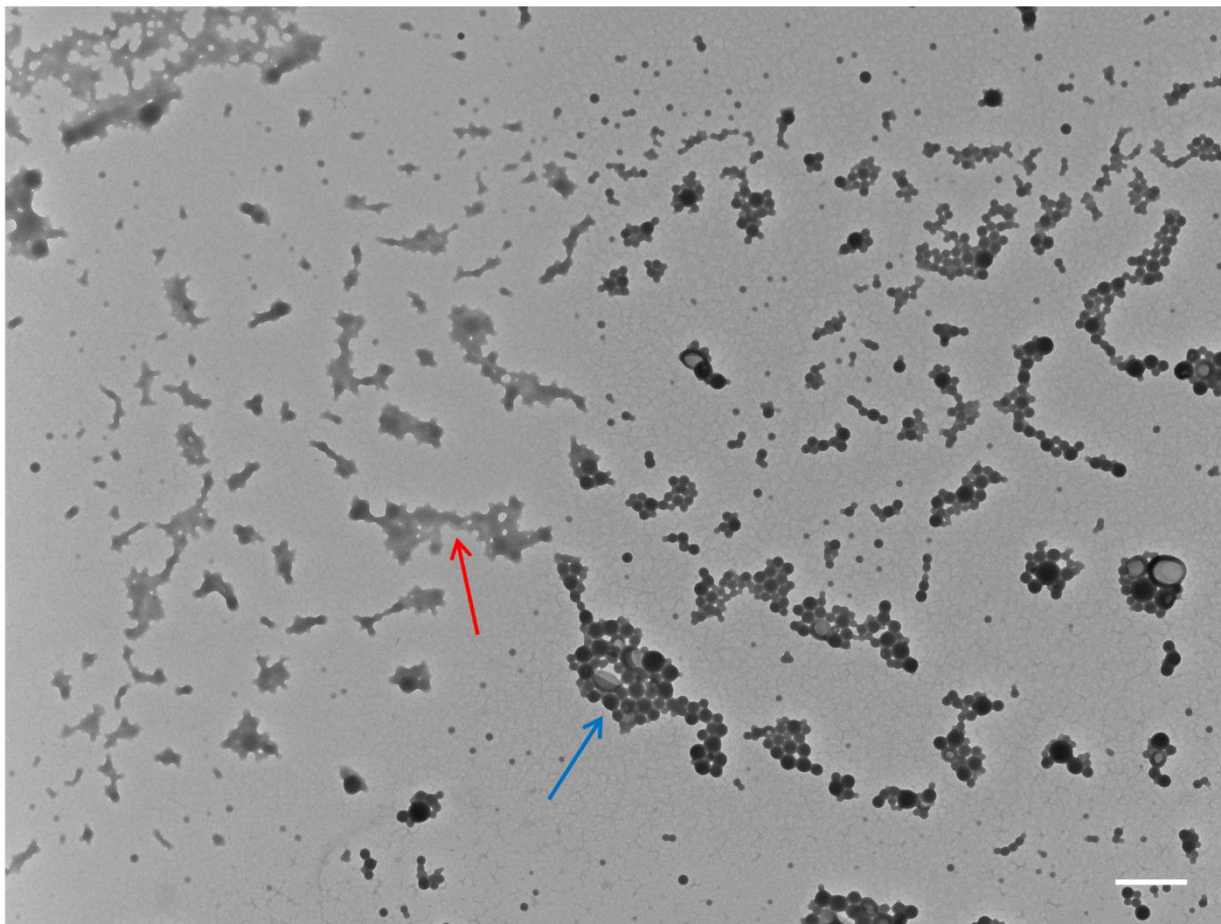


Figure 4.3. TEM of PLGA NPs under prolonged exposure from electron beam. PLGA NPs were exposed to the electron beam for a prolonged duration, resulting in sample degradation (red arrow at left). The FOV was shifted immediately before micrograph acquisition, minimizing degradation of PLGA NPs on the right half of the micrograph (blue arrow at right). Scale bar is one micron.

Unexpectedly large increases particle size and PDI after coating with silica suggested aggregation of SiLGA NPs during DLS measurements. The large PDI of SiLGA NPs was not due to variability in the thickness of the silica layers because TEMs confirmed uniform coating. No appreciable particle aggregation occurred prior to silica coating because TEMs showed complete layers around individual, rather than aggregated, PLGA NPs. The low PDI of PLGA NPs also suggested that aggregation

prior to coating was unlikely. Interestingly, aggregated SiLGA NPs did not appear to affect NTA measurements, perhaps because NTA samples were 100 to 1000 times more dilute than DLS samples, reducing the relative proximity of SiLGA NPs and their tendency to aggregate.

4.5.2 *Enzyme loading of SiLGA NPs*

Chitosan (CS) is a common surface coating for PLGA NPs, reportedly slowing the burst release of SN38, ferulic acid, epirubicin, and paclitaxel^{282-284, 289}. Given my similar goal of controlling cargo release from PLGA NPs, I wanted to compare the loading efficiency and content of the hybrid SiLGA NPs to reported values for hybrid CS-PLGA NPs. I measured the loading efficiency and content of PLGA and SiLGA NPs that encapsulated the enzyme, penicillinase. Nitrocefin, a chromogenic substrate, was used to measure penicillinase activity. Nitrocefin contains a β -lactam ring that β -lactamases like penicillinase hydrolyze, causing a chromogenic shift in peak absorbance from 380 nm to 486 nm. To generate the activity standard in panel e of **Figure 4.4**, nitrocefin was added to a serial dilution set of known concentrations of penicillinase. The activity of each dilution was plotted against penicillinase concentration. A best-fit line ($R^2 = 0.985$) through the plotted data served as the activity standard. I measured the enzymatic activity of wash buffers to estimate the amount of penicillinase lost during NP synthesis. The concentration of penicillinase in each wash buffer was calculated using the standard curve. Concentrations were multiplied by respective wash buffer volumes to estimate the total penicillinase lost during NP

synthesis. Approximately 0.55 mg of penicillinase was not encapsulated during the synthesis of PLGA NPs. An additional 0.36 mg of penicillinase was lost during the formation of SiLGA NPs.

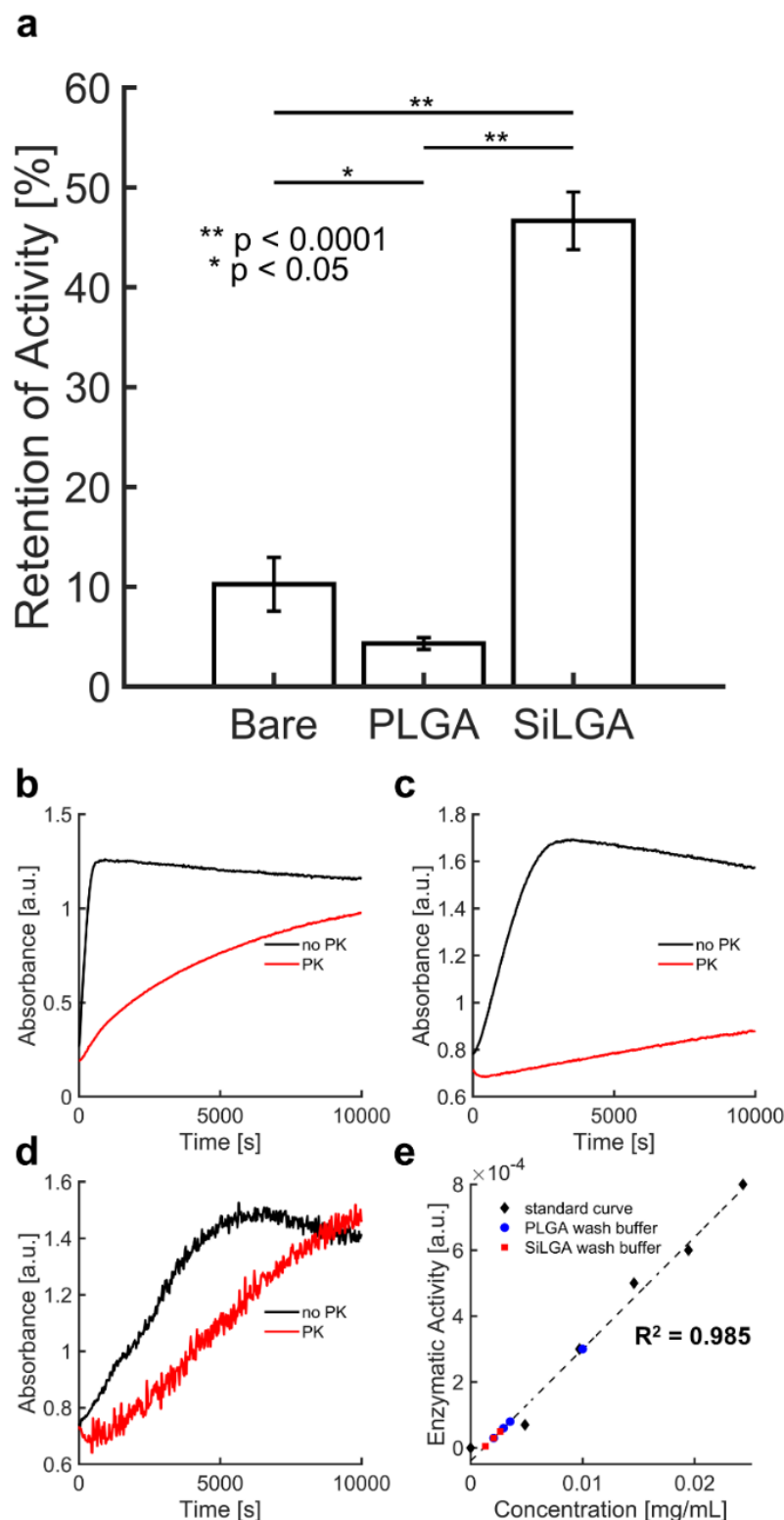


Figure 4.4. Penicillinase loading and retention of activity after treatment with proteinase K. The silica layer protects penicillinase from proteinase degradation *in vitro*. Bare penicillinase, penicillinase-encapsulated PLGA NPs, and penicillinase-encapsulated SiLGA NPs were incubated with and without Proteinase K (PK) prior to the addition of nitrocefin. The vertical axes in panels a through c indicate the spectral shift in light absorption that occurs when penicillinase catalyzes nitrocefin. The vertical axis in panel d is the percent decrease in the enzymatic activity due to the presence of PK. Enzymatic activity is measured as the slope of the linear region of each contour in panels a through c. a) SiLGA NPs offer the most protection for penicillinase, which retained 46.7% $\pm$ 2.9% enzymatic activity after incubation with PK. PLGA NPs without the silica layer were ineffective at protecting against degradation *via* PK, retaining only 4.33% $\pm$ 0.58% enzymatic activity. Bare penicillinase retained 10.2% $\pm$ 2.7% enzymatic activity after PK treatment. Error bars in panel d

represent one standard deviation of the mean across triplicates. Each contour in panels a through c represent the average of technical duplicates. b) Representative curves showing a substantial reduction in the enzymatic activity of unencapsulated penicillinase

when exposed to PK. c) Representative curves suggest that encapsulation of penicillinase in PLGA NPs does not shield penicillinase from proteinase degradation. d) Representative curves demonstrate that encapsulation of penicillinase in SiLGA NPs successfully protects against degradation from PK. e) Nitrocefin was added to a serial dilution set of penicillinase to generate a standard curve for enzymatic activity (black diamonds; $R^2 = 0.985$). Nitrocefin was then added to buffer used to wash PLGA (blue circles) and SiLGA (red squares) NPs. Enzyme loading efficiency and content of the NPs were calculated indirectly from the amount of penicillinase lost during washing.

Enzyme loading efficiency is defined as the amount of enzyme encapsulated in NPs divided by the amount of enzyme added during synthesis, typically expressed as a weight percentage²⁹⁰. Enzyme loading content is defined as the amount of enzyme encapsulated in NPs divided by the starting mass of the NPs²⁹⁰. The initial amount of enzyme added during synthesis was 9.33 mg, and the starting mass of the NPs was 90 mg (81 mg PLGA, 9 mg (+)-dimethyl L-tartrate). The majority (0.55 mg; 60%) of penicillinase lost during the synthesis of SiLGA NPs was recovered in the wash buffer of precursor PLGA NPs; the remainder (0.36 mg; 40%) was recovered in the wash buffer of SiLGA NPs (**Figure 4.4**). The enzyme loading efficiency of PLGA NPs was calculated to be approximately 94% ($= (9.33 \text{ mg} - 0.55 \text{ mg}) / 9.33 \text{ mg} * 100\%$). The enzyme loading content of the PLGA NPs was determined to be approximately 9.8% ($= (9.33 \text{ mg} - 0.55 \text{ mg}) / 90 \text{ mg} * 100\%$). The enzyme loading efficiency of SiLGA NPs was approximately 86% ($= (9.33/2 \text{ mg} - 0.55/2 \text{ mg} - 0.36 \text{ mg}) / (9.33/2 \text{ mg}) * 100\%$). The enzyme loading content of the SiLGA NPs was approximately 8.9% ($= (9.33/2 \text{ mg} - 0.55/2 \text{ mg} - 0.36 \text{ mg}) / (90/2 \text{ mg}) * 100\%$), excluding the mass of the silica. NTA estimated the concentration of penicillinase PLGA NPs to be approximately 10^{14}

NPs/mL as resuspended after washing. As formulated, the concentration of penicillinase SiLGA NPs was measured to be approximately 7×10^{13} NPs/mL.

These results suggest that enzymes can be loaded into NPs with high efficiency. SiLGA NPs achieved a similar loading efficiency (86% versus 87.1%) and content (8.9% versus 6.42%) to the optimal CS-PLGA NP formulations reported by Lu *et al*²⁸². SiLGA NPs exhibited higher loading efficiency (86% versus 74.17% $\pm$ 9.21%) and content (8.9% versus 4.2% $\pm$ 0.82 %) than CS-PLGA NPs synthesized by Alibolandi *et al*²⁸⁹. SiLGA NPs also showed higher loading efficiency than CS-PLGA NPs reported by de Lima *et al*²⁸⁴ (50% $\pm$ 4%) and Chen *et al*²⁸³ (72.0% $\pm$ 3.5%). The enzyme loading efficiency of penicillinase-PLGA NPs (94%) was only 5% lower than a previous report by Singhal *et al*²⁸⁰, which used a similar protocol to synthesize catalase-PLGA NPs. Other published formulations of PLGA NPs reported slightly lower loading efficiencies: 84.1% $\pm$ 3.4% by Chen *et al*²⁸³, 65.8% by Lu *et al*²⁸², and 60% $\pm$ 3% by de Lima *et al*²⁸⁴. The loading content of penicillinase-PLGA NPs (9.8%) was higher than that of paclitaxel-loaded PLGA NPs (5.07%) reported by Lu *et al*²⁸². Penicillinase-loaded SiLGA NPs demonstrated a loading efficiency that was similar to, if not higher than, those of several reported drug-loaded hybrid CS-PLGA NP formulations intended to slow burst release kinetics.

Enzyme loading contents of approximately 10% are common for inert drug carriers like PLGA and SiLGA NPs²⁹⁰. Loading content suggested that encapsulated enzymes could be delivered at approximately one-tenth the concentration of unencapsulated enzymes, assuming equal masses of each were administered. While higher loading content would reduce the number of NPs necessary to deliver

therapeutic enzymes and the likelihood of systemic toxicity, SiLGA offers potential advantages that could offset the reduction in deliverable enzyme concentration by prolonging enzyme activity.

4.5.3 SiLGA NPs protect enzymes from proteolysis in vitro

One such advantage of SiLGA NPs is the ability to protect enzymatic cargo from proteolysis while retaining enzymatic activity. It has been suggested that proteolytic degradation of PEGylated enzymes *via* proteinases might reduce therapeutic efficacy over repeated dosages in cancer patients²⁹¹. With IV injection being a common mode of administration, enzyme-based therapies are susceptible to proteolysis from a variety of blood proteases. Viable encapsulation platforms should shield enzymatic cargo from proteolysis while retaining enzyme activity. It was intended that pores in the silica layer would be sufficiently small to prevent inward diffusion of proteases and outward diffusion of enzymatic cargo, yet large enough to permit inward diffusion of small-molecule substrates for catalysis.

To test the ability of SiLGA NPs to shield enzymatic cargo from proteolysis while retaining enzymatic activity, unencapsulated and encapsulated formulations of penicillinase were incubated with proteinase K (PK) for 4.5 hours at 37 °C. Nitrocefin was added to each sample after PK treatment to measure penicillinase activity. Panel a of **Figure 4.4** demonstrated that penicillinase encapsulated in SiLGA NPs retained 46.7% +/- 2.9% enzymatic activity after incubation with PK, which was a ten-fold improvement over PLGA encapsulation. Panels b through d of **Figure 4.4** show

representative curves for penicillinase activity. SiLGA NPs do not show a smooth contour in panel d due to NPs settling during incubation and over the course of the assay; however, SiLGA NPs exhibited much higher retention of enzymatic activity after PK treatment than PLGA-encapsulated and unencapsulated penicillinase, as witnessed by the moderate reduction the slope of the linear region in panel d. This observation confirmed the hypothesis that pores in the silica layer were sufficiently small to allow inward diffusion of nitrocefin yet large enough to inhibit or prevent inward diffusion of PK. Unencapsulated penicillinase retained 10.2% $\pm$ 2.7% activity after PK treatment, suggesting that a longer incubation period might have been necessary for complete inactivation of penicillinase.

Interestingly, PLGA NPs offered no protection for enzymatic cargo as retention of enzymatic activity after PK treatment (4.33% $\pm$ 0.58%) was on-par with unencapsulated penicillinase. One explanation could be that PLGA NPs hydrolyzed during the incubation period, releasing penicillinase into solution. PLGA degrades over time in aqueous solutions as ester linkages between glycolic acid and lactic acid monomers hydrolyze^{273, 292}. Cargo release from PLGA particles generally undergoes three phases driven by hydrolysis degradation: an initial burst release of cargo, a sustained “lag” period with minimal release, and a final burst release^{292, 293}. Full hydrolysis of PLGA particles can span mere days to several months depending on factors including storage temperature, pH, monomer ratios, and molecular weight²⁹². Elevated temperature is known to increase the rate at which PLGA hydrolyzes, especially as temperatures approach or exceed the glass transition temperature^{292, 294}. Arguably the most important consideration is the lactide-to-glycolide monomer ratio²⁷³.

Degradation rates generally increase as glycolide content increases^{292, 295}; however, PLGA with a monomer ratio of 50:50 lactide-to-glycolide is widely known to have the fastest rate of hydrolysis at any given molecular weight²⁷³. I used PLGA with a 50:50 lactide-to-glycolide monomer ratio, suggesting that these PLGA NPs likely experienced a fast rate of hydrolysis. Incubation at 37 °C likely expedited hydrolysis, especially considering that the glass transition temperature of PLGA in this study is only 10 °C higher (between 46 °C and 50 °C) as reported by the manufacturer. Therefore, hydrolysis of PLGA NPs is likely to have prompted the release of penicillinase into solution during PK treatment, explaining the sizable reduction in penicillinase activity.

SiLGA NPs were more effective at shielding enzymatic cargo from proteolytic cleavage *in vitro* than unencapsulated and PLGA-encapsulated penicillinase; however, protection against proteolysis was incomplete. The partial reduction in penicillinase activity after PK treatment of SiLGA NPs in panel d of **Figure 4.4** has two possible explanations. It is feasible that pores in the silica layer were not small enough to completely prevent the inward diffusion of PK or outward diffusion of penicillinase, thereby reducing but not eliminating enzyme inactivation by proteolysis. Alternatively, PLGA beneath the silica layer might have partially hydrolyzed during the incubation period, releasing a portion of penicillinase cargo into solution for proteolytic inactivation by PK.

Data from panel c of **Figure 4.4** suggested that hydrolysis degradation of PLGA NPs led to the complete release and deactivation of penicillinase cargo during PK treatment. The partial, rather than complete, reduction in penicillinase activity after PK treatment of SiLGA NPs is significant because it suggests silica might have slowed

PLGA degradation and subsequent release of penicillinase cargo into solution. Importantly, silica appeared to slow PLGA hydrolysis while shielding encapsulated penicillinase from proteolytic inactivation because enzymatic activity was measurable with and without PK treatment. This interpretation of data is consistent with reported reductions in rates of cargo release from CS-PLGA NPs, suggesting that silica could potentially improve the half-life of encapsulated enzymes after delivery by slowing the characteristic burst release of cargo. The combined features of proteolytic protection and controlled cargo release could be beneficial in the clinical administration of therapeutic enzymes.

4.5.4 SiLGA NPs slow the burst release of enzymatic cargo in vivo

I hypothesized that the silica layer reduces the rates of PLGA hydrolysis and initial burst release, thereby increasing IM retention of protein cargo and improving the half-life of encapsulated therapeutic enzymes. To test this hypothesis, unencapsulated and encapsulated formulations of bovine serum albumin-conjugated Cy7 (BSA-Cy7) were injected intramuscularly into mice, mimicking a common delivery mode for asparaginase in the treatment of acute lymphoblastic leukemia (ALL).

Panels a through c in **Figure 4.5** show the first two phases of cargo release for PLGA and SiLGA NPs. PLGA NPs exhibited an initial burst release of BSA-Cy7 over the first day, followed by a sustained lag period of minimal release through the fifth day. Burst release from SiLGA NPs occurred over the first two days, followed by a similar lag period. Importantly, there is a significant difference ($p < 0.05$) between PLGA and

SiLGA in terms of IM levels of BSA-Cy7 after one day in both immunodeficient and immunocompetent mice, suggesting that silica slowed the initial burst release kinetics of protein cargo after IM injection. After the second day, there was no difference between PLGA and SiLGA, suggesting that silica coating did not improve long term survival of encapsulated BSA-Cy7; however, both PLGA and SiLGA NPs successfully increased IM retention of BSA-Cy7 relative to unencapsulated BSA-Cy7 for twenty-one days post-injection ($p < 0.05$). These results demonstrate that SiLGA NPs slow burst release kinetics of enzymatic cargo while protecting the payload from immune clearance and proteolysis for an extended period.

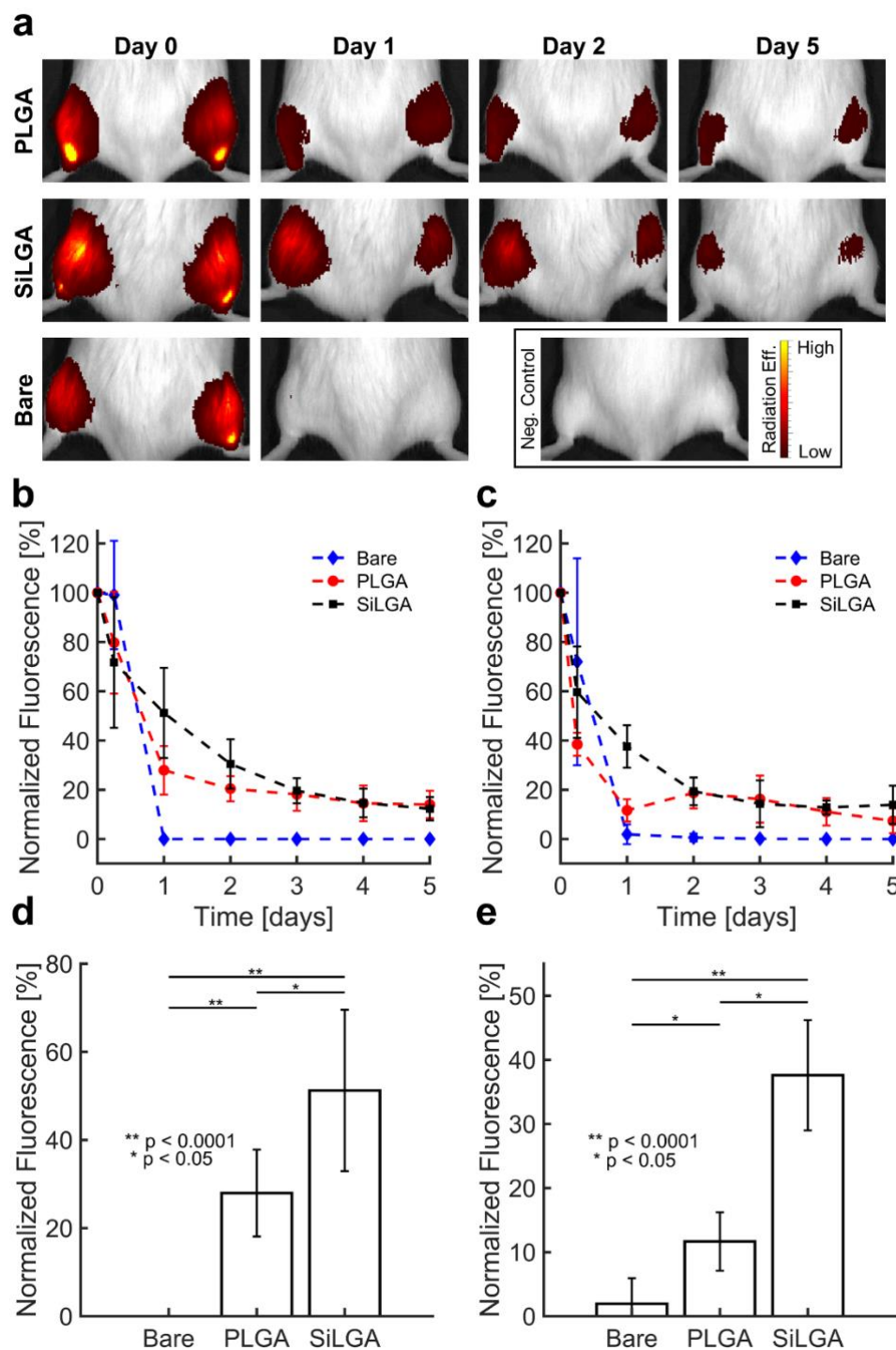


Figure 4.5. SiLGA NPs slow burst release kinetics of BSA after IM injection in mice. BSA-Cy7 conjugate was encapsulated in PLGA and SiLGA NPs prior to IM injection in immunocompetent (B6a) and immunodeficient (NSGTM) mice. a) Radiation efficiency of BSA-Cy7 PLGA NPs (top), BSA-Cy7 SiLGA NPs (middle), and unencapsulated BSA-Cy7 (bottom) in the hind legs of immunocompetent mice (B6a) as imaged over a five-day span. Saline was injected into the negative control shown in panel a at Day 0. b) Normalized fluorescence levels of BSA-Cy7 injected into B6a mice

as quantified from image data over a five-day span. c) Normalized fluorescence levels of BSA-Cy7 injected into NSGTM mice as quantified from image data over a five-day span. d) Normalized fluorescence levels of unencapsulated (bare) BSA-Cy7, BSA-Cy7 PLGA NPs, and BSA-Cy7 SiLGA NPs in B6a mice after one day. SiLGA NPs retain the highest amount of BSA-Cy7 and exhibit statistically significant differences between PLGA NPs and unencapsulated BSA-Cy7. e) Normalized fluorescence levels of unencapsulated (bare) BSA-Cy7, BSA-Cy7 PLGA NPs, and BSA-Cy7 SiLGA NPs in NSGTM mice after one day. SiLGA NPs retain the highest amount of BSA-Cy7 and exhibit statistically significant differences between PLGA NPs and unencapsulated BSA-Cy7. Error bars represent one standard deviation above and below the mean normalized fluorescence level. Three mice were injected in each hind leg yielding six injection sites. Means and standard deviations were calculated across all six injection sites. One outlier injection site was removed from analysis at all points along the PLGA contour in panels c (red circles) and e.

The controlled release of enzymes into circulation could be highly beneficial for patients with ALL. ALL blasts are auxotrophic for asparagine (Asn), meaning they do not express Asn synthetase and cannot synthesize Asn²⁹⁶. Asn is critical for cancer cell proliferation. As such, ALL blasts depend on environmental Asn²⁹⁶ for survival. A variety of asparaginase-based medications are prescribed to ALL patients, targeting environmental Asn. Asparaginase starves the tumor of a metabolic nutrient that is essential to their growth in a type of treatment called AADT.

Elspar is the commercial name for *E. coli*-derived asparaginase, an FDA-approved enzyme used to treat ALL patients. Elspar requires a taxing administration of three times per week *via* IM or IV injection. PEGylated versions of Elspar, like Oncaspar (pegaspargase) and Asparlas (calaspargase pegol), improve the elimination half-life of asparaginase and reduce the frequency administration. Unfortunately, many patients develop sensitivities to PEG and/or *E. coli*-derived asparaginase. Sensitized patients must follow desensitization regimens in order to continue asparaginase treatment²⁷².

Suggested desensitization regimens often involve asparaginase *erwinia chrysanthemi*, the active enzyme in FDA-approved treatments like Erwinaze and Rylaze, which were initially developed for patients who develop allergic reactions or other sensitivities to *E. coli*-derived asparaginase.

SiLGA NPs could significantly enhance asparaginase-based treatments in the fight against ALL. Encapsulation of asparaginase within SLGA NPs would ostensibly slow burst release after IM injection, prolonging enzyme half-life and reducing the frequency of administration. Feasibly any of the FDA-approved asparaginases could be loaded into SiLGA NPs using the water-oil-water double emulsion and sol-gel silica deposition described in the Methods. It is unlikely that PEGylation of asparaginase would prohibit encapsulation with SiLGA as PEG is soluble in water. A hypothetical formulation of pegaspargase or calaspargase pegol encapsulated within SiLGA NPs might benefit from both a slow burst release at the injection site and a prolonged circulation half-life. While I have not analyzed clearance of SiLGA NPs after IV injection, data suggest that SiLGA NPs could also offer some protection against asparaginase proteolysis *via* blood proteases.

SiLGA NPs offer new strategies for asparaginase desensitization as well as a more manageable administration schedule for patients, potentially improving quality of life. Importantly, successful encapsulation of asparaginase in PLGA NPs has been demonstrated^{297, 298}. The benefits of coating PLGA particles with silica could extend to asparaginase as well as a wide variety of other therapeutic enzymes and particle formulations shown in **Tables 1 and 2**. Future work will examine the impact of silica coating on the burst release kinetics of different molecular weights and monomer ratios

of PLGA, the use of gatekeeper systems^{286, 299, 300} to further control the release of enzymatic cargo, and the surface functionalization of silica for targeted delivery.

4.5.5 DEP isolation of SiLGA NPs could enable further characterization of pharmacokinetics

Figure 4.6 shows the DEP isolation of PLGA and hybrid SiLGA NPs loaded with BSA-conjugated AF594 and BSA-conjugated AF647 from buffer and plasma, respectively. Qualitative data indicates that there is sufficient contrast in the dielectric properties of PLGA and SiLGA NPs relative to suspending media to generate DEP forces that enable NP isolation from static fluid. Overall, these data show that DEP chip technology is a viable tool for characterizing and optimizing formulations of PLGA and SiLGA NPs, and that DEP could potentially be used to characterize DDS pharmacokinetics after IV delivery.

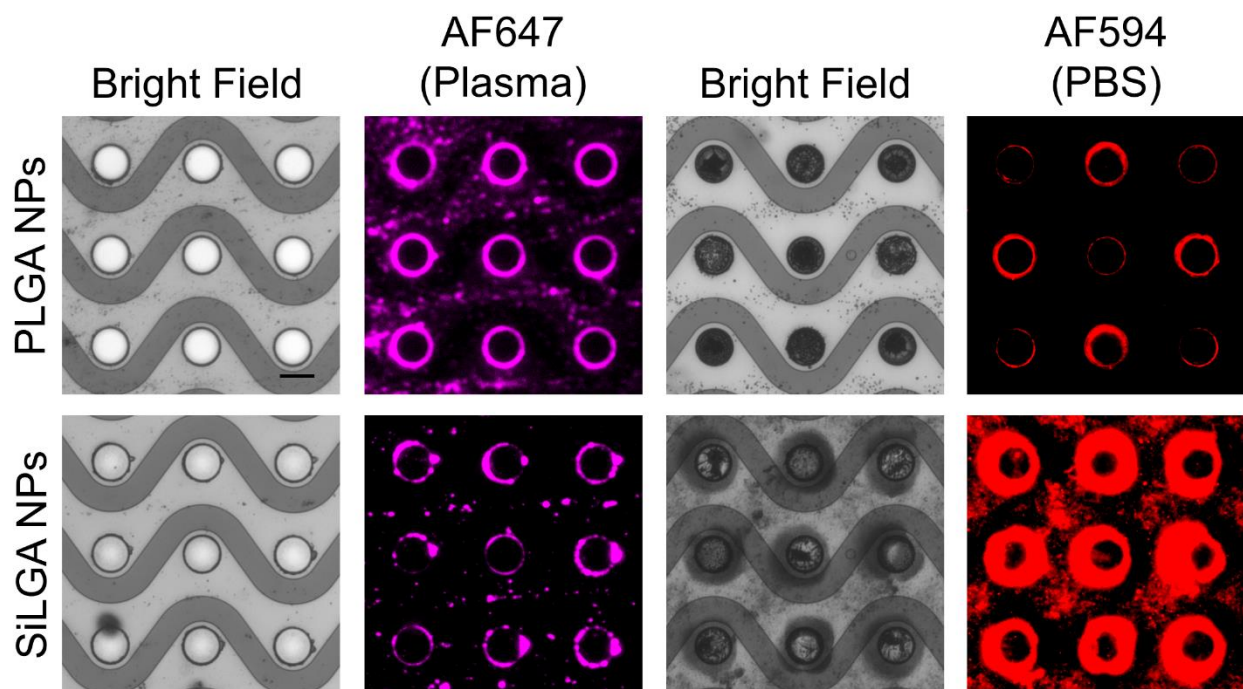


Figure 4.6. DEP collection of BSA-loaded SiLGA and PLGA NPs. BSA-AF594 was loaded into SiLGA and PLGA NPs (pink), which were spiked into human plasma and recovered using the DEP chip. BSA-AF647-loaded SiLGA and PLGA NPs (red) were isolated directly from PBS using DEP. Scale bar is 60 μm .

DEP isolation of NP DDSs from high conductance fluids offers new avenues for NP characterization. For instance, on-chip activity assays could be used to confirm enzyme encapsulation. If small molecule substrates inwardly diffuse through the silica layer into the NP core, fluorescent products should remain within NPs long enough to detect fluorescence at high-field regions prior to outward diffusion outside the NPs. Temporal analysis of fluorescence patterns at the high-field region may indicate the inward diffusion of chromogenic substrates, the catalysis of substrates to fluorescent products, and the outward diffusion of products into the fluidic chamber. These data would offer unique insights into the catalysis rates of encapsulated enzymes. In addition, quantitative techniques from Chapter 2 could generate standard curves for

enzymatic activity versus NP concentration. Using these standard curves, one could determine the therapeutic half-life of DDSs after IV injection for the patient-specific optimization of drug administration and dosing.

Considering limitations on collection volume, DEP could be especially useful for analyzing blood samples from mice to characterize NP clearance after IV injection. In an effort to optimize the design of NP DDSs, several NP formulations (*e.g.* combinations of molecular weights, monomer ratios, coatings, sizes, *etc.*) could be administered to mice through IV injection. NPs could be recovered from those samples using DEP at multiple timepoints after injection. Circulating levels of each formulation could be determined by measuring levels of the encapsulated fluorescent cargo at high-field regions on the DEP chip. Alternatively, NP concentrations could be determined using chromogenic substrates that are specific to encapsulated enzymes, as previously described. The higher the on-chip fluorescence level of the formulation, the less susceptible those NPs were to clearance through that timepoint. This information could be useful in assessing the viability of different NP formulations as IV DDSs.

4.6 Conclusion

This study introduces a new drug delivery platform for the protection and controlled release of enzymes. Enzymes are encapsulated in PLGA NPs using a water-oil-water double emulsion technique. PLGA NPs act as scaffolds onto which uniform, porous layers of silica are deposited, forming SiLGA NPs. The activity of encapsulated enzymes is retained due to the porosity of the silica layer. Small molecule substrates

permeate the silica and are subsequently catalyzed by encapsulated enzymes.

Successful coating of silica was confirmed using TEM, DLS, and NTA. I also measured the enzymatic activity of wash buffers to determine the amount of enzyme lost during particle synthesis. Loading efficiency and content were calculated using measurements of enzyme loss during synthesis. SiLGA NPs offer encapsulated enzymes substantial protection against proteolytic cleavage *in vitro* compared to PLGA NPs. The addition of silica also slows the initial burst release of enzymatic cargo *in vivo* after IM injection, possibly by reducing the rate of PLGA hydrolysis. The advantages of SiLGA NPs could easily extend to a variety of FDA-approved therapeutic enzymes that exhibit a short elimination half-life *in vivo*.

Future work will examine the impact of polymer molecular weight, polymer monomer ratios, and gatekeeper systems on the controlled release of enzymatic cargo from SiLGA NPs. FDA-approved formulations of asparaginase will be encapsulated within SiLGA NPs and administered to mice *via* IV and IM injection. Circulating Asn levels will be monitored to determine whether encapsulation enhances asparaginase activity after delivery. Future work will also utilize DEP chip technology to elucidate rates of Asn catalysis and to identify new formulations of SiLGA NPs that exhibit viable circulation half-lives.

Chapter 5. Conclusions and future directions

5.1 Conclusions

The studies outlined in this thesis establish quantitative techniques and multiplex assays for the proteomic profiling of exosomes and other extracellular vesicles collected directly from plasma using dielectrophoresis-based particle separation technology for liquid biopsy-based early cancer detection. These studies also explore the development of a therapy for treating early-stage nonresectable tumors *via* a hybrid nanoparticle drug delivery system designed to enhance cancer-dependent amino acid depletion.

The clinical utility of DEP chip technology depends on acquiring reliable quantitative data from liquid biopsy biomarkers. The first project (Chapter 2) described the design of a software algorithm to quantify fluorescently-labeled biomarkers collected at high-field regions on the DEP chip and development of an internal standard to reduce chip-to-chip variability due to differences in collection efficiency. These techniques allow direct comparisons between samples from different patients, which is necessary for clinical blood-based.

The second project (Chapter 3) utilized the quantitative techniques from Chapter 2 to establish two methods for cycIF profiling of proteomic content in cell-derived NPs and exosomes from cancer patient plasma. These techniques demonstrated the multiplex assessment of biomarker levels and could enable future implementation of target panels with the sensitivity and specificity necessary for population-wide screening.

Furthermore, the full benefits of early cancer detection are seen when viable treatment options with manageable side effects are available for the treatment of early-stage tumors. To that end, my final project (Chapter 4) developed and characterized a DEP-responsive hybrid SiLGA NP DDS that was intended to enhance the activity of therapeutic enzymes used to treat nonresectable tumors. DEP is a viable tool for characterizing pharmacokinetics and biodistribution in order to optimize the design of these NP DDSs.

Overall, these projects demonstrate the utility of DEP chip technology as a platform for isolating, quantifying, and characterizing exosomes and drug-delivery NPs, which serve critical roles in the detection and treatment of early cancer. In this chapter, I summarize results from each project and outline future work.

5.2 Summary

5.2.1 Quantification used to define DEP detection limit, dynamic range, and collection efficiency

Biomarkers collected from liquid biopsies using DEP are immunofluorescently labeled to identify associated target proteins. I designed an image processing algorithm that quantifies fluorescence levels of immunolabeled target proteins. This algorithm identifies ROIs from a brightfield image based on the geometry of the microelectrode array at the base of the DEP chip. Unusable ROIs that contain optical artifacts are automatically removed, and the set of usable ROI locations is mapped onto the

corresponding fluorescence image. The algorithm calculates the noise level of the BG region adjacent to each usable ROI and subtracts that noise level from each pixel within the ROI. The average noise-subtracted pixel intensity for each ROI is then calculated. ROIs that contain contaminating fluorescent artifacts are removed from downstream calculations. The algorithm then calculates the mean of the average noise-subtracted pixel intensity across all usable ROIs, yielding a single numeric value referred to as “fluorescence intensity”. The algorithm is fully automated and capable of analyzing multiple images successively without user input. All data generated during image analysis are saved as reports for the user, which can be accessed for downstream analysis.

Reports contain information about data quality that are necessary to interpret fluorescence intensity values: “ROI contribution” and pixel saturation rate.

Characterization of the algorithm revealed that values with an ROI contribution of at least 90% and a pixel saturation rate of less than 1.5% fell within the linear dynamic range spanning 1 a.u. to 2000 a.u. Therefore, the algorithm established a lower limit of detection of 1 a.u. and an upper limit of 2000 a.u. Full automation of the algorithm eliminated analytical user bias. Implementation of a complementary analytical technique, referred to as the internal standard, reduced the variability in fluorescence intensity caused by differences in chip collection efficiency. By measuring levels of the internal standard, fluorescence levels of biomarkers collected on the same DEP microelectrode array can be adjusted for the relative strength of the particular chip. Use of the internal standard reduced variability across technical replicate samples.

5.2.2 *CyclIF enables multiplex proteomic profiling of biomarkers collected using DEP*

Previous studies that used DEP chip technology reported analysis of up to two biomarkers per sample, limited by the number of fluorophores and optical filters available for fluorescence imaging. My work to design such an assay employed cyclIF imaging to profile the proteomic content of biomarkers collected on the DEP chip, thereby applying a tissue biopsy analytical technique to increase the multiplicity of liquid biopsy assays. Six target proteins were identified. Antibodies against each target protein were conjugated with either AF488 or AF647. A pair of one AF488-conjugated antibody and one AF647-conjugated antibody were incubated with biomarkers collected using DEP. After washing, imaging, and quantifying fluorescence in each channel, fluorophores were quenched using a combination of chemicals and high-intensity light exposure. The sample was then immunolabeled with another pair of antibodies against two different target proteins, and the process was repeated. Three rounds of cyclIF profiling were achieved, tripling the maximum number of target proteins analyzed per small-volume liquid biopsy using DEP chip technology. Quantitative techniques from Chapter 2 confirmed complete quenching and determined that the placement of pairs in the stain order affected fluorescence levels of certain target proteins.

5.2.3 Encapsulation within SiLGA NP protects enzymes against proteolysis and slows their release

Once detected, early-stage cancers require therapies that are less toxic and more targeted than broad-spectrum chemotherapeutics, which are usually used to treat later-stage tumors. One such technique is amino acid depletion therapy, which has some associated challenges. Cancer patients receiving therapeutic enzymes for amino acid depletion are directly affected by low circulation half-lives and immune responses to therapy. I encapsulated enzymes within a hybrid NP DDS to protect enzymatic cargo from proteolytic degradation and to slow the initial rate of cargo release after delivery. The hybrid NP DDS consisted of silica and PLGA, two biocompatible and biodegradable materials. The SiLGA NPs were sub-200 nm, exhibited high loading efficiency, and elicited a strong response to DEP forces when suspended in buffer and plasma. PLGA encapsulation appeared to have no protective effect against proteolytic degradation of enzymatic cargo; however, enzymes encapsulated within SiLGA NPs retained almost half of their activity after incubation with proteinase K. The protective effect suggested that holes permeating the silica layer were sufficiently large to permit inward diffusion of substrate molecules yet sufficiently small to hinder inward diffusion of proteinases and/or outward diffusion of enzymatic cargo. Data also suggested that enzymatic cargo was able to withstand the encapsulation and coating processes. While both PLGA and SiLGA NPs slowed the rate of cargo release after IM injection in mice relative to unencapsulated protein, SiLGA PLGA NPs exhibited a slower rate of cargo release one day after injection. Reduced cargo release and protection against proteolysis could

feasibly enhance asparaginase delivery in ALL patients, potentially reducing the frequency of administration and improving therapeutic efficacy. Furthermore, the response of PLGA and SiLGA NPs to DEP highlights potential downstream applications for monitoring the circulation and half-life of NP DDSs after IV delivery.

5.3 Future Directions

The techniques for quantification and multiplex labeling of liquid biopsy samples presented in this work are intended to identify changes in blood-based biomarker profiles over time, ultimately characterizing differences between common malignant and non-malignant patient trajectories. Here, I present additional labeling, detection, and quantification strategies consistent with themes from Chapters 2 and 3 that could further improve high-multiplicity DEP assays, and I describe future experiments pertaining to oligo cyc-DEP. Furthermore, I assess the utility of blood-based immune biomarkers in identifying immune escape, which is a critical timepoint for early cancer detection. Preliminary data highlights a novel application of DEP in the early detection of peritoneal metastasis, as early detection of metastatic regions is an important aim of early cancer detection that is often overlooked. I also outline future experiments to characterize the clinical utility of hybrid SiLGA NPs as a DDS, which include the encapsulation of FDA-approved asparaginase and the use of DEP chip technology to optimize enzymatic cargo release.

5.3.1 Antibody-oligo conjugates for on-chip cycIF

With regard to the work presented at the end of Chapter 3, next steps would be to determine the advantages and disadvantages of oligo cyc-DEP over cyc-DEP. A method is presented for the quick hybridization and dehybridization oligo-fluorophore conjugates to complementary oligo-antibody conjugates, which label proteins on NPs that are collected using DEP. Oligos conjugated to antibodies are referred to as DSs, while complementary oligo-fluorophore conjugates are called imager strands.

As shown in Chapter 3, fluorescence imaging of LNCaP cells confirmed that three unique imager strands hybridize complementary DSs bound to FASN, CD9, and PGK1 antibodies. To assess whether these hybridizations are specific, additional experiments need to evaluate the cross-reactivity of all imager strands against all DSs and validate hybridization of three additional oligo pairs that correspond to the remaining target proteins in **Table 3.1**. Experiments must also evaluate the cross-reactivity between pairs of imager strands that are introduced in the same round of staining. After assessing cross-reactivity in the oligo library, hybridization parameters (e.g. temperature, time, concentration of imager strands, presence of blocking agents, etc.) and dehybridization parameters (e.g. concentration of ethylene carbonate, duration, dynamic washing versus static incubation, etc.) need to be optimized for on-chip cycIF. Oligo cyc-DEP assays will be performed on the same samples that were analyzed using cyc-DEP. Analogous datasets will enable direct comparison between the two methods for on-chip cycIF. An objective evaluation of relative advantages and disadvantages will indicate which method is more suitable for a clinical environment.

It is expected that the oligo-based method will be well-suited for the clinic, while the chemical quenching method will be more feasible for research labs. Due to the added specificity of complementary oligo hybridization, all targets can be immunolabeled in a single incubation period using the oligo-based method. This single incubation will almost certainly require less time, reduce sample loss, and minimize the impact of round order on observed fluorescence levels. Furthermore, complete hybridization and dehybridization can be achieved within minutes. Preliminary data therefore suggests that the oligo-based method will yield clinically viable sample-to-answer times and more reliable quantification. A six-target panel could be analyzed in under six hours using the oligo-based method. This same panel would require over twenty-four hours using the chemical quenching method. The primary advantage of the chemical quenching method is that it can be achieved using commercially available supplies without the need to design unique oligo sequences and chemically modify antibodies. A secondary advantage is that the chemical quenching method is generally more affordable than the oligo-based method; however, the financial benefits come at the expense of longer sample-to-answer times.

5.3.2 Automation of DEP assays

This thesis presented the kinds of quantitative techniques that would be necessary for the clinical translation of DEP chip technology. One of the functional benefits of the algorithm in Chapter 2 is its automation, which eliminated analytical user bias and substantially reduced user burden. Benefits of automation should extend to

sample preparation (e.g. by centrifuging whole blood, collecting plasma, adding the internal standard, diluting antibodies for on-chip immunoassays, etc.) and conducting the assay (e.g. by transferring the sample to the microfluidic chambers, applying alternating current, washing, blocking, immunolabeling, imaging, quenching, and eluting the sample for downstream analysis). Automation of sample preparation, on-chip immunoassays, and image analysis would enable DEP chip technology to be scaled massively. Human error would be substantially reduced and conducting assays would require little training or time on behalf of users, ultimately making DEP data more robust. Automation could also reduce the cost per assay, permitting analysis of additional technical replicates, which would improve overall data quality.

5.3.3 Narrow emission-band fluorophores for DEP analysis

Chapter 3 demonstrate two-channel cyclIF imaging of biomarkers collected using DEP. One way to increase the number of target analytes per round of on-chip cyclIF staining would be to use fluorophores with narrower, discrete emission bands like quantum dots³⁰¹. For example, instead of labeling two targets in each round with a pair of antibodies (e.g. one conjugated to AF488 and another conjugated to AF647), three or more targets could be labeled using three or more respective antibody-fluorophore conjugates. This would require each fluorophore to have unique emission spectrum that neither prompts, nor overlaps with, the emission of other fluorophores. Overlap in excitation spectra should not be problematic, assuming use of proper optical filters. A larger library of spectrally isolated fluorophores would require unique optical filters

corresponding to each respective fluorescence channel. Depending on the emission peaks, custom optical bandpass filters may be necessary.

5.3.4 Hyperspectral imaging for DEP analysis using large fluorophore libraries

In hyperspectral imaging³⁰², fluorescence emission spectra spanning a wide range of wavelengths are acquired at every pixel in the field-of-view using a series of narrow-band fluorescence images. This is in contrast to conventional fluorescence imaging in which each pixel represents the amount of light that passes through the particular broad-band optical filter, or fluorescence channel. The spectral resolution is significantly higher in hyperspectral imaging than conventional fluorescence microscopy, eliminating the need for unique broad-band optical filters that correspond to particular fluorophores. The main benefit of hyperspectral imaging is its immediate and broad applicability to a variety of fluorophore libraries.

While the image processing algorithm in Chapter 2 was designed for conventional fluorescence images, modifications could be made to a portion of the algorithm to accommodate hyperspectral imaging. The first modification would be a pre-processing step that assembles fluorescence spectra across a set of narrow-band fluorescence images at each pixel location. The second modification would be a new approach to measuring BG noise levels. The spectra for each BG pixel that is locally adjacent to an ROI would be averaged, creating a smooth composite BG spectrum. The composite BG spectrum would then be subtracted from the spectrum at each pixel in the adjacent ROI. The BG-subtracted spectrum of each ROI pixel would be averaged,

and the resultant composite spectra for each ROI would be then averaged. This last step would generate a smooth, noise-subtracted spectrum that represents the average pixel across all usable ROIs in the hyperspectral image.

This overall composite ROI spectrum would contain up to n local maxima corresponding to the relative amounts of n analytes labeled with n non-overlapping fluorophores. Quantification could be as straightforward as measuring the intensities of local maxima in the overall composite ROI spectrum. Hyperspectral imaging could be used with either method for on-chip cyclIF profiling of captured biomarkers presented in Chapter 3 and with any library of fluorophores like narrow emission-band quantum dots.

5.3.5 Temporal analysis for phenotyping blood-based biomarker profiles

Doctors recommend blood tests to patients at least once per year during routine checkup appointments. It is essential that blood-based panels for cancer screening be included in these annual tests. This is because temporal, patient-specific analysis of circulating biomarkers will be necessary to establish blood phenotypes that parallel malignant neoplastic progression, which is a dynamic process taking place over several years. Rates of change in biomarker concentrations over time might reflect rates of neoplastic tissue growth or the onset of specific processes involved in tumorigenesis. Temporal monitoring of these rates of change will enable retrospective analysis, which will be the foundation for population-wide blood-based screening. The ability to differentiate malignant and precancerous or healthy phenotypes will require pattern recognition in biomarker profiles of patients that follow similar disease trajectories. Once

those phenotypes have been established, a physician can compare a patient's biomarker profile with established phenotypes to assess the patient's likeliest disease trajectory. Downstream screening, diagnosis, and treatment options could be planned years in advance. While multi-omic, high-multiplicity assays will likely be required to achieve the sensitivity and specificity necessary for blood-based cancer screening at the population level, it does no good to cast a wide net without checking that net frequently. Due to its ease of use and low-volume requirement, DEP would lend itself well to frequent blood-based screening strategies.

5.3.6 Monitoring the immune system for indications of immune escape

Neoplastic lesions that are ultimately eliminated by or in indefinite equilibrium with the immune system are not a primary concern for early detection. In fact, early detection methods should avoid these tumors due to their lack of malignant potential and likelihood of prompting overdiagnosis, overtreatment, and misallocation of resources. The tumors of interest in early detection are those with the potential to escape immune equilibrium and undergo unchecked proliferation. While the tumors that "escape" immunity may be relatively small and difficult to detect, one could instead search directly for changes to the immune system that indicate immune escape, like the downregulation of anti-tumor activation ligands or upregulation of anti-tumor suppression ligands that target immune cell receptors.

Tumors expressing ligands that bind activating receptors on Natural killer (NK) cells are known to shed those ligands as free or exosome-bound proteins into

circulation, ostensibly as a smokescreen that desensitizes NK cells from recognizing and eliminating the tumor. This process has been observed for a variety of ligands that bind the NKGD2 receptor and B7H6, which binds NKp30 receptor isoforms. The elevated concentration of soluble ligands in circulation may correlate with the downregulation of those ligands at the surfaces of tumor cells³⁰³ and/or the downregulation of receptors on the surfaces of NK cells³⁰⁴, both of which could feasibly lead to decreased anti-tumor immunity. Importantly, elevated circulating levels of these soluble ligands might be the first blood-based indication of a tumor with malignant potential. Studies should be conducted to determine whether current technology is capable of detecting those ligands, and whether significant changes in ligand concentration have prognostic or diagnostic value. Specifically, immune ligands associated with exosome release are an attractive target for DEP detection. Alternatively, routine profiling of transcripts for immune cell receptor isoforms has demonstrated clinical prognostic value^{305, 306} and might be the most feasible route for monitoring tumor escape. In either case, multi-omic, multiplex panels of immune biomarkers should be analyzed temporally as a part of routine blood-testing. DEP is a technique that could be employed to detect these types of biomarker panels.

5.3.7 Strategies for DEP detection of early metastasis

Early detection of metastasis is a valuable yet often overlooked. While a primary goal of early detection is the identification of early-stage pre-metastatic tumors with malignant potential, locating borderline metastatic tumors prior to metastasis and/or

finding metastasis earlier could also increase the likelihood of successful treatment⁴. DEP-based collection could be employed in techniques like peritoneal lavage cytology (PLC)³⁰⁷⁻³¹³, which demonstrates the clinical utility of detecting metastasis earlier and informs treatment after primary tumor resection. In PLC, fluid is injected into a patient's peritoneal cavity typically during resection surgery. The wash buffer is analyzed for cancer cells, which indicate metastatic regions that were undetectable or absent at the initial diagnosis.

Adjunctive PLC has demonstrated prognostic significance in PDAC, gastric, and ovarian cancer, despite high rates of false positives and false negatives. For instance, approximately 10% of gastric cancer patients with negative PLC results ultimately exhibit peritoneal recurrence³¹⁴. Interestingly, molecular analysis peritoneal wash fluid from cancer patients improves predictions of peritoneal recurrence³¹⁰, as shown through RT-PCR amplification of mRNA for CEA³¹⁵ and multiplex molecular analysis³¹⁶ of peritoneal wash buffer. Despite these improvements, false results hinder the widespread clinical adoption of PLC, and novel approaches to molecular profiling of peritoneal wash fluid are needed.

To the best of my knowledge, exosomes and other EVs present in peritoneal wash fluid have not been analyzed or assessed for prognostic value in cancers that benefit from PLC analysis. Preliminary data in **Figure 5.1** suggests that exosomes and other EVs in peritoneal wash fluid might be viable biomarkers for DEP collection and downstream analysis. CD63/RFP-transfected A375 human melanoma cells were injected subdermally into the ears of mice (n = 3). Fluorescent xenograft tumors grew over several weeks, and some metastasized to the peritoneal cavity. Upon sacrificing

the mice, peritoneal cavities were washed with buffer in a manner similar to PLC. DEP was then applied to undiluted wash buffer. CD63/RFP⁺ fluorescent material was detected in wash buffer from the mouse with the largest metastatic tumor burden using DEP. Interestingly, fluorescent material was undetectable in the two mice without observed peritoneal metastases. This trend was consistent across two repeat experiments (each n = 3). Across three experiments, fluorescent material was detected in wash buffer from one mouse with peritoneal metastases and absent in wash buffer from two mice without peritoneal metastases.

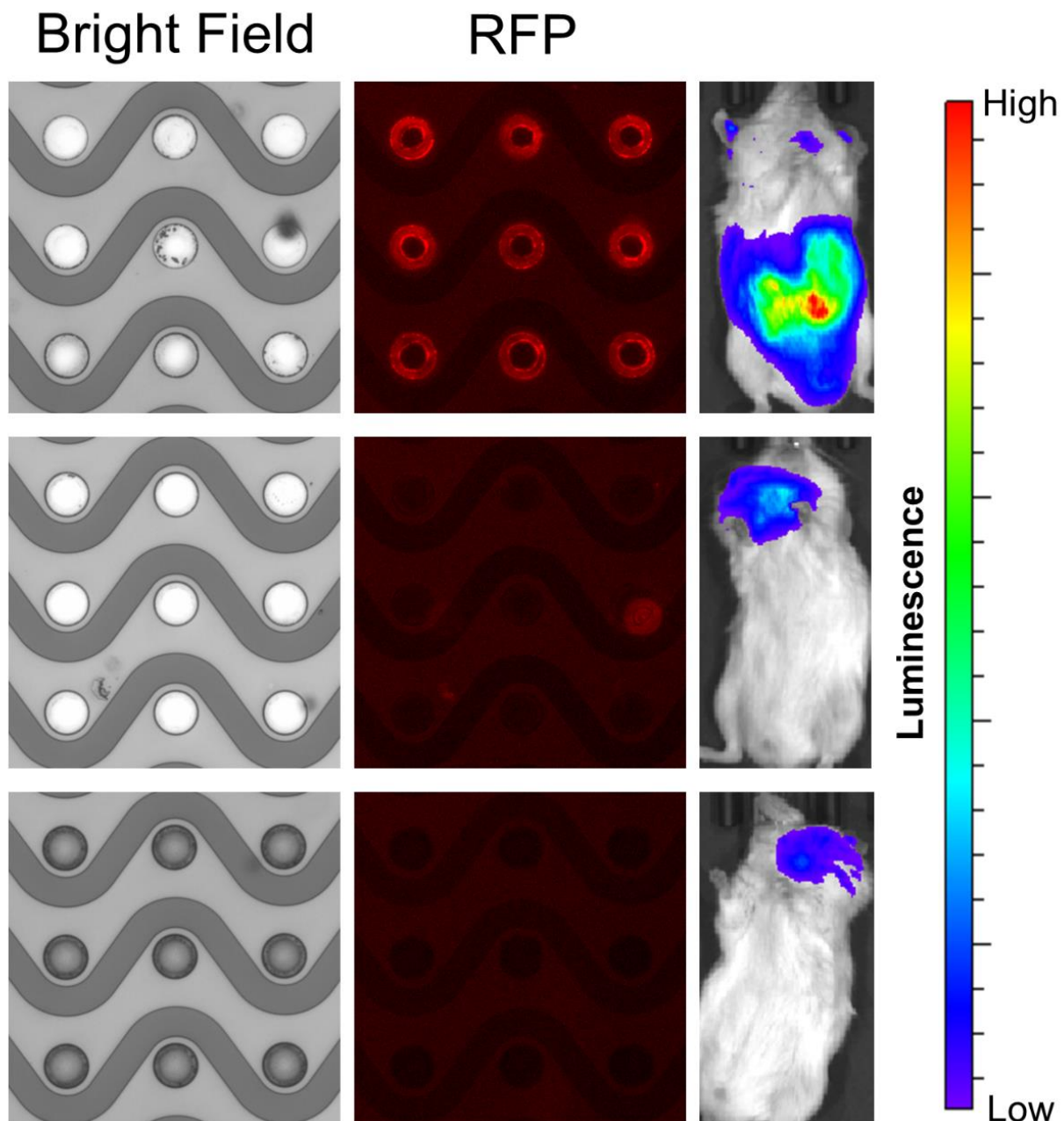


Figure 5.1. Detection of CD63/RFP+ material collected from peritoneal wash fluid. CD63/RFP-transfected A375 human melanoma cells were injected subdermally into the ears of mice ($n = 3$). Xenograft tumors grew over several weeks. Upon sacrificing the mice, peritoneal cavities were washed and analyzed using DEP. Fluorescent material was collected on the chip in one of the three mice (top), as observed through the RFP/DsRed optical filter. Detect of fluorescent material correlated with the presence of peritoneal metastasis. No fluorescent material was detected in wash fluid from mice without peritoneal metastases (middle; bottom). Trends were consistent in two repeat experiments.

There are several reasons why exosomes and other EVs likely constituted a portion, if not a majority, of the collected material. The material was not immunofluorescently labeled before or after collection, indicating that fluorescence likely originated from RFP expressed in the xenograft. RFP and CD63 were co-transfected and should have therefore been co-expressed. Furthermore, CD63 is a known marker for exosome-enriched EV subpopulations, and tumor cells secrete exosomes at elevated rates. Considering that DEP forces are significantly stronger on larger particles like exosomes than smaller particles like free proteins, it is likely that peritoneal metastases released fluorescent exosomes or other fluorescent EVs into the wash buffer at detectable levels. These data suggest that a novel application for DEP in molecular profiling. When coupled with PLC, DEP could potentially benefit the early detection of exosomes and other EVs associated with peritoneal metastasis in PDAC, ovarian, and gastric cancer patients.

5.3.8 Optimization of SiLGA NPs for enhanced enzymatic activity

In its many formulations, asparaginase is arguably the most common enzyme for systemic cancer treatment. Therefore, future experiments should focus on asparaginase encapsulation. PEGylated and non-PEGylated formulations of asparaginase will be encapsulated within PLGA and SiLGA NPs. All structural characterization and loading calculations for asparaginase-loaded NPs will be performed in a manner consistent with the data shown in Chapter 4. Future work should assess the retention of asparaginase activity after incubation with PK using an appropriate chromogenic substrate like a

modified Asn. To generate drug release curves, one could monitor the outward diffusion of encapsulated asparaginase over time using a dialysis membrane. Future *in vivo* experiments could deliver encapsulated formulations of fluorescent asparaginase intramuscularly. IM levels of asparaginase could be monitored using IVIS imaging. Circulating levels of fluorescent asparaginase and Asn could be monitored periodically using fluorescence and chromogenic assays. Similar experiments could be performed to assess the feasibility of IV delivery. Upon sacrificing mice, biodistribution studies should analyze predominant routes of NP clearance.

Other experiments should focus on optimizing the monomer ratio and molecular weight of PLGA to slow hydrolysis. Based on biodistribution data, NP size should be optimized to reduce clearance. By functionalizing the silica layer at the NP surface, gatekeeper systems could further control the release of enzymatic cargo. Alternatively, surface functionalization could enable targeted delivery or selective uptake of SiLGA NPs at the tumor. Furthermore, it would be interesting to model the DEP response of a multilayered core-shell NP like the SiLGA DDS presented in Chapter 4 and to compare simulations with experimental data.

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