Title
Microfluidic Approaches to Multiplexing Heterogeneous Protein Assays

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Microfluidic Approaches to Multiplexing Heterogeneous Protein Assays

by

Rachel E. Gerver

DISSERTATION

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Abstract

While genetic sequencing and other technologies have enabled the investigation of DNA and RNA at an unprecedented scale, new tools are needed to more rapidly advance proteomics research. New protein detection technologies can enable improved disease biomarker identification, more efficient antibody epitope mapping for vaccine development, better point of care diagnostic assays, and accelerated drug discovery. Microfluidic approaches to protein measurements can offer advantages over benchtop methods in terms of faster assay times, increased multiplexing, reduced consumption of sample and reagents, compatibility with full automation, and potential for implementation even in locations with limited lab infrastructure.

This dissertation presents the development of three novel microfluidic platforms for multiplexed protein detection in biological samples:

1) A microfluidic Western blot for low-molecular-mass proteins that enables the separation of proteins down to 6.5kDa with 40% higher separation resolution and a >100-fold improved signal to noise ratio in small-pore-size gels compared to previous approaches.

2) A microfluidic device for synthesizing spectrally encoded microspheres for large scale parallel peptide synthesis and other biological multiplexing applications with the potential to code up to millions of uniquely identifiable microspheres through the use of lanthanide nanoparticles.

3) Initial development towards a microfluidic tool for PSA glycan specific isoform identification for improved prostate cancer diagnosis and prognosis.
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Chapter 1: Introduction

Microfluidics offers opportunities for increased multiplexing in the detection of proteins whether it be with size-based\textsuperscript{1-5} or isoelectric point\textsuperscript{6-8} separations or the ability to probe large numbers of samples in parallel.\textsuperscript{9,10} Microfluidic tools enable the rapid detection of biological targets while utilizing significantly smaller sample volumes and reagent consumption than traditional bench-top assays. The laminar flow in microfluidic channels also allows for more precise control over the fabrication of small scale features.\textsuperscript{11-13} Combining these capabilities in microfluidics opens the door to high throughput and high multiplexed assays for biological discovery and diagnostic applications. This dissertation describes the development of a microfluidic device for synthesis of spectrally encoded beads for parallel peptide synthesis and biological multiplexing applications, innovations to extend the detectable molecular range of a microfluidic Western blot assay down to lower-molecular-mass targets, and initial work towards a microfluidic tool to identify glycan specific isoforms on prostate specific antigen for prostate cancer diagnosis and prognosis.

Traditional benchtop assays including slab-gel Western blotting and slab-gel isoelectric focusing combined with immunoprobing are powerful tools for identification of proteins in complex biological samples such as blood, saliva, or cell/tissue lysates.\textsuperscript{14-17} The separation by molecular weight or isoelectric point provides greater specificity and higher multiplexing than antibody probing alone and also enables the identification of unique isoforms that may bind to the same antibody. The drawback to these approaches is that the separation step adds significant time and labor to the process as compared to ELISAs, which are routinely run in 96-well plates in fully automated benchtop systems. The use of microfluidic approaches that include protein separation and immunoprobe within a single integrated channel can enable fully automated systems that offer the specificity and multiplexing of Western blotting with the high throughput and hands-free operation of an ELISA, giving researchers the
opportunity to run larger numbers of samples to accelerate proteomic discovery. This full automation would be substantially more challenging in a slab-gel configuration where multiple transfer steps require hands-on interaction including sample loading, separation, transfer to a membrane, and antibody probing.

Microfluidic approaches also offer substantial reduction in sample consumption. The microfluidic separations in this dissertation separate nanoliters of sample instead of the microliters required in a slab-gel IEF or Western blot. This can enable far more tests to be run on a limited supply. Further advances in interfacing with microfluidic channels could allow samples as low as 1-10nL to be effectively separated and immunoprobed, in contrast to the 1-20µL typically recommended for use in a benchtop slab-gel Western blot configuration. The shorter distances in a microfluidic channel and the higher electric fields enabled by the high surface area to volume allow more rapid separations that can be completed in seconds or minutes instead of hours. Advances in microfluidics that enable µWestern blotting to extend down to lower MW targets as well as development towards a microfluidic IEF assay to identify glycan specific isoforms of prostate specific antigen as a tool for cancer diagnosis and prognosis will be presented.

Multiplexed detection of many targets in a sample of interest can enable the use of much smaller sample volumes and a higher throughput pipeline. For example, the use of 100,000 uniquely barcoded 50 µm microspheres with attached peptides can enable the simultaneous detection of 100,000 targets with beads that take up a volume of < 10µL. In contrast, detecting these targets individually in a traditional 96-well plate ELISA would require over 1000 plates and over 100mL of sample volume if utilizing 1µL sample per well. Existing commercially available encoding schemes, such as Luminex beads spectrally encoded with fluorescent dyes are only available with up to 500 unique codes, limiting the scale of proteomics studies utilizing this approach. Academic publications, while promoting higher numbers of theoretical codes, are limited in applicability for large scale parallel
peptide synthesis either due to issues of code stability, high cost per encoded microcarrier, long time scales needed to generate each code, large microcarrier size, or incompatibility with FMOC peptide synthesis reagents. New advances in encoding methods are needed to enable large scale ($10^5$-$10^6$) parallel peptide synthesis for protein-peptide binding studies. As will be described in this dissertation, utilizing a microfluidic device to mix in ratios of lanthanide nanoparticles into polymer droplets enables synthesis of spectrally encoded spheres with microcarrier production on the order of milliseconds in a fully automated platform with material costs on the order of $1/\text{gram}$ and a theoretical code capacity in the millions. These microspheres can then be utilized for large scale parallel peptide synthesis and subsequent protein-peptide binding assays.

Through novel approaches to microfluidic devices, proteomics research can be further advanced by enabling rapid, multiplexed, specific detection of proteins and protein isoforms in low sample volumes. This opens the door to improved biomarker discovery, more efficient antibody epitope mapping for vaccine development, better point of care diagnostic assays, and easier mapping of signaling pathways throughout biology. New analytical tools are needed to fully realize the potential of proteomics research as genetic sequencing technology has done for the genomics revolution.
References


Chapter 2: Microfluidic Western Blotting of Low-Molecular-Mass Proteins

The following section (3.1) of the dissertation is reproduced from “Microfluidic Western blotting of low-molecular-mass proteins” in Analytical Chemistry by Rachel E. Gerver and Amy E. Herr. Amy E. Herr supervised research on the project and research was conducted by Rachel E. Gerver. This section will be followed by a more in-depth discussion of the development of the low-molecular-mass µWestern blot leading to the work described here, as well as additional data not yet published, and recommended future steps in development.

2.1 Publication on Microfluidic Western Blotting of Low-Molecular-Mass Proteins
Rachel E. Gerver and Amy E. Herr

2.1.1 Abstract

We describe a microfluidic Western blot assay using a Tris tricine discontinuous buffer system suitable for analyses of a wide molecular mass range (6.5 – 116 kDa). The Tris tricine µWestern is completed in an enclosed, straight glass microfluidic channel housing a photopatterned polyacrylamide gel that incorporates a photoactive benzophenone methacrylamide monomer. Upon brief UV light exposure, the hydrogel toggles from molecular sieving for size based separation to a covalent immobilization scaffold for in-situ antibody probing. Electrophoresis controls all assay stages, affording purely electronic operation with no pumps or values needed for fluid control. Electrophoretic introduction of antibody into and along the molecular sieving gel requires that the probe must traverse through (i) a discontinuous gel interface central to the transient isotachophoresis used to achieve high performance separations and (ii) the full axial length of the separation gel. In-channel antibody probing of small molecular mass species is especially challenging, as the gel must effectively sieve small proteins while...
permitting effective probing with large molecular mass antibodies. To create a well-controlled gel interface, we introduce a fabrication method that relies on a hydrostatic pressure mismatch between the buffer and polymer precursor solution to eliminate the interfacial pore-size control issues that arise when a polymerizing polymer abuts a non-polymerizing polymer solution. Combined with a new swept antibody probe plug delivery scheme, the Tris tricine μWestern blot enables 40% higher separation resolution, destacking of proteins down to 6.5kDa, and a 100-fold better SNR for small pore gels, expanding the range of applicable biological targets.

2.1.2 Introduction

Western blotting comprises an indispensable analytical tool for both research and clinical laboratories.1–6 In conventional Western blots, slab-gel electrophoresis forms the basis for protein sizing. Antibody probing is conducted after transfer of protein bands from the small pore-size polyacrylamide gel (i.e., 19nm to 140nm pores for 3.5-10.5% total monomer (%T) and 0.5-10% cross-linker (%C) gels)7 to a larger-pore size polymer membrane (e.g., PVDF or Nitrocellulose with 200nm to 450nm size pores).8 Proteins are immobilized on the membrane via hydrophobic interactions.9 Immobilization of protein bands on a large pore-size membrane facilitates antibody-based probing of the immobilized species with large molecular mass antibodies.1 In effect, the design of conventional Western blotting decouples pore-size demands required for effective molecular sieving during polyacrylamide gel electrophoresis (PAGE) from specifications for effective probing.

Microfluidic design affords faster assay times, smaller sample volumes and easier integration with automation systems than conventional slab-gel systems. Building on these advantages, protein separations from capillaries,10 microfluidic chips,11 and a microarrayer12 have been interfaced to blotting membranes for probing. In another approach to full integration, a capillary system supports the protein separation (mass or pI) and subsequent photocapture and immunoprobing of antigens on the capillary
The commercial capillary Western blot completes in 3 hrs. While an advance in integration, the assay sees low protein immobilization efficiencies (~0.01%) and substantial hardware (fluid pumping, high voltage, and robotic control for multiplexing). In an alternate approach using a planar glass microdevice, protein separation and probing steps are integrated on chip yielding assay times as fast as 3 min. A microchamber patterned with functionalized polymers forms the basis for assay integration. Nevertheless, the approach requires complex fabrication protocols that limit throughput and multiplexing capabilities.

Using a planar microfluidic device, our group reported on a single microchannel Western technique that unifies protein separation and probing in a single microfluidic channel. The simple single channel design provides a basis for scale up and multiplexing. In order to integrate the assay stages and obtain high immobilization efficiencies, the microWestern makes dual-use of the separation axis: the axis forms the molecular sieving dimension during PAGE and also forms the path for antibody probe introduction (e.g., during probing, antibody is introduced through the protein separation gel).

While the in-channel probing strategy underpins completion of Western blotting in a single microchannel, the approach poses a particular challenge for small molecular mass species. The gel must both effectively sieve small proteins during PAGE and also allow large antibodies to electromigrate through the gel pores during probing. As such, the standard Tris glycine microchannel Western blot is limited to analyses of proteins larger than ~21kDa, as smaller species remain stacked between the leading and terminating electrolyte in the 7.5%T gel. While use of a higher %T gel allows destacking of smaller MW species, the small pore-size gel traps antibodies at the separation gel interface, thus substantially reducing immunoprobing signals. To expand the applicability of microfluidic Western blotting to smaller molecular mass proteins, we describe a new fabrication technique that reduces confounding interactions of the gel with large antibody probes, as well as transition to assay conditions relevant to smaller species.
2.1.3 Materials and Methods

**In-channel gel fabrication** Microchannel designs are completed in-house, then fabricated using standard wet glass etching at a glass foundry (PerkinElmer).\(^{20}\) Separation channels are 1 cm long, 10 µm deep and 70 µm wide. Each well pair is connected by three parallel separation channels for technical triplicates. Prior to gel fabrication, channel walls are functionalized with acrylate monomers, as previously described,\(^{21}\) to enable gel cross-linking to the channel walls.

The separation gel precursor solution is composed of acrylamide/bisacrylamide at a ratio of 37.5:1 (Sigma-Aldrich A3699) diluted to a final %T between 8 and 12 as indicated in the text. To enable protein photocapture, 1.5mM N-[3-[(4-benzoylphenyl)formamido]propyl]methacrylamide (BPMAC, C\(_{22}\)H\(_{22}\)N\(_2\)O\(_3\) 350.2 g/mol) is added from a stock solution of 100mM in DMSO. BPMAC is synthesized in house.\(^{22}\) The gel precursor buffer is 500mM Tris HCl titrated to pH 8.45 for the Tris tricine discontinuous buffer system and 375mM Tris HCl titrated to pH 8.8 for the Tris glycine discontinuous buffer system, consistent with the Tris HCl pH typically used in slab gels for each respective buffer system.\(^{23}\) These components are degassed in a sonicator under vacuum for 3 min. After degassing, sodium dodecyl sulfate (SDS) (0.1% final concentration) and Triton X-100 (0.1%) are added along with the initiators riboflavin5’ monophosphate (0.0006%) (F1392, Sigma-Aldrich), TEMED (0.05% vol/vol) (T9281 Sigma-Aldrich), and ammonium persulfate (0.015%) (A3678 Sigma-Aldrich). Gel precursor is applied to one well, with capillary action wicking the solution into the dry microchannels. In the original protocol,\(^{19}\) after gel precursor loading, glass chips are submerged in a petri dish with gel buffer precursor solution with SDS (0.1%) and Triton X-100 (0.1%) to prevent flow in the channels and remove gel precursor from the wells. In the alternate protocol utilized here (unless otherwise noted), the gel precursor in the well is replaced with buffer solution after gel loading but before submerging the chip, so as to generate a buffer/gel precursor interface part way through the channel (detailed in the Results and Discussion section). For the comparison of antibody plug to antibody front probe approaches, additional buffer is added to the opposite well to
subsequently migrate the gel precursor back towards the center of the channel and establish a buffer/gel precursor interface on both ends of the gel in the channel. While submerged, chips are photopolymerized using a collimated blue LED source (470nM M470L2, Thorlabs) at 300 lumens (Sper Scientific 840022 Advanced Light Meter) for 6 min. After fabrication, the chip is stored in gel buffer solution with 0.1% SDS and 0.1% Triton X-100 until use. In the case of the DHEBA gel, the acrylamide/bisacrylamide solution is replaced with acrylamide monomer and a N,N’-(1,2-Dihydroxyethylene)bisacrylamide (DHEBA) cross-linker (Sigma Aldrich 294381) at a molar ratio equivalent to the 37.5:1 bis/acrylamide for a final concentration of 12%T, 3.5%C acrylamide/DHEBA.

**Sample and antibody preparation** A protein ladder is used to optimize the assay, with the ladder consisting of Alexa 488 conjugated proteins: B-galactosidase (116kDa) (Sigma-Aldrich G8511) (labelled in house), Bovine Serum Albumin (66kDa), (Life Technologies A13100, prelabelled), Ovalbumin (45kDa) (Life Technologies 34781, prelabelled), C-reactive protein (25kDa) (labelled in house), and Aprotin (6.5kDa) (Santa Cruz Biotechnology sc-3595) (labelled in house) is utilized. Proteins are labelled using Alexa Fluor 488 Protein Labelling Kit (Life Technologies A10235) following the package protocol.

Prior to Western blotting, the sample is prepared in a buffer consisting of 2% SDS, and 100mM of the reducing agent dithiothreitol (DTT), then heated for 3min at 90C. Finally, 500mM Tris HCl pH 6.8 is added to bring the sample to 50mM Tris HCl pH 6.8 prior to analysis. Target sample consists of purified PSA (Calbiochem 539834) at either 300nM or 600nM concentration, as indicated in the text, and probed using a polyclonal PSA antibody (Fisher Scientific AF1344). Antibodies are labelled using Alexa 568 (Life Technologies A10238) following the package protocol.

**Sample separation** Voltage is applied to the chip using platinum electrodes attached to a custom built, eight channel high voltage power supply with current/voltage feedback control. To load sample onto the chip, 2.3µL of sample is pipetted into a well and electrophoresed into the channel at 1.5µA
(~11V) (1.0µA, ~11V for Tris glycine) for 80 s. The well is then washed out with the terminating electrolyte run buffer consisting of 0.1% Triton X-100, 0.1% SDS, 3% DMSO with either 1X Tris glycine (25mM Tris, 192mM glycine Bio-Rad 161-0734) or 1X Tris tricine (100mM Tris, 100mM tricine, Sigma-Aldrich T1165) and a fixed current applied across the channel to stack the injected plug, via transient isotachophoresis, and then size the sample species. Sizing uses a fixed current of 1.5uA for Tris tricine and 1.0uA for Tris glycine systems, both of which result in a voltage ramp of ~25-55 V/cm during separation. For Tris glycine separations, 0.3uA is applied during stacking (~4-8V/cm ramp) followed by 1uA for separation once the proteins entered the gel, as this yields slightly improved stacking and separation performance compared to applying a 1uA continuous current. For the Tris tricine system, a 1.5uA current is applied during both the stacking (~12-25V/cm ramp) and sizing phases.

**Protein blotting** Protein capture on the photoactive gel is performed using a Hamamatsu Lightening Cure LC5 UV source through a light guide, with the gel exposed for 30-45 s at 100% intensity. After photocapture, unconjugated proteins are electrophoresed out of the channels by applying a reverse voltage for 10min at 100V/cm. During this step, both wells are filled with Tris tricine SDS buffer. The Tris tricine SDS buffer is then replaced with Tris glycine buffer (no SDS) in both wells for an additional 10min washout at 100V/cm in the same direction.

**Antibody probing** Following the second wash step, electrophoresis is used to drive probe antibodies (500nM) through the protein decorated gel. Two antibody probing schemes are utilized: a plug of antibody (a ‘top hat’ concentration distribution) and a front of antibody (a ‘step function’ concentration distribution). In the antibody plug scheme, the concentration distribution is defined by first electrophoretically loading an antibody concentration front into the channel (7 min at 200V/cm) from one well. To create the plug, the electric field is set to zero and the loading well is thoroughly washed with Tris glycine buffer via gentle aspiration. After the well is devoid of antibody, the electric field is re-applied (200V/cm), defining the back of the antibody plug, which is then allowed to migrate
along the separation axis. In the continual antibody loading scheme, antibodies are electrophoretically loaded (200V/cm) along the separation axis until the axial signal is uniform (~54 min). Both wells are then thoroughly washed with Tris glycine buffer via gentle aspiration and a reverse polarity voltage is applied to electromigrate unbound antibodies out of the channel.

For both probing schemes, fluorescence images are collected every 2 min using automated time lapse imaging controlled via Metamorph. Monitoring allows determination of the wash time that yields a maximum signal-to-noise ratio (SNR).

**Imaging and image analysis** Chips are imaged on an inverted epi-fluorescence microscope (Olympus IX-50) using a 10X objective (Olympus UPlanFLN, NA 0.3) with CCD camera (CoolSNAP HQ2, Photometrics), filter cubes (XF102-2 and XF100-3 (Omega Optical, Brattleboro, VT)), and automated x-y stage.

Background values are calculated by taking the raw image values and subtracting the autofluorescence signal from regions adjacent to the channel of interest. Prior to antibody loading, the fluorescence intensity in the channel is approximately equal to the surrounding glass, so channel intensity higher than the surrounding glass is likely due to non-specific antibody adsorption in the channel. To estimate the standard deviation of the background signal in the channel, we measure the signal standard deviation in two 50 pixel (192µm) regions. The location of the two regions is > 1 peak width away from the probe signal in both channel directions.

The separation resolution (SR) is defined as the distance between peak maxima divided by four times the average standard deviation of two neighboring ladder protein concentration distributions, as per convention. The concentration distribution metrics are calculated via least squares fitting to assumed Gaussian distributions (MATLAB).
The photocapture efficiency of protein immobilization is estimated by measuring the area under the curve of each protein peak after UV exposure and comparing two conditions: the peak signal intensity before electrophoretic washout of mobile species and the same signal after electrophoretic washout of mobile species. Area under the curve is calculated using the built in MATLAB least squares fit Gaussian algorithm. Importantly, after UV exposure in the gel, we have observed fluorescence intensity recovery of the Alexa 488 dyes over several minutes (data not shown), so images are taken immediately before and immediately after washout to minimize fluorescence intensity recovery. Fluorescence intensity recovery could lead to overestimation of the capture efficiency.

2.1.4 Results and Discussion

Conducted in a single enclosed microchannel, the microfluidic Western blot (Figure 2-1) is a multi-stage assay comprised of: polyacrylamide gel electrophoresis (PAGE), protein blotting via photocapture, and probing with antibodies. The present study addresses three central performance considerations necessary to optimize a single-channel microfluidic Western blotting assay for low-molecular mass proteins.

Firstly, to expand the molecular mass applicability of in-channel microfluidic Western blotting to include low molecular mass species, we implement a discontinuous Tris tricine buffer system. Originally developed by Schägger and Von Jagow, the Tris tricine system offers improved destacking of small MW species in a given pore-size PAGE gel, as compared to the Laemmli Tris glycine system. The basis for the extended molecular mass range of the Tris tricine system stems from the higher electrophoretic mobility of the tricine terminating electrolyte, as compared to the lower mobility of the commonly used glycine terminating electrolyte.

Secondly, to mitigate the reduced effective PA gel pore-size often observed at a gel/buffer or gel/gel interface, we introduce a new gel fabrication method. Importantly, careful control of interfacial gel pore-size is essential to avoid accumulation of material (i.e., antibody probe or sample) at the interface.
We also consider an alternative hydrophilic cross-linker (DHEBA) that offers flexibility in forming and cleaving cross-links. To assess performance across these systems we utilize immunoprobing of PSA, a 28 kDa protein, important to prostate cancer screening diagnostics.

Thirdly, to reduce background signal on the gel, unwanted accumulation of antibody material at gel interface, and total assay duration in the antibody probing step we implement antibody probing using a swept antibody plug (along the separation axis) as compared to earlier approaches that relied on continuous introduction of a concentration front.

**Separation step: ITP for low-molecular mass proteins.** Prior to PAGE, transient isotachophoresis affords both sample enrichment and low injection dispersion. A discontinuous buffer system establishes the ITP protein stack. A discontinuous pore-size gel (step change from large to small pore-size) transitions the assay from ITP to PAGE. As the ITP sample stack enters the separation gel, proteins slow relative to the terminating electrolyte and, thus, proteins de-stack and separate in the molecular sieving gel.
separation gel, any proteins small enough to migrate faster than the terminating electrolyte will remain stacked, which can prevent effective separation of small molecular mass species, particularly in large pore-size (low %T) gels.

As demonstrated by Schägger and Von Jagow\textsuperscript{23}, a Tris tricine terminating electrolyte ITP system allows effective destacking of smaller molecular mass proteins in a given pore-size gel. The isoelectric point of tricine is lower than that of glycine, resulting in tricine having a higher mobility for a given gel pH. Proteins that electromigrate faster than the terminating electrolyte will remain stacked. Consequently, a higher mobility terminating electrolyte enables destacking of smaller molecular mass species in a given pore-size gel.

Figure 2-2. Optimization of discontinuous buffer system for low molecular mass PAGE. (A) PAGE kymograph of Tris glycine (top) and Tris tricine (bottom) discontinuous buffer systems in a 12% discontinuous gel. PAGE is operated under a fixed current of 1.5uA for Tris tricine and 1uA for Tris glycine, yielding ~25-55V/cm voltage ramp during each separation. (B) ITP sample stacking intensity profiles for protein ladder stack in open-channel regions for both the Tris glycine (upper) and Tris tricine (lower) systems at initial sample loading and minimum sample width. During stacking, a 1.5uA fixed current is applied for Tris tricine (~12-25V ramp) and a 0.3uA fixed current (~4-8V ramp) for Tris glycine (as lower current yielded better stacking). Inset shows ITP stacking in a 4%T stacking gel for the Tris glycine system, added to reduce putative EOF-induced dispersion. (C) Inverted fluorescence micrographs and corresponding intensity profiles of sizing in the Tris glycine (top, open-channel loading no 4%T gel) and Tris tricine (bottom) systems. In both cases, the 25kDa ladder protein is at the 1.5mm separation distance position.
pore-size gel. In this system, we seek to optimize PAGE for a protein ladder spanning 6.5-116kDa mass range. Previous work with our in-channel microfluidic Western blotting performed well across a 21-116 kDa mass range in a 7.5%T, 2.7%C acrylamide/bisacrylamide gel and utilized the conventional Tris glycine trailing electrolyte.\textsuperscript{19}

We first compare the stacking and separation performance of the Tris glycine and Tris tricine discontinuous buffer systems, both with Tris HCl as leading electrolyte (Figure 2-2A). ITP stacking is conducted in an open channel (free solution) region abutting a 12%T discontinuous gel for PAGE. For the conventional Tris glycine system, we observe a 440 µm (CV = 1.6%, n = 3) ITP stack (Figure 2-2B) in free solution and a 200 µm stack at the gel interface. The Tris tricine system presents an ITP stack of 70 µm (CV = 9.1%, n = 3) in free solution and a 50 µm stack at the gel interface (CV = 7.8%, n = 3). For Tris glycine, the injected plug is stacked 3.6x, based on comparison of full width half maximum (FWHM) for the loaded sample plug width to the minimum stacked width (CV = 2.6%, n = 3). In contrast, the Tris tricine system yields a stacking factor of 17.8x (CV = 5.6%, n = 3). In the case of the Tris glycine system, a minimum free solution stack width is achieved within 35 s in the first 1.1 mm of the channel. With the Tris tricine system, the free solution stack width continues to decrease as the sample migrates towards the gel interface. With a gel interface located 4.4 mm from the well inlet, a minimum free solution stack width is achieved in 100 s and at a location just before entering the gel.

We hypothesize that the larger stack width observed in the Tris glycine system may be due to the greater difference in conductivity between Tris glycine and the leading electrolyte, Tris HCl, as compared to the Tris tricine system. The buffer conductivities are measured as: 1.3 mS/cm for the Tris tricine run buffer, 0.47 mS/cm for the Tris glycine run buffer, 5.1 mS/cm for the 500mM Tris HCl pH 8.45 gel buffer in the Tris tricine system, and 3.1 mS/cm for the 375mM Tris HCl pH 8.8 gel buffer in the Tris glycine system. We hypothesize that the enhanced conductivity difference measured for the Tris glycine system, as compared to the Tris tricine system, may result in a substantial mismatch in electroosmotic flow (EOF).
between the leading and terminating electrolyte. Consequently, EOF generated in the open-channel region during ITP may be contributing dispersion to the Tris glycine system.\(^{27}\)

To test the EOF dispersion hypothesis, we replace the open channel region with a 4\%T stacking gel. The presence of even a large pore-size gel should reduce EOF and any associated dispersion. In the modified Tris glycine system (Figure 2B), we observe an ITP stack width of 31 µm (CV = 13.1\%, n = 3). The ratio of the change in FWHM (from injected sample plug to the stacked plug) for OVA and CRP (BSA de-stacks in stacking gel) is 23.2x (CV = 9.7\%, n = 3) with Tris glycine and 4\%T loading gel, notably higher than the 5.2x stacking previously observed with the Tris glycine and no loading gel (i.e., free solution) system for the same proteins. The Tris glycine system with stacking gel offers stacking similar to that observed in the Tris tricine system (27.9x stacking for BSA, OVA, CRP) in free solution. These observations suggest that EOF-induced dispersion in the open channel/PA gel Tris glycine system may reduce ITP stacking capability.

**Separation step: PAGE for low molecular mass proteins.** We next compare the PAGE performance of the Tris tricine to the conventional Tris glycine system (Figure 2-2C). We first observe a total separation time of 153s for Tris tricine and 143s for Tris glycine. PAGE assay completion is defined as the time of arrival of the 25kDa ladder protein to the 1.5 mm position on the separation axis from when the sample first enters the separation gel. Secondly, the SNR for the Tris glycine system is notably lower than that of the Tris tricine system, as expected given the transient ITP behaviors described in the previous section. We attribute the slightly lower protein peak area under the curves (AUCs) for the Tris glycine system (as compared to the Tris tricine system) to ~25\% less sample material loaded in the Tris glycine system.

Lastly, the Tris tricine system is observed to provide ~40\% more SR than the Tris glycine system, under otherwise similar conditions. The SR between the two lowest molecular mass species (CRP and AP) is \(SR_{\text{CRP-AP}} = 0.66\) (CV = 2.3\%, n = 3) for Tris glycine and \(SR_{\text{CRP-AP}} = 0.91\) (CV = 3.0\%, n = 3) for Tris tricine. Considering the larger proteins, \(SR_{\text{BSA-OVA}} = 0.78\) for Tris glycine (CV = 2.4\%, n = 3) and \(SR_{\text{BSA-OVA}} = 1.04\) for...
Tris tricine (CV = 3.3%, n = 3). Whereas, $SR_{\text{OVA-CRP}} = 0.63$ (CV = 0.9%, n = 3) for Tris glycine and $SR_{\text{OVA-CRP}} = 1.2$ (CV = 3.0%, n = 3) for Tris tricine. In sum, our observations suggest that both the greater sample preconcentration and lower injection dispersion attained with the Tris tricine system affords higher PAGE separation performance for the molecular mass range considered here.

**Blotting: Protein immobilization efficiency.** In lieu of physical sample transfer from a gel to a hydrophobic blotting membrane, the microfluidic Western blot uses UV photocapture of proteins in the channel via a benzophenone functionalized polyacrylamide gel.\textsuperscript{19} While protein is not physically transferred \textit{per se}, mass is indeed immobilized – the critical aspect of a blotting step. For a 45 s UV exposure in the Tris tricine system, we observe a BSA capture efficiency ($\eta_{\text{BSA}}$) of 65.7% (CV = 8.1%, n = 3). For the Tris glycine system, we observe $\eta_{\text{BSA}} = 51.6\%$ (CV = 6.8\%, n = 3). Capture efficiencies for photo-immobilization in the Tris tricine system are observed to be species dependent, as reported previously:\textsuperscript{22} $\eta_{\text{OVA}} = 48.9\%$ (CV = 4.8\%, n = 3), $\eta_{\text{CRP}} = 35.3\%$ (CV = 11.5\%, n = 3) and $\eta_{\text{AP}} = 63.0\%$ (CV = 14.1\%, n = 3). Raw data and intensity profiles used to calculate these values are shown in Figure S-1 of the Supplemental Information.

**Antibody probing: Discontinuous gel interface.** As the microfluidic Western blot is completed in a single enclosed microchannel, introduction of probing antibody makes use of directed electromigration of antibody down the separation axis. An implication of this scheme is that the large probe antibody electromigrates through the molecular sieving gel – here optimized for a wide molecular mass range separation which, importantly, includes low molecular mass species. To evaluate the impact of probe introduction into the 12\%T sieving gel after sizing, we utilize a fluorescently labeled polyclonal antibody against prostate specific antigen (PSA), Figure 2-3A. A plug of antibody is loaded into the channel for 7 min and then electromigrated long the separation axis (both at 200V/cm).
Figure 2-3. A larger pore-size gradient at the open-channel/gel interface reduces unwanted size-exclusion effects during probing. (A) Inverted fluorescence micrographs show antibody probing across a gel with smaller pore sizes at the interface\(^1\) (left) and for a gel with a gradient to larger pore sizes at the interface (right), both with 12\% T gels utilizing DHEBA cross-linker and 600nM purified PSA sample. Gel interface is marked with black arrow; expected location of the PSA major isoform is indicated with an (*). (B) Inverted fluorescence kymographs of a 116-6.5kDa ladder separation in an 8\% T (top) and 12\% T gel (bottom) with a Tris tricine discontinuous buffer. Right panel shows the ladder when the 25kDa marker is 1.5mm into the gel. In the 8\% T gel, the small 6.5kDa marker migrates faster than the stack and so rejoins the stack a short distance into the gel. 12\% T enables destacking and separation of full 116-6.5kDa ladder. (C) Schematic depicting fabrication protocol yielding a short larger-to-bulk pore-size gradient at the separation gel interface.
Using this directed electromigration of probe to immobilized antigen, we observe interfering antibody accumulation near the open-channel/12%T gel interface, when using the original fabrication protocol. The background signal near the gel/free solution interface is higher than the probe signal at the immobilized PSA, resulting in an SNR of <3 even with a sample containing 600nM of PSA. High antibody background at the interface occurs regardless of directionality of antibody loading relative to the interface. In contrast, a fabrication method enabling the generation of larger pore sizes at the interface relative to the bulk of the gel generates a clear antiPSA probe signal for the 28kDa primary isoform. We also observe additional minor peaks at a lower and higher MW. We attribute the smaller MW peak to known biological cleavage PSA isoforms and/or sample degradation and the larger MW peak to proPSA and/or PSA aggregates (Figure 2-3A). Validation of the µWestern was completed via conventional Western blot. We observed signal at both larger and smaller MW positions, relative to the 28 kDa PSA peak position (Figure S-2 in SI), consistent with the µWestern blot results for the same sample. The lower MW PSA cleavage isoforms present as a single peak in the µWestern due to the short separation distance used compared to conventional slab gel (2mm vs. 8cm).

The preferential accumulation of antibody probe material at the open-channel/gel interface is attributed primarily to size exclusion at the interface. The size-exclusion is exacerbated by difficulty in controlling gel pore-size at an open channel interface. Briefly, monomer and cross-linker from unpolymerized regions of the microfluidic channel diffuse into the polymerizing region during fabrication, thus establishing smaller pore-sizes at the gel/free solution interface relative to the pore-size in the bulk of the gel. The small pore-size occurs when either photomasking or oxygen inhibition from the wells is used to create the gel interface. Effective preconcentration and separation requires a region of free solution (or much larger pore sizes) adjacent to a separation gel to enable good sample stacking and destacking. The smaller effective pore-size at the open-channel/gel interface presents an issue for probing, particularly for higher %T gels, as it is this accumulation of high antibody background at the interface that
obscures the probe signal from the antigen, greatly reducing assay sensitivity. Simply utilizing a lower %T gel does not allow for destacking of smaller molecular mass species, thus limiting the assay to larger proteins. As shown in Figure 2-3B, an 8%T gel results in ineffective de-stacking of the small molecular mass protein aprotinin (7kDa with label), with aprotinin migrating faster than the stack and, thus, rejoining the stack a short distance into the gel. The lower %T gel also results in lower SR between the other destacked ladder proteins at a given distance into the gel.

To overcome antibody probe size exclusion at the open-channel/ gel interface and enable immunoprobing even in high %T gels, we seek to eliminate the interfacial small pore-size artifact by establishing a short gradient of larger-to-bulk pore-size at the interface. We hypothesized that eliminating the unpolymerized gel precursor abutting the polymerizing region would inherently eliminate the source of additional monomer and cross-linker that yield smaller pore-sizes at the interface than the bulk of the gel. To achieve the configuration prior to blue light photopolymerization, the channel is first filled with gel precursor solution from a well (Figure 2-3C). Next, gel precursor solution is removed from the well, thus leaving two empty wells and a channel filled with gel precursor solution. One well is then filled with buffer solution (3µL). Owing to the hydrostatic pressure mismatch generated between the wells, the buffer then flows into the channel. After a fixed duration (c.a. 15 s), the entire chip is submerged in buffer, thus eliminating the hydrostatic pressure head and the resultant flow in the channel. The entire chip is illuminated with a blue LED for 6 min to photopolymerize the gel. The duration between buffer loading into the well and subsequent submersion of the chip in buffer establishes the location of the interface gradient in the channel. The time interval between establishing multi-fluid configuration in the channel and photopolymerization establishes the pore-size gradient characteristics (i.e., pore-size gradient length and steepness) due to diffusion of gel precursor into the free solution region. Longer durations between chip submersion and photopolymerization would lead to a longer region of the larger pore size gradient at the interface. For the examples here, the chip is illuminated with blue light immediately (< 1 min) after
submerging to minimize diffusion and generate a relatively sharp interface. As shown in Figure 2, this fabrication method enables excellent stacking and separation performance when using the Tris tricine discontinuous buffer system.

Using the fabrication technique, we observe an interface located ~4.4mm from the edge of the input well for a 15 s equilibration time (i.e., duration between buffer loading into the well and subsequent submersion of the chip in buffer). Variation in the position of the gel interface between triplicate channels sharing a well is 1.1% (n = 4) and the CV of the gel interface position between lanes not sharing a well on a chip is 4.8% (n = 4). By observing the location of the buffer with a fluorescent dye tracer included, the position of the fluid-fluid interface within the channel is observed to be linearly dependent on time ($R^2 = 0.999$). The fluid interface migrates at the same velocity (< 1% difference) regardless of whether the well is filled with buffer or gel precursor, indicating the position is dictated by hydraulic pressure alone and differences in viscosity and surface tension are negligible between the buffer and the gel precursor. Further, we note that fabrication of a stacking gel by one-step UV exposure is also possible using this approach, simply by loading a 4%T, 2.7%C gel precursor solution into the well instead of buffer solution.

This fabrication method yields the antibody probe performance shown on the right hand side of Figure 2-3A, which provides a clear antiPSA probe signal for the PSA isoforms. For both Figure 3A results, the use of a DHEBA cross-linker, in place of bisacrylamide, is utilized as a proof of concept. DHEBA is a more hydrophilic cross-linker that is cleavable in highly acidic or basic conditions.34,35 Further development with DHEBA may enable greater design flexibility in the μWestern blot system by allowing for an increase in pore size between the separation and probe stages of the assay.

**Antibody probing: Swept plug scheme.** Our broad molecular mass range microfluidic Western blot19 utilizes electromigration to drive a front of primary antibody into and then along the entire separation axis to achieve probing. In practice, this front introduction method is applied for 20 min to fill
the channel with antibody followed by a 20 min antibody washout in the reverse direction in a 7.5%T, 2.7%C gel. We seek to establish and validate an alternative transport approach for probing to: (i) reduce assay time and complexity, (ii) provide uniform antibody incubation times across a gel, (iii) enable a consistent protocol regardless of gel length or pore-size, and (iv) reduce non-specific antibody background in the gel, which increases substantially with increasing %T gels when using an antibody front probe approach.\textsuperscript{19} To achieve these goals, we investigate electrophoretic introduction of a well-defined plug of 500nM antibody into the gel, then along the entire separation axis (7 min, \( E = 200 \text{V/cm} \)), Figure 2-4A.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure24.png}
\caption{Antibody probing scheme impacts background signal. (A) Inverted fluorescence micrographs for electrophoresis of antibody probe via swept plug introduction (upper) and continuous front loading (lower). Loading images use 50ms exposure time; washout images use 300ms exposure time. Antibody loading concentration is 500nM; \( E = 200 \text{V/cm} \). (B) Inverted fluorescence micrograph shows protein ladder and final probe results for PSA for each method. The PSA primary isoform is indicated with a (*)}.\end{figure}
Selection of the 7 min antibody loading time for the plug scheme is informed by results of a kinetic model indicating that >94% of immobilized antigen would be bound assuming $k_{\text{on}} = 2 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ and $k_{\text{off}} = 5 \times 10^{-4} \text{s}^{-1}$ for an antiPSA mAb.\textsuperscript{36} For the configuration under study, the antibody plug requires ~22 min to traverse a 4.5 mm long 12%T, 2.7%C gel with (E = 200V/cm). We observe maximum SNR after an additional 50 min of antibody washout via electrophoresis of clear buffer into the channel (E = 200V/cm).

During antibody probing, we observed both the major antibody probe peak and two low concentration, large molecular mass peaks electromigrating along the microchannel axis. As the low concentration peaks continually migrate during the time lapse imaging, we hypothesize that the peaks are contamination in the stock antibody probe solution (i.e., aggregation\textsuperscript{37}). As the spurious peaks are continuously mobile, whereas the probe antibody is immobile at the target peak location, mobility is used to differentiate the signal sources. We suggest a filtration step to remove aggregates from the stock antibody solution, which would reduce assay time by minimizing the probe washout duration.

With a 7 min sweep of antibody plug along the separation axis, we observe an SNR = 263 (CV = 11.4%, n = 3) and average background intensity in the gel of 24 RFU (CV = 3.2%, n = 3). In comparison, continual antibody loading yields an SNR of 131 (CV = 18.8%, n = 3) and a more than 2-fold higher average antibody background intensity in the gel of 54 RFU (CV = 16.3%, n = 3) for the same sample, which contains 300nM of PSA (Figure 4B). Thus, the swept antibody plug technique results in higher SNR due to a lower background and lower standard deviation of the background, as compared to continually loading antibodies into the gel.

We further note that the swept antibody plug scheme offers uniformity in antibody incubation time with target for all targets, regardless of molecular mass. In other words and in contrast to a continuous antibody front introduction approach, high molecular mass proteins immobilized near the head of the separation channel will be incubated with the antibody probe for the same duration as small
proteins immobilized near the end of the separation axis. Varying the loading time of the swept plug would allow optimization for different antibody-antigen binding kinetics by increasing or decreasing the incubation time.

The µWestern channel design also supports optimization to meet a desired separation resolution and/or a desired assay time. For example, using a longer separation length could enable improved separation resolution, with the tradeoff of a longer assay time as the antibodies must traverse the full separation length for probing. Conversely, using an even shorter gel length than the 5mm gel presented here would reduce the total assay time.

2.1.5 Conclusions

Overall these improvements to the microfluidic Western blotting system in (i) Tris tricine discontinuous buffer, (ii) fabrication to generate larger interfacial pores, and (iii) antibody plug probe enable the detection of a lower MW range of proteins. The Tris tricine discontinuous buffer system enables improved separation resolution and the destacking of smaller MW species for a given pore size gel. Control over gel interfacial pore size through fabrication optimization is critical for enabling both good stacking for high separation resolution as well as good antibody transport for high signal to noise resolution probing. Furthermore, utilization of an antibody plug sweep probe approach reduces antibody consumption while improving assay time and signal to noise resolution. Through continued future development with more hydrophilic and cleavable cross-linkers such as DHEBA, with effective separation and probing initially demonstrated here, we plan to further push the bounds of sample molecular weight, sensitivity, and assay speed. The approaches presented here expand the applicability of the µWestern to a wider variety of diagnostic and basic biological research applications.
2.1.6 Supplemental Information

Figure S-1: Capture efficiency calculation. As described in Materials & Methods, capture efficiency is calculated by comparing the area under the curve of each ladder peak immediately before and after washout using Gaussian fits. (A) Overlaid image intensity plots before and after washout. (B) Gaussian fits to image intensity plots before and after washout utilizing built in MATLAB least squares fit algorithm. (C) Tables with the Gaussian fit area under the curve for each ladder protein in relative fluorescence units.
Figure S-2: Slab gel Western blot A 10%T Tris tricine slab gel WB was run to validate the µWestern blot data. Three conditions were run on the slab gel WB: (1) PSA alone (300nM), (2) AF488 µWestern ladder alone (OVA, CRP, and AP) and (3) PSA (300nM) + µWestern ladder (same sample used in µWestern). The fluorescent signal from the 6.5kDa AP is not visible on the PVDF membrane. From these results we observe no non-specific interaction between the antiPSA and the µWestern ladder markers as evidenced by no antiPSA probe signal seen in the lane without PSA and by similar signal profile in the two lanes with PSA and with and without the µWestern ladder. We observe that the polyclonal antiPSA recognizes a number of different PSA cleavage isoforms smaller than the primary peak as well as producing a PSA signal at a larger MW than the primary isoform. The greater separation resolution on the slab gel allows these smaller MW cleavage forms to be resolved as multiple peaks on the slab gel, whereas this presents as a single peak on the µWestern due to the shorter separation distance (2mm vs. 8cm). The µWestern probe results from Figure 3A are shown below for comparison. The traditional Western blot was run using a 10%T Tris tricine discontinuous buffer SDS-PAGE system following the standard protocol of the Life Technologies XCell II Mini Cell with an unlabeled antiPSA polyclonal (same as µWestern, Fisher Scientific AF1344) and HRP anti-Goat secondary with chemiluminescent substrate for detection.
2.2 Antibody Probe Optimization

One of the major challenges with the µWestern blot platform is obtaining robust antibody probe results. This is partially due to high levels of antibody background seen in the gel, particularly at the gel interfaces. Antibody background levels are even higher with higher %T gels as originally demonstrated by Hughes et al.\textsuperscript{19} Size based separation of lower-molecular-mass species in a discontinuous buffer system requires a decrease in pore size through the use of a higher %T gel, to enable destacking through effective sieving. Use of an 8%T gel does not enable destacking of proteins down to 6.5kDa even with a Tris tricine run buffer (Figure 2-3). As a result, further development for antibody probing was required to enable the use of these higher %T gels, which are critical for effective separation and probing of lower MW targets.

Simply using the original gel fabrication and probe protocols utilized in a 7.5%T gel in Hughes et al.\textsuperscript{19} and applying them to a 12%T gel, results in the probe performance shown in Figure 2-5, where the antibody background at the interface is higher than the signal at the expected PSA location and antibody background is high throughout the gel. The smaller pore sizes generated at the interface from the diffusion of monomer from unpolymerizing into polymerizing regions of the channel results in a smaller pore size than the bulk of the gel,\textsuperscript{33} leading to trapped sample and antibody at the interface where sample is loaded, obscuring the signal from the target immobilized PSA band. As can be seen in Figure 2-5, the antibody background at the interface is higher than the signal at the expected probe location (denoted by an ‘*’). Continuously loading antibody to fill the channel and then reversing the electric field polarity to electrophoretically wash out the antibody also leads to substantially longer assay times (> 1 hour for antibody probe) due to the smaller pore size and high background throughout the gel.
A number of different antibody probe methods were compared utilizing this gel fabrication method, which involves filling the channel with gel precursor and allowing oxygen inhibition from the wells to establish a gel interface in the channel. This results in ~600µm of free solution prior to the gel interface, similar to the 400µm region seen in Hughes et al.\textsuperscript{19} Four antibody probe methods are compared in Figure 2-6: antibody plug probe (left) or continuous antibody loading (right) with antibody loaded from either the same side of the channel as the sample separation (lower) or the opposite side of the channel (upper). None of these methods result in probe performance with SNR > 3 due the high background in all cases at the gel/free solution interface on the side of the gel where the sample is loaded and separated. Running longer separations to bring the target further from the gel interface could help, but would result in lower signals due to diffusion reducing the local antigen concentration.

The same antibody probe methods are compared in Figure 2-7 on gels fabricated using the new
fabrication method outlined in Figure 2-3, which generates larger pore sizes at the interface by establishing a region of buffer solution in the channel next to the region of gel precursor prior to polymerization. All probe methods utilizing this gel fabrication technique lead to SNR > 3, with the highest SNR found when utilizing a 7min antibody plug loaded at 200V/cm on the side of the gel opposite to sample loading and separation (top left box, Figure 2-7). It should be noted that the antiPSA target antigen is further into the microfluidic channel in Figure 2-7 than Figure 2-6 because adding free solution into the channel results in a gel interface a further distance from the well, but the separation distance into the gel is similar in both cases. This increased electromigration distance does not increase diffusive losses in this case as sample remains fully stacked throughout the free solution region of the channel from ITP when using a Tris tricine discontinuous buffer system. In the case of the Tris tricine terminating electrolyte, local concentration continues to increase as the sample migrates through the free solution region with a maximum reached as the sample enters the gel.
Figure 2-6: Inverted fluorescence micrographs of antiPSA probe performance utilizing previously published$^{19}$ gel fabrication method. Four conditions are compared: antibody plug probe (left) or continuous antibody loading (right) with antibody loaded from either the same side (lower) or the opposite of the channel (upper) as the sample loading and separation. In all cases antibodies are electromigrated at 200V/cm in 1x Tris glycine buffer. Arrows indicate direction of initial antibody migration. Continuous antibodies were loaded for 44min and then washed out with a reverse polarity voltage for 2 hours. Plug antibodies were loaded for 7min at 200V/cm and then migrated for 2 hours. (12%T, 3.5%C DHEBA gel)
Figure 2-7: Inverted fluorescence micrographs of antiPSA probe performance utilizing new gel fabrication method, which generates larger pore sizes at the interface. Four conditions are compared: antibody plug probe(left) or continuous antibody loading(right) with antibody loaded from either the same side(lower) or the opposite of the channel(upper) as the sample loading. In all cases, antibodies are electromigrated at 200V/cm in 1x Tris glycine buffer. Continuous antibodies are loaded for 30min then washed out with a reverse voltage for 80 minutes. Plug antibodies loaded for 7min at 200V/cm and migrated across the gel for 80 min. (12%T, 3.5%C DHEBA gel).
Initial experiments using lower antibody concentrations (15nM) resulted in lower antibody background, but reducing the antibody concentration leads to reduced forward reaction rates as illustrated by the kinetic binding equations below. Depending on the sample protein concentration and the $k_{on}/k_{off}$ rates of the antibody-antigen pair this could lead to long assay times and/or failure to reach equilibrium binding. Desiring to still maintain a rapid microfluidic Western blot and a long quantitative linear range, alternative binding approaches were investigated, including the use of a plug probe.

Antibodies electrophoresed through the gel will bind with immobilized target antigen at rates governed by the $k_{on}/k_{off}$ value of the pair:

$$\text{Ab} + \text{P} \underset{k_{off}}{\overset{k_{on}}{\longleftrightarrow}} \text{C}$$

Where $\text{Ab}$ is the antibody used to probe for a target of interest, $\text{P}$ is the protein target of interest and $\text{C}$ is the complex formed when antibody binds to antigen. $k_{on}$ is the kinetic association rate for the particular antibody-antigen pair and $k_{off}$ is the rate of dissociation for the antibody-antigen pair. These rates vary for different antibodies and can also vary with temperature, buffer, and other factors.

The change in complex formation over time is governed by the following equation:

$$\frac{\partial [C]}{\partial t} = k_{on} \ [Ab][P] - k_{off} [C]$$

As can be seen from this equation, the rate of complex formation $k_{on}[Ab][P]$ is based on the concentration of antibody and immobilized target protein. The amount of target protein is largely determined by the sample used with some increase possible based on optimization of sample stacking and separation in the µWestern assay. The antibody concentration is set by the µWestern user based
on the antibody dilution loaded into the well. In a typical slab gel Western blot, high antibody dilutions of 1:1000-1:10000 are often used. With a slab gel, the membrane is often incubated overnight on a shaker with the antibody solution. The long time scales (> 12 hours) allows the system to reach equilibrium and achieve adequate binding even with low antibody concentrations. In contrast, the µWestern is designed to be a rapid assay, and therefor requires higher complex formation rates ($k_{on}[Ab][P]$) to achieve high signal in short periods of time (ideally < 1 hour).

A computational model of these binding kinetics utilizing the $k_{on}$ and $k_{off}$ rates measured in Kapil and Herr\textsuperscript{36} for a PSA mAb, illustrates the impact of antibody concentration on time to equilibrium for the system and maximal fraction of antigen bound (Figure 2-8). Use of a 500nM antibody plug enables >90% equilibrium binding with only a 7min plug probe. Reducing the antibody plug concentration reduces the bound concentration at equilibrium and increases the time to equilibrium.

![Simulation results of fraction of antigen bound over time as a function of antibody concentration. (300nM immobilized antigen, $k_{on} = 2E4$, $k_{off} = 5E-4$)](image)
Knowledge of the $k_{on}$ and $k_{off}$ of an antigen-antibody pair in gel can enable system optimization for various targets by varying both the antibody concentration and plug load time. This can lead to improved LLOD and assay time for various targets of interest. Further future optimization around use of unlabeled primary and labeled secondary as well as optimal concentrations of each could further improve signal to background of the assay. The use of a 7 minute plug probe versus continuous loading of antibody was found to lead to improved signal to standard deviation of the background while reducing assay complexity, user intervention, and overall assay time (Figure 2-4 and Figure 2-7).

Another advantage of the plug probe is that continuous loading of antibody followed by reverse migration can easily lead to false negatives on the assay if the antibody has not migrated across the full channel prior to reversing the voltage. The free dye present with the antibody can make it challenging for the user to determine when the antibody has fully migrated across the gel even under circumstances where the assay is being continually monitored by the user during the antibody probe step. Differences in antibody clogging at the interface, target antigen concentration, and overall immobilized sample concentration can impact antibody mobility, leading to variations in the length of time for the antibody to migrate across the full gel. Furthermore, portions of the gel closer to the antibody loading side can have significantly longer antibody incubation times than the lower MW proteins on the opposite end of the gel, making it difficult to accurately quantitatively compare concentrations of different isoforms.

Loading an antibody plug in excess of the target protein concentration and migrating that plug across the full length of the gel enables a more uniform antibody incubation for all regions of the gel, although not as uniform as could be achieved with antibody loading and washout perpendicular to the axis of separation.
2.3 Stacking and Separation Performance in Tris Tricine and Tris Glycine

Experiments comparing separations with Tris glycine and Tris tricine terminating electrolytes showed consistently better stacking and separation performance in the Tris tricine system. This was true for a variety of gel buffer molarities, %T’s, and both gel fabrication methods (large vs. smaller pores at the interface) as seen in Figure 2-9 and Figure 2-10.

The wider full width half maximum (FWHM) of samples entering the gel in the Tris glycine discontinuous buffer system leads to broader bands of lower local concentration in the gel and reduced separation resolution (Figure 2-9 and Figure 2-2). Obtaining equivalent separation resolutions with Tris glycine would require loading much lower sample volumes to reduce the stack width, which can reduce assay sensitivity. Figure 2-11 shows the free solution stack up until the sample (BSA, OVA, CRP) begins to enter the gel for Tris glycine and Tris tricine (both with Tris HCl at 500mM as the leading electrolyte (pH 8.8 and 8.45 respectively), similar results are seen when Tris HCl 500mM pH 8.45 is used as the leading electrolyte with 1x Tris glycine terminating electrolyte (Figure 2-15). The free solution stack is much broader with Tris glycine and minimal stacking occurs prior to the sample reaching the discontinuity of the gel interface.
Figure 2-9: Inverted fluorescence kymographs of ladder separations with a 1x Tris glycine discontinuous buffer and Tris HCl pH 8.8 gel buffer. Gel %T, %C, cross-linker, and gel buffer molarity vary as indicated. 1µA fixed current separations.
Figure 2-10: Inverted fluorescence kymographs of ladder separation with a 1x Tris tricine discontinuous buffer. (1.5µA fixed current)

Figure 2-11: Inverted fluorescence kymographs of ladder stacking (BSA, OVA, CRP) in 1x Tris glycine and 1x Tris tricine terminating electrolytes. The varying lateral positions of the free solution stack in the image are due to shifting of the scope to keep the stack in the camera field of view.

After experimentally observing differences in free solution stacking, simulations in Simul 5 were run to compare stacking performance between Tris tricine and Tris glycine discontinuous buffer.
systems. (Figure 2-12 - Figure 2-15). The simulation results did not show better stacking with tricine than glycine in contrast to the experimental results, and while the Tris tricine experimental results closely matched the Simul 5 simulations the Tris glycine experimental results did not match the simulation well in either sample preconcentration or shape of stack. Simul 5 is a 1D simulation, suggesting 2 or 3-dimensional effects, such as electroosmotic flow (EOF) may be leading to the reduced stacking and sample preconcentration observed with Tris glycine.

We hypothesized that the greater conductivity difference between Tris glycine and the gel buffer as compared to Tris tricine and gel buffer (1.3 mS/cm for the Tris tricine run buffer, 0.47 mS/cm for the Tris glycine run buffer, 5.1 mS/cm for the 500mM Tris HCl pH 8.45 gel buffer in the Tris tricine system, and 3.1 mS/cm for the 375mM Tris HCl pH 8.8 gel buffer in the Tris glycine system), could result in a greater mismatch in electroosmotic flow (EOF)\(^{27}\) between the leading and terminating electrolyte resulting in sample recirculation in the free solution portion of the channel that increases the peak width. EOF occurs in a channel when the surface acquires a net electric charge, leading to a Debye layer (i.e., electric double layer) to form in the region near the interface. When an electric field is then applied to the fluid, as occurs in our system for sample loading and separation, the net charge in the electric double layer induces fluid motion. With an open channel free of gel, the fluid will typically move along the channel length, but in our case when there is a gel blocking fluid flow further down the channel this can lead to recirculating flows that create greater dispersion in the sample. Previous work in our lab by Duncombe and Herr\(^{38}\) utilizing fluorescently labelled beads in a free-standing gel system showed recirculating flows with sample loading in a free solution region next to a gel interface, which decreased stacking performance.
Figure 2-12: Simul 5 simulation results of free solution stacking of 3 proteins with 1x Tris tricine terminating electrolyte. Initial protein concentration is 500nM and final is 4.5µM for lowest mobility species. 500mM Tris HCl pH 8.45 leading electrolyte.

Figure 2-13: Fluorescence intensity plot of ladder proteins (BSA, OVA, CRP) at minimum stack width in free solution with 1x Tris tricine terminating electrolyte. (500mM Tris HCl pH 8.45 leading electrolyte). Initial sample full width at half maximum was ~1mm.
Figure 2-14: Simul 5 simulation results of free solution stacking of 3 proteins with 1x Tris glycine terminating electrolyte. Initial protein concentration is 500nM and final is 10µM for lowest mobility species. 500mM Tris HCl pH 8.45 leading electrolyte.

Figure 2-15: Fluorescence intensity plot of ladder proteins (BSA, OVA, CRP) at minimum stack width in free solution with 1x Tris glycine terminating electrolyte. (500mM Tris HCl pH 8.45 leading electrolyte). Initial sample full width at half maximum was ~1mm.

To test the electroosmotic flow hypothesis for Tris glycine, a stacking gel was added and stacking and separation performance compared for the two systems. As can be seen in Figure 2-16, the addition of a stacking gel leads to substantially improved stacking and separation resolution for the BSA (66kDa),
OVA (25kDa), and CRP (25kDa) ladder. Differences in stacking and separation resolution for Tris Tricine with and without a stacking gel are more minimal (Figure 2-17).

**Tris Glycine Separations**

- **No Stacking Gel**
- **4%T Stacking Gel**

![Image of kymographs showing ladder separation with and without stacking gel.](image)

*Figure 2-16: Inverted fluorescence kymograph of ladder separation with 1x Tris glycine terminating electrolyte with and without a stacking gel. 12%T, 2.7% acrylamide/bis separation gel and 4%T, 2.7% acrylamide/bis stacking gel. 1µA fixed current resulting in a ~25-55V/cm ramp. 375mM Tris HCl pH 8.8 gel buffer.*
Figure 2-17: Inverted fluorescence kymograph of ladder separation with 1x Tris tricine terminating electrolyte with and without a stacking gel. 12%T, 2.7% acrylamide/bis separation gel and 4%T, 2.7%C acrylamide/bis stacking gel. 1.5µA fixed current resulting in a ~25-55V/cm ramp. 500mM Tris HCl pH 8.45 gel buffer.

2.4 BPMA Concentration and Separation Resolution

BPMA groups cross-linked into the gel enables the use of a single gel for both separation and blotting, eliminating the need for a separate blotting membrane as is typically used for slab-gel Western blots. While critical for the µWestern assay, the BPMA groups were found to also negatively impact separation performance (Figure 2-18) and increase risk of gel breakdown in a concentration dependent manner (Figure 2-19). Interestingly, this phenomena was found to be more pronounced for the N-[3-[(3-Benzoylphenyl)formamido]propyl] form custom synthesized by PharmAgra labs (3-Benzo) than the positional isomer N-[3-[(4-benzoylphenyl)formamido]propyl] synthesized in house22 (4-Benzo). More
analysis would need to be done to determine the cause for this phenomena, but potential mechanisms include a higher purity in the professionally synthesized BPMA or a differential impact in chain termination or polymerization inhibition based on BPMA isomer.

Figure 2-18 below shows separation kymographs for BPMA in varying concentrations for both the 3-Benzo and 4-Benzo isomers in an 8%T, 2.7%C acrylamide/bisacrylamide gel. We hypothesize that the performance degradation may be due to chain termination by BPMA and/or polymerization inhibition, but further studies would need to be conducted for a conclusive mechanism. Functionally, decreasing the BPMA concentration improves gel stability and separation performance and was critical for achieving adequate µWestern performance. For the low-molecular-mass µWestern research, the 4-Benzo isomer synthesized in lab was used at 1.5mM BPMA, half the previously published concentration. Eliminating BPMA entirely leads to even better separation resolution, as shown in Figure 2-18, suggesting further improvements may be possible in the system. Preliminary studies also suggest that higher concentrations of BPMA reduces gel stability, leading to increased risk of gel breakdown with applied electric field during both sample separation and antibody probing (Figure 2-19).
Figure 2-18: Kymographs showing separation of BSA, OVA, and TI with varying concentrations of two different BPMA isoforms in 8%T, 2.7%C acrylamide/bisacrylamide gels. (3µA fixed current resulting in 20-60V/cm ramp)
Figure 2-19: BPMA concentration and gel stability (4-Benzo). Separations were run at 3µA fixed current (20-60V/cm ramp). Post separation wash steps at 100V/cm.

<table>
<thead>
<tr>
<th>BPMA Concentration</th>
<th>Description</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5mM BPMA</td>
<td>Gel stable for all steps. Substantially better separation performance than other conditions and lower background.</td>
<td></td>
</tr>
<tr>
<td>3mM BPMA (original protocol)</td>
<td>Gel stable during separation (shown), but gel damage during 100V/cm wash steps.</td>
<td></td>
</tr>
<tr>
<td>4.5mM BPMA</td>
<td>Significant gel breakdown during separation.</td>
<td></td>
</tr>
<tr>
<td>6mM BPMA</td>
<td>Significant gel breakdown during separation.</td>
<td></td>
</tr>
</tbody>
</table>

It was also observed during development that BPMA capture efficiency does not decrease with repeated UV exposure (Figure 2-20). A gel exposed to UV for 45 sec at 100% with the Hamamatsu has similar capture efficiency to a gel without UV exposure. This phenomena could be utilized in new assay formats requiring multiple capture steps, such as a sandwich barcode assay with a high Kd between target and the immobilized protein (such as a lectin) or to maintain a higher percentage of bound primary antibody if using a secondary antibody in the µWestern. This phenomena could also be potentially utilized to increase capture efficiency with lower concentrations of BPMA in the gel through extended UV exposure.
2.5 Capture Efficiency and Photobleaching Recovery

During the course of assay development, it was observed that the fluorescently labelled ladder markers recovered some fluorescence intensity after losing signal during UV exposure for the benzophenone photocapture stage of the assay. Understanding and accounting for this is critical to ensure quasi-quantitative measurement of capture efficiency. Otherwise analysis may lead to physically impossible calculated capture efficiencies in excess of 100%. Capture efficiency is calculated by integrating the fluorescence intensity for each ladder marker 1) after UV exposure and before washout and 2) after washout. UV exposure leads to a significant drop in fluorescence intensity some of which recovers over time.

*Figure 2-20: BPMA capture efficiency with repeated UV exposure (n = 3 each condition).*
An increase in fluorescence intensity over minutes has been observed in the µWestern even when no buffer exchange occurs, as shown in Figure 2-21 below. Further analysis would need to be done to fully understand and characterize the mechanism. To minimize impact on overestimation of capture efficiency for publication, lanes were imaged with 2 min timelapse imaging with signals compared immediately before and immediately after washout, typically a 6 min interval.

![Figure 2-21: Fluorescence signal recovery after UV exposure.](image)

2.5 Cleavable Cross-Linkers for µWestern Blot

Decreasing the pore-size of the acrylamide gel matrix improves separation performance, particularly for low-molecular-mass species, but leads to longer antibody electromigration times across the gel, can lead to higher antibody background, and with very small pore-sizes can exclude antibody from the gel.
entirely. This can place limits on the applicable targets for the µWestern relative to bench-top slab gels that utilize a transfer to a larger pore-size membrane. Desiring to avoid these restrictions, cleavable cross-linkers were evaluated to enable a change in pore-size between the separation and probing steps of the microfluidic assay.

Chemically cleavable cross-linkers include N,N’–diallyl-tartar-diamide (DATD) which is cleavable by oxidation, N,N’–bisacrylylcystamine (BAC) which has a disulphide bond that is cleavable by DTT or b-mercaptoethanol, ethylene diacrylate (EDA) which undergoes alkaline hydrolysis, and N,N’-(1,2-Dihydroxyethylene)bisacrylamide (DHEBA) which can undergo cleavage by either oxidation or alkaline hydrolysis. UV photocleavable cross-linkers including ones utilizing 2-nitrobenzyl acrylate (2-NBA) have also been developed in the literature.

Acrylamide gels utilizing varying ratios of DHEBA cross-linker with and without bis were found to polymerize using the standard blue light riboflavin polymerization method used for the µWestern. Initial testing with EDA failed to produce polymerized gels using the published procedure or extended blue light exposure times up to 20 minutes, likely due to the slower reaction kinetics of EDA compared to DHEBA cross-linkers. DHEBA had also previously been found in the literature to be more stable than EDA under typical SDS-PAGE discontinuous gel pH’s, so further research utilized the DHEBA cross-linker for the low-molecular-mass µWestern. BAC was not investigated due to concerns of non-specific interactions with antibodies, which was found experimentally by Todd Duncombe in work with free-standing gels, and 2-NBA was not tested as this cross-linker was not commercially available. DHEBA cross-linked gels were comparable in separation resolution and capture efficiency to bis cross-linked gels when used in the µWestern.

In tests of bulk acrylamide/DHEBA gel breakdown 8%T, 2.7%C gels were found to break down to a viscous liquid in 1 hour with 5.25M NaOH or 1M NaOH, but require overnight incubation with 1M
Glycine NaOH pH 10.4. The addition of heat sped up breakdown with 1M Glycine NaOH as did use of a higher pH 1M Glycine NaOH (pH 12). An acrylamide/DHEBA gel incubated with 1M Glycine NaOH pH 10.4 at the elevated temperature of 70°C was also found to break down at a similar rate to 1M NaOH resulting in a viscous liquid after ~1 hour. Bulk gel breakdown was evaluated qualitatively by testing for loss of rigid gel structure of gels soaking in buffer solutions in eppendorf tubes. DHEBA gels are base cleavable at amidomethylol bonds as indicated in Figure 2-22.

**Figure 2-22: DHEBA alkaline cleavage**

Electrophoresing 5.25M NaOH into an acrylamide/DHEBA gel in the microfluidic channel led to the elimination of fluorescence signal from the immobilized ladder markers (Figure 2-23) and after 1 hour of incubation also generated what appeared to be EOF within the channels during antibody loading, suggesting a breakdown of the gel. Antibody probing failed to produce a signal at the expected location with the use of NaOH cleavage so other gel cleavage approaches were investigated.

**Figure 2-23: Time lapse fluorescence micrograph of immobilized ladder markers during electrophoresis of 5.25M NaOH through an acrylamide/DHEBA gel in a microfluidic channel.**
1M NaOH also seemed to destroy the gel even with 50/50 DHEBA/bis and prevented effective electromigration of antibodies into and out of the gel. For the timelapse antibody migration image shown below (Figure 2-24), after the overnight 1M NaOH incubation, 1x Tris Glycine was loaded into the wells and the NaOH washed out for 10min at 50V/cm prior to any antibody loading. When an electric field was applied to electrophoresis antiOVA into the channel the plug migrates from negative to positive electrode as expected, but halfway through the channel some of the antibody and/or free dye changes direction and migrates towards the negative electrode (Figure 2-24). Unexpected electromigration was observed during two other experiments with NaOH cleavage of a DHEBA cross-linked gel in a glass chip. The anomalous electromigration is hypothesized to arise from EOF induced migration or changes to antibody charge with gel interaction. No antibody probe signal was observed at the target antigen location in any of the gels treated with 1M NaOH. Due to these antibody electromigration issues and the lack of antibody probe signal against immobilized proteins after introduction of NaOH, alternative cleavage methods were further investigated.

Overnight incubation with 1M Glycine NaOH pH 10.4 enabled positive antiPSA probe results with a high signal at the expected 28kDa location (Figure 2-25), in contrast to overnight incubation with 1M NaOH, but also led to high background compared to a DHEBA gel soaked overnight in 1x Tris glycine (Figure 2-26) or probed immediately after separation (Figure 2-27). All results were with 14%T, 3.5%C acrylamide/DHEBA gels. While initial probe results with DHEBA cleavable gels were promising, more development would need to be done to enable faster assay times and/or improved SNR with DHEBA
versus standard bis cross-linked gels. DHEBA cross-linked gels have been demonstrated to enable separation performance, capture efficiency, and antibody probe on par with bis cross-linked gels (Figure 2-3), but statistically significant improvements in antibody probe SNR or faster overall assay times through the use of DHEBA gels with de-crosslinking has not yet been demonstrated.

Figure 2-25: Inverted fluorescence micrograph of 14%T, 3.5%C acrylamide/DHEBA gel probed with antiPSA after overnight incubation with 1M Glycine NaOH pH 10.4
Figure 2-26: Inverted fluorescence micrograph of 14% T, 3.5%C acrylamide/DHEBA gel probed with antiPSA after overnight incubation with 1x Tris glycine.
Antibody mobility was higher and SDS treated proteins slightly lower for a given %T/%C gel for DHEBA vs. bis cross-linker (Figure 2-28). This change in mobility is hypothesized to arise from the increased hydrophilicity of DHEBA gels compared to bis cross-linked due to the additional –OH groups in DHEBA. More studies would need to be done to better characterize the system, but this characteristic of a gel matrix would be theoretically beneficial for the μWestern given the need to effectively sieve SDS treated proteins, but still allow probing with antibodies. More testing could be done to see if a reduction in gel hydrophobicity can reduce antibody background in the gel.
2.6 Future Directions

Looking ahead, there is significant room for improvement of the µWestern blot assay. Three primary areas of improvement that may be needed for the µWestern blot for future research are 1) better sensitivity and specificity including a lower LLOD and improved separation resolution for improved specificity between isoforms, 2) reduced labor time per data point, and 3) reduced sample and antibody consumption. As shown in Figure S-2 Gerver & Herr\textsuperscript{41} the separation resolution is lower than for a slab gel due to the shorter separation distance (~2mm vs. 8cm). Depending on the target isoforms of interest greater separation resolution may be needed to accurately identify targets. While the
electrophoretic portion of the assay can be run in < 1 hour, the total assay workflow including chip prep and gel fabrication lead to substantially longer hands-on time (Figure 2-29).

The assay itself requires substantial manual intervention due to the use of a single well on either side of the channel and the lack of any fluidic pressure control manifolds overlaying the chip. Automation of fluid exchanges through the use of pressure driven flow control or multiple input wells driven electrophoretically would enable hands-free operation of a full μWestern blot, offering true advantages over a slab-gel Western blot. While a number of commercial systems have been developed to automate various portions of the slab-gel Western blot such as electrophoresis, rapid membrane transfer, and automated blocking and probing steps after sample has been transferred to the membrane, the need for transfer between gel and membrane prevents easy full automation of the traditional slab-gel assay, necessitating at least brief user intervention at various points of the assay. While not currently automated, a full assay taking place in a single microfluidic channel requiring only the electrophoretic exchange of buffers and reagents and a brief UV exposure for blotting allows relatively straightforward system automation, a critical component for more widespread use of the assay and opening the door to integration with other high throughput systems such as 96 well plate screening assays. Through this automation, the labor of a μWestern could be brought significantly below that of a traditional slab Western blot for similar information obtained.
Figure 2-29: Total μWestern blot workflow for running a chip with 4 lanes.

Figure 2-30: μWestern sample and reagent consumption. Left image reprinted from Gerver and Herr.*1

Table 2-1 offers some suggestions for improvement and which of three areas (probe performance, labor time per data point, and sample & antibody consumption) is likely to be improved by each of these changes.
<table>
<thead>
<tr>
<th>Areas for improvement</th>
<th>Improved probe performance (sensitivity, specificity)</th>
<th>Labor time per data point</th>
<th>Sample &amp; antibody consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher multiplexing</td>
<td>X</td>
<td></td>
<td></td>
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<tr>
<td>Better chip interfacing (smaller wells, all components pre-loaded, microfluidic manifold)</td>
<td>?</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Automation (Tecan, pressure driven microfluidic manifold, components pre-loaded)</td>
<td>?</td>
<td>X</td>
<td></td>
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<tr>
<td>Reduce assay &amp; total workflow times (shortening or removing steps)</td>
<td></td>
<td></td>
<td>X</td>
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<tr>
<td>Decouple pore size needed for separation from pore size during antibody probing (open gel pores (de-cross-linking), transfer to lower %T gel for probing, transfer to membrane)</td>
<td>X</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Conduct studies on antibody probe optimization (concentration, duration, primary &amp; secondary, alternative labels/detection)</td>
<td>X</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Move away from probing and washout along the separation axis</td>
<td>X</td>
<td>X</td>
<td>?</td>
</tr>
<tr>
<td>Improved separation resolution (better stacking, longer gels)</td>
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<td></td>
</tr>
<tr>
<td>Improved sample preconcentration (better stacking, membrane preconcentration, integrated sample prep)</td>
<td>X</td>
<td></td>
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*Table 2-1: Areas for improvement on the μWestern blot*

### 2.7 References


Chapter 3: Programmable Microfluidic Synthesis of Spectrally Encoded Microspheres for Parallel Peptide Synthesis and Biological Multiplexing Applications

3.1 Project Motivation

While genomics technologies have enabled the prediction of the amino acid sequences of proteins produced by a number of organisms, it is impossible to computationally determine the protein-protein interactions based on this data alone, and empirical approaches are needed to find peptide binding motifs. Discovering these peptide motifs is important for both the treatment of diseases and advancing the understanding of basic biological processes. For example, the antigen binding targets for a wide variety of antibodies remain unknown, including both anti-self antibodies implicated in causing autoimmune disease and protective antibodies that provide immunity against pathogens. Discovering these epitopes could help develop effective tolerizing treatments or biomarker detections for autoimmune disease\(^1\) or new potential vaccine targets in the case of infectious diseases such as malaria.\(^2\) Beyond the direct application to diseases, linear peptide binding motifs are critical for signaling pathways and are not well mapped for the majority of receptors and enzymes.\(^3\) Creation of a large synthetic linear peptide display library would allow both more efficient mapping of a number of these unknown sites and an assessment of how post translational modifications affect the binding of certain peptides. In addition, the generation of non-biological components such as peptoids could allow for the development of better binding mimetics for pharmaceutical applications.\(^4\)

Existing peptide display technologies are not practical for the unbiased display of peptides spanning the whole proteome of humans or parasites such as malaria. Millions of 10 to 15-mer peptides would be needed to span these proteomes and existing technologies are not even within an order of magnitude of generating a library of this scale. Current peptide library generation technologies are either limited to small numbers of specified peptide sequences (up to \(~10^4\) for planar arrays) or allow for
much larger numbers of peptides through random processes in phage display or split-and-merge combinatorial synthesis, but a random library will only cover a small subset of the actual peptide sequences in an organism as a 10-mer has $20^{10}$ (over ten trillion) possible permutations.

3.2 Project Overview
The goal of this project is to develop a microfluidic platform to enable the inexpensive and rapid generation of libraries of tens of thousands to millions of unique linear peptides each attached to a uniquely barcoded bead. Having a unique barcode for each bead that will contain a specific peptide allows us to both specify a peptide to be synthesized on each bead for an inexpensive parallel batch synthesis process and provides fast peptide read out of biological binding assays. By spectrally barcoding beads using variable ratios of lanthanides, rare earth metals that are excited in the UV and have distinct and narrow emissions in the visible, a large number of uniquely barcoded beads that are compatible with both synthetic peptide synthesis and biological binding assays can be inexpensively and rapidly generated. Generating a massive defined peptide library on small microcarriers allows for inexpensive peptide scans across proteomes while requiring only small sample quantities for binding assays.

3.3 Collaborators
The technology for generating this system spans a number of fields and experts with diverse backgrounds from both UCSF and Lawrence Berkeley National Lab contributed to the project:

- Joe DeRisi (UCSF faculty, Principle Investigator on the project)
- Brian Baxter (DeRisi lab postdoc, lanthanide nanoparticle synthesis and polymer chemistry)
- Camilo Andres Diaz Botia (research assistant in Rafael’s lab, contributed to device fabrication, design, and testing, COMSOL modeling, and developed Simulink code to enable automated control of the microfluidic devices)
• Polly Fordyce (DeRisi lab postdoc, development of microfluidic bead imager/sorter and imaging of beads for publication)
• Rachel Gerver (UCSF/UC Berkeley bioengineering PhD student, development of microfluidic device to generate spectrally encoded beads and synthesis of spectrally encoded beads used in publication)
• Rafael Gómez-Sjöberg (LBNL scientist, advisor on microfluidics development and developed initial proof of concept device for generating single color (and off-chip mixed) PEG-diacrylate beads and developed initial chemistry and UV exposure parameters for PEG-DA bead polymerization, designed surrounding microfluidic device control system at UCSF and LBNL, developed Simulink code to enable automated control of microfluidic devices)
• Brett Helms (LBNL scientist, lanthanide nanoparticle development)
• Kurt Thorn (director UCSF Nikon Imaging Center, spectral code reading algorithms and imaging optimization, imaging of beads for publication and data analysis of imaged beads, construction of optical image system)
• Ron Zuckerman (LBNL scientist, peptide synthesis and mass spec expertise)

3.4 Initial project aims
AIM 1: Develop a microfluidic bead synthesizer device capable of generating stable, optically barcoded beads. Initial goal is to generate 1000-10,000 unique codes, with the ultimate goal of achieving 1 million unique barcodes. This device must allow for the precise control of lanthanide quantities into each bead, mixing of lanthanides to get uniform distribution within a bead and between beads of the same code, generation of uniform droplets and polymerization into solid beads. All of this must be automated and occur on a fast time scale in order for the system to be scalable to a million bead peptide display library.
**AIM 2: Generate peptide display libraries on optically barcoded beads and validate system.**

Validation will include making sure that beads and barcodes are stable with FMOC synthesis reagents and compatible with biological binding assays. Peptides will be validated for quality by checking the peptide sequences on a subset of beads using mass spectrometry. The system will also be validated by using known antibody and linear antigen pairs and checking the ability to both accurately read out fluorescent signal and bead barcode from these bound antibodies and demonstrate lack of non-specific absorption on negative peptide control beads. Initially this work will be done on a small scale with simple binding assays. Another team (Polly Fordyce and Kurt Thorn) is developing a device for bead imaging/sorting to allow for batch parallel peptide synthesis. Once this device is working, the bead sorter can be used to develop much larger scale peptide libraries. Mass spec peptide confirmation combined with repeated bead imaging will be used to make sure that bead barcodes remain identifiable from before the addition of the first amino acid through to the end of the biological binding assay. Once the system is validated with this preliminary testing, patient blood samples will be obtained from Lupus and Rheumatoid Arthritis patients with known epitopes, provided by Paul Utz at Stanford, and tested to confirm that our system can pick up these epitopes from a large scale scan across parts of the human proteome.

### 3.5 Prior Work in Peptide Library Generation

Generating linear 10-mer peptides to span the entire proteome of a pathogen such as malaria or across the human proteome requires the creation of millions of unique peptide sequences. No existing technology allows for generation of a deterministic library within even an order of magnitude of this scale. Very large randomized libraries (>10^7) can be generated using phage display^1 or a split-and-merge combinatorial approach on unlabeled beads, but random libraries will sample only a subset of the actual linear peptide sequences in a proteome.
The largest deterministic peptide libraries (~10⁴) are currently generated in a planar array format, where peptides can be either purified or synthesized separately and then attached to the array, produced on-chip with synthetic chemistry using mechanical deposition of materials⁵, a photolithography-based deprotection scheme⁶, or electric field patterning⁷, or synthesized from DNA strands using cell-free transcription and translation.⁸ However, these planar peptide array synthesis processes become more time consuming and costly as the scale of the array increases, making the generation of large libraries prohibitively expensive. With a bead-based approach, only 20x10 reactions are required for a 10-mer library regardless of the number of unique peptides. The primary added time and cost with increasing peptide library size in a bead-based system comes from the generation of additional encoded beads and the time needed to sort the beads prior to the addition of each amino acid. Our approach minimizes these times and costs to make the system highly scalable.

3.6 Bead Barcode Technical Requirements

An effective microcarrier encoding method to allow for an automated million bead based peptide synthesis system and assay platform should meet the following requirements:

1) A very large code space (>10⁵)
2) Robust and stable code readout (>99.99% accuracy) with multiple reads (>15) over weeks
3) Inexpensive and fast to produce beads of each code (< $0.001 and <10 seconds average per code)
4) Fast code readout and sorting (<0.2 sec per bead)
5) Small microcarriers ( <100uL for a million beads) to minimize sample volumes for testing
6) Compatible with FMOC synthesis process
7) Compatible with biological peptide binding assays including use of fluorescent tags to detect binding

While not an absolute requirement, the following specifications simplify the use of the microcarriers in bioassays and peptide synthesis:

8) Relative density of 1-1.5 compared to water so that the particles stay mixed with the solution for improved reaction kinetics for bioassays

9) Porous structure to allow for diffusion of synthesis reagents through all beads in a batch during the FMOC synthesis process

Existing encoding technologies are limited in their ability to simultaneously meet all of the above requirements.

3.7 Existing Encoding Methods

Spectrally encoded beads have typically relied on quantum dots or organic dyes to generate multiple colors and intensity levels. Organic dyes are subject to photobleaching, which will change the intensity levels over time and with repeated code readouts. Organic dyes also have extremely wide emission bands, limiting the number of colors and range of intensity values that can be used, different excitation wavelengths for each color, and spectral overlap between excitation and emission. Quantum dots initially seemed more promising with papers claiming theoretical code spaces in excess of a million codes, but they have been plagued with challenges that have limited the practical number of codes that have been generated. Due to the overlap between the emission and excitation wavelengths of quantum dots, FRET can occur between QDs in a bead, leading to intensity shifts between colors and making it extremely difficult to control the spectral code of a bead during fabrication. Commercially available quantum dots also have emission peaks twice as broad as those of the lanthanides (25-40nm full width at half maximum vs. 10-20nm), limiting the number of unique colors and intensity levels that can be resolved due to spectral overlap. Small shifts in QD size during QD fabrication will also shift the
peaks, whereas the lanthanide emission peaks are extremely stable and independent of size, doping level, and other manufacturing parameters. Existing commercial bead systems such as Luminex, which utilizes both organic dye and quantum dot encoded beads, have limited their assays to 500 unique beads.  

A couple of groups have developed encoded microcarriers using lanthanides, but neither approach is easily scalable to a million peptide library. Dejneka et al. have developed lanthanide-doped glass bars for bioassays, using stripes of different colored lanthanides as the barcode with a high theoretical code space >10^6, but each code must be separately fabricated by hand with a glass extrusion process, making the system difficult to scale up to millions of codes in practice. Another group has encoded polystyrene spheres by adding 6nm layers of different lanthanides using a layer by layer process with alternating layers of oppositely charged polyelectrolyte. While a large number of codes could be generated in parallel using a split and mix combinatorial process, the thin lanthanide layers and interaction between the lanthanides and the polyelectrolyte interlayers, limits the intensity range and ultimate code space than can be generated with this approach versus our proposal of encapsulating lanthanides in the bulk of the bead.

So far, spatial encoding schemes have been able to generate the highest number of theoretical codes, with some claiming in excess of a billion codes by combining both space and color. This includes spatial codes formed by photolithography of polymers, aluminum bars shaped by photolithography and dry etching, electrochemical deposition of metals with different reflectivities, and selectively photobleaching a barcode into fluorescently labeled polystyrene spheres. While each of these systems meets the requirement of >10^6 codes, they all have limitations in meeting the other requirements including exceeding the microcarrier size limits, not directly compatible with FMOC synthesis, or potential issues with code stability over repeated code readouts.
3.7.1 Spectral Encoding

Existing spectral encoding methods have generally used quantum dots, fluorescent organic dyes or lanthanides. Combining these components in defined ratios enables the generation of spectrally unique signatures that can be used for identification. The number of unique spectral signatures that can be generated for a given spectral system is dependent on the number of resolvable intensity values for each component to the power of the number of components with a distinct spectral channel. For example, 5 unique dyes that can each be resolved reliably at 8 different intensity values would lead to a code space of $8^5 = 32,768$. A challenge with many spectral encoding methods is the need for robust spectral read out in a variety of conditions and over repeated measurements. Fabrication can also pose a challenge as generation of a large number of beads with a given target spectral code requires a low CV on intensity of each spectral channel to enable a greater number of non-overlapping intensity values for each spectral channel. Also, different spectral encoding methods have different spectral emission bandwidths, which can limit the number of spectral channels that are non-overlapping. Some emitters can have shifts in intensity and/or emission bandwidth based on buffer conditions, prior excitations, and other conditions that can make generation of a robust code space difficult.

A number of groups have used quantum dots to spectrally encode spheres, with most claiming a theoretical code space in excess of 1 million and sorting times of ~1000 beads per second.\textsuperscript{20,10,21} One group has addressed the FRET issues of quantum dots by doping each color of QD in a different layer separated by an empty 10nm layer of silica to space QDs of different colors far enough apart to prevent FRET.\textsuperscript{11} Their method uses a batch synthesis process where each layer is added separately in a 200mL flask to produce each code individually, taking many hours to produce each code. This method could potentially be scaled up to large numbers of codes by using a random split-and-mix process where for each QD colored layer, 10 flasks are available corresponding to a given intensity level. Using seven
colors of quantum dots, $10^7$ different codes could be produced in 7 steps using 10 different synthesis flasks allowing for the synthesis of millions of beads in only a few days.

Battersby et al. claim the ability to generate millions of unique signatures by randomly adding fluorescent dyes in a layer by layer split-and-merge synthesis process and observing the signatures with up to 11 detectors along nine different fluorescent parameters and two light scattering parameters. Beads that do not have unique optical signatures are discarded and the remaining beads used as solid supports for directed compound synthesis. Beads can be sorted very rapidly using flow cytometry. They also propose using a similar method with active barcoding during the combinatorial synthesis process by using small (0.5-2.5µm) colloidal particles with different ratios of organic dyes that will adhere to the solid support at each step of the synthesis process. While an impressively large theoretical code space is described for both these methods it is not known how stable the codes would be through an FMOC peptide synthesis process.

3.7.2 Spatial Encoding

So far, spatial encoding schemes have been able to generate the highest number of theoretical codes, with some claiming in excess of a billion codes by combining both space and color. This includes spatial codes formed by photolithography of polymers, aluminum bars shaped by photolithography and dry etching, electrochemical deposition of metals with different reflectivities, and selectively photobleaching a barcode into fluorescently labeled polystyrene spheres.

A spatial encoding scheme in small aluminum rods fabricated by sputtering aluminum onto silicon wafers and forming patterns using a photolithography process followed by a dry etch has been developed with a theoretical code space in the millions. The main advantage of this process is that millions of uniquely and robustly encoded carriers with small volumes can be made in a few days at a very low cost. The main drawbacks of this approach is the high density of aluminum (2.7g.cm$^3$)
compared to water, making the kinetics of bioassays less desirable and the incompatibility of aluminum as a support for peptide synthesis without further modifications to provide binding sites for amino acids.

A system with a very large theoretical code space has been demonstrated by Lee et al. using a color tunable magnetic material. The color changes in the presence of different magnetic fields and a particular color can be permanently frozen in place by mixing the particles with a polymer and using a UV polymerization process. The group has generated particles with 10 spatially separated barcodes with eight distinct colors for a code space of $8^{10}$, over a billion codes, in both hexagonal and bar shapes. All of the particles in the paper are >200µm, but the authors indicate that the minimum size of a feature element can be reduced to a few micrometers, indicating the possibility of producing bar shapes with 8 colors and 7 stripes in the 50µm range to generate over a million codes. Each particle is synthesized individually and each part of the particle must be polymerized separately with a changing magnetic field to generate the different colors so each microcarrier takes about 1 second to generate. The polymer used is PEG-diacylate, as that allows fast UV polymerization, but that is not compatible with FMOC synthesis reagents, so the particles would need to be sealed and coated in another solid support material for peptide synthesis applications.

Braeckmans et al. has demonstrated the potential capability to encode millions of unique optical barcodes into 45µm fluorescent microspheres by using selective photobleaching of spatial barcodes into the spheres using a modified confocal microscope retrofitted with a high power laser. Coding capacity is only limited by the number of stripes, stripe widths, and stripe intensity values that can be generated and optically resolved in the center plane of the sphere. They have demonstrated readable bars of various widths > 1µm separated by 1µm and in 8 different resolvable intensity values. While they claim very fast code generation on the order of 100-1000 microspheres per second is feasible, code read out is difficult due to the need to either orient the spheres in a particular direction to read out the barcode or to systematically scan through large parts of the interior of the sphere to find the code. They have also
created these microspheres embedded with ferromagnetic particles (CrO$_2$) in a layer by layer process and magnetizing the particles by applying an external magnetic field after barcoding.$^{23}$ Applying a smaller magnetic field later allows them to reorient these particles for proper readout. The authors state the materials remain magnetized for “a period after the material is no longer in the field”, but do not indicate how long the magnetization lasts or even how much time has elapsed between their applied magnetization and demonstrated code readout in the paper.$^{23}$ For effective use for the synthesis of millions of unique peptides and the subsequent bioassays, this applied magnetization would need to be stable for weeks. The authors indicate that use of larger particles sizes of CrO$_2$ allows for stronger induced magnetized, but reduces the readability of the code due to increased shadows in the central plane of the barcode. More information would be needed to know whether or not spatial codes embedded in spherical particles present a potentially robust scheme for automated peptide synthesis.

Pregibon et al. demonstrate a spatial encoding method using a microfluidic device for continuous flow lithography. They use two parallel streams, one with a fluorescent dye for encoding and the other with an acrylate-modified probe, and polymerize particles with 30-ms bursts of UV light through a photomask inserted into the field-stop position of the microscope to generate a 2D dot coding scheme which can theoretically generate over a million codes ($2^{20}$). The main limitation of this method is the large particle size needed to encode that many dots, which in this case was $180\text{um} \times 90\text{um} \times 30\text{um}$. Large particle sizes are problematic for our application because of the additional sample volumes that would be needed to accommodate millions of these microcarriers in a bioassay.

Thin metal rods with alternating stripes of metals with different reflectivities can reach very large coding capacities > $10^6$ in a small rod size, 1um x 5um, with the coding capacity primarily limited by the minimum length of stripe that can be read by the light microscope and the number of different metals with unique distinguishable reflectivities.$^{18}$ Rods are synthesized using an electrochemical
deposition process which can produce large quantities of rods simultaneously, but each code much be
generated in a separate batch process making it difficult to scale up to millions of codes in practice."}

3.8 Implementation

A large spectral code space ($10^6$), can be achieved by doping each of six lanthanides into
polymer beads at ten possible intensity levels. As seen in Figure 1, the seven different lanthanides
(Cerium (Ce)/Terbium (Tb), Thulium (Tm), Samarium (Sm), Holmium (Ho), Europium (Eu), Erbium (Er),
Dysprosium (Dy)) have unique and narrow emission peaks, providing the potential for easier
determination of multiple intensity levels than has been achievable with spectral mixing of fluorescent
dyes and quantum dots that have wider emission peaks and overlap between emission and excitation
wavelengths. The seventh lanthanide will be used as a fixed reference standard in all the beads to
reduce errors with spectral readout of the intensity levels of the other six. Lanthanides can all be excited
at the same wavelength in the UV, so only one excitation source is needed. Brian Baxter, a chemist in
the DeRisi lab, has developed lanthanide nanoparticles for this application.

![Image of lanthanide nanoparticle emission spectra](Image generated by Kurt Thorn)

Figure 3-1: Lanthanide nanoparticle emission spectra. (Image generated by Kurt Thorn)

Imaging of lanthanides requires longer time scales than quantum dots or fluorescent dyes, so is
not compatible with a flow-cytometry sorting system. Instead the beads are illuminated in the UV and
imaged in a microfluidic device on a scope with a set of filters optimized for the emission peaks of the
lanthanides. After imaging hundreds of beads simultaneously in a serpentine channel, they will be sorted one at a time into the appropriate bin for the next amino acid in the sequence for batch parallel peptide synthesis. Because the beads will be imaged 10 times for a 10-mer, this imaging process needs to occur quickly in order to produce a million unique peptides in a reasonable amount of time. With just 0.2 seconds of imaging/sorting per bead, it would take over 55 hours just to sort one million beads for each amino acid in the sequence. This time can be minimized by using multiple sorters in parallel, increasing the number of beads in the camera field of view, and working to increase the emission intensity of lanthanides to minimize the exposure time needed for each filter. The sorting and imaging device is being developed by Polly Fordyce, Kurt Thorn, and Rafael Gómez-Sjöberg.

The polymer beads with embedded lanthanides were synthesized using a microfluidic device to allow for precise control over both the bead size and ratios of lanthanides. Such a device needs to be capable of precisely controlling the quantity of each lanthanide, efficiently mixing together the lanthanides to get uniform distribution within beads and between beads of the same desired code, and generating solidified beads of the target size (50µm).

Beads were developed with a mixture of amino-PEG-acrylamide and PEG-diacrylamide in water at 330mg/mL, which is expected to be directly compatible with the FMOC peptide synthesis process, as well as with PEG-diacrylate at 330mg/mL. PEG-acrylamide displays fairly low UV absorption and fluorescence, can be readily thermally polymerized with the addition of APS, and prior to polymerization, remains a liquid at room temperature so can flow through microfluidic channels. The initial publication utilized PEG-diacrylate beads, because they were easier to synthesize due to faster polymerization kinetics and reduced issues with lanthanide nanoparticle aggregation that occurred in the presence of APS with some of the lanthanide nanoparticle wrappers.

3.9 Device Development for Microfluidic Synthesis of Microspheres
Several design iterations of the spectrally encoded bead synthesizer were developed and tested prior to arriving at the design utilized in publication. All of the designs utilized a two stage approach for spectrally encoded microsphere fabrication. In stage (1) (Figure 3-2) lanthanide nanocrystals in prepolymer solution are loaded from the inputs and mixed in the herringbone mixer while the flow exits through the waste output. In stage (2) (Figure 3-3) the waste output valve is closed, the lanthanide input valves are closed and flow is controlled through a water input to push the prepolymer lanthanide solution through the T-junction. The prepolymer solution meets an oil phase at this junction enabling the formation of precisely controlled droplets. These droplets can either by polymerized on-chip utilizing UV light or off-chip in a thermal water bath. On-chip UV light was utilized for PEG-diacylate beads whereas off-chip thermal polymerization was utilized with PEG-acrylamide beads due to the slower reaction kinetics. All of the microfluidic bead synthesizer chip designs developed through the course of this dissertation utilized this basic two stage process. Simulink was used to control all of the on-chip valves and off-chip pressure controllers. This enabled full hands-free walk away operation for bead synthesis once fluid lines and control lines were connected to the device.
Figure 3-2: First stage of microfluidic bead synthesizer operation: mixing together the lanthanide prepolymer solutions.
Initial device development was based on the original bead synthesizer device designed by Rafael Gomez-Sjöberg for synthesis of single color beads. This device, shown in Figure 3-4, produced beads utilizing pre-mixed prepolymer solution loaded into input (A) and oil with span 80 loaded into input (B). Both of these inputs contained high resistance channels to enable precise control of flow rates for stable droplet production at the T-junction (C). Both the oil and pre-polymer inputs also contained variable resistors activated through the opening and closing of on-chip valves consisting of a thin PDMS membrane into a rounded channel. These were controlled by pressurizing water filled control lines underneath the flow layer, a technology originally developed by the Quake lab. After droplet formation at the T-junction, drops slow down upon entering the wider and taller light blue channel to the left of the T-junction. In this region, UV light illumination polymerizes the PEG-diacylate droplets containing lanthanide nanoparticles. The prepolymer solution consisted of 153µL of water, 666µL of PEG-DA (for a final v/v of 66% PEG-DA concentration), and 181 µL of Irgacure solution in methanol at
0.33mg/mL (for a 6% w/v final Irgacure concentration). This had resulted in the successful polymerization of beads containing lanthanides as shown in Figure 3-4. This device operated in a single stage process.

Figure 3-4: Initial bead synthesizer design for single color beads (without on-chip lanthanide mixing) developed by Rafael Gómez-Sjöberg prior to my arrival on the project.

Figure 3-5: PEG-diacylate beads spectrally encoded with lanthanide nanoparticles. (Image generated by Kurt Thorn)
Subsequent designs focused on enabling on-chip mixing of different lanthanide input streams to form spectrally encoded beads of controlled ratios of lanthanides. This requires accurate control of different lanthanide flow rates, efficient on-chip mixing, and stable production of droplets. The development required to achieve these goals is described in the subsequent sections.

3.9.1 On-Chip Mixing of Lanthanides

The small channel cross-sectional areas in microfluidic devices lead to laminar flows that can make mixing challenging. In a straight smooth channel, mixing will occur by diffusion alone. From the Stokes-Einstein equation for estimation of diffusion coefficient:

\[ D = \frac{k_B T}{6 \pi \eta a} \]

Where \( a \) is the particle radius, \( T \) is temperature, \( k_B \) is the Boltzmann constant, and \( \eta \) is the dynamic viscosity. For lanthanide nanocrystals the estimated diffusion coefficient from the Stokes-Einstein equation is estimated to be \( 1.38 \times 10^{-23} \times 298/(6 \times 3.14 \times 0.0023 \times 10^{-7}) = 9.5 \times 10^{-13} \text{ m}^2/\text{s} \) (range \( 1 \times 10^{-12} \) to \( 1 \times 10^{-13} \) depending on lanthanide particle size and polymer concentration/size). Without chaotic mixing the characteristic time scale of diffusion over a 50\( \mu \text{m} \) distance would be \( t = (5 \times 10^{-5})^2/9.5 \times 10^{-13} = 44 \) minutes, substantially longer than is feasible for efficient code generation.

Instead modifications need to be made to the channel to enable efficient on-chip mixing. A staggered herringbone channel was chosen due to ease of fabrication in PDMS and efficiency of mixing. This mixing channel architecture, originally developed in the Whitesides lab by Stroock et al.,\(^{25} \) relies on alternating chevron grooves in the channel. These grooves lead to recirculating flows within the channel to enable chaotic mixing, significantly decreasing the channel length and time needed to fully mix within a microfluidic channel. An image of these grooves patterned at the entrance to the mixing channel for
the microfluidic bead synthesizer is shown in Figure 3-6. This shows the first 1.5 cycles with each cycle containing 8 grooves in each orientation.

Figure 3-6: Herringbone groove patterning at entrance to mixer channel.

The number of herringbone cycles needed to achieve CV < 0.1 within a fluid filled channel can be estimated from Williams et al.\textsuperscript{26}:

\begin{equation}
I_{0.1} = 1.73\log(Pe) - 2.16
\end{equation}

This yields a predicted cycle length of 10 herringbone cycles, for a mixing channel width of 200µm, with a cycle consisting of a row of herringbone grooves in one orientation followed by a row of herringbone grooves in the mirror image orientation. The initial device design utilized 10 herringbone cycles, but failed to enable complete mixing. This was partially due to the challenge of effectively patterning grooves on top of the mixing channel in SU-8. Grooves were missing on parts of the channel and some of the walls were sloped on the herringbones, narrowing the width of the grooves and
negatively impacting the flow recirculation. Additional grooves were lost during PDMS casting as PDMS would become trapped in between the grooves and stick to the SU-8 molds. A few changes were made to address these fabrication issues 1) the grooves were designed to be wider (65µm in the first iteration, 80 µm in version 2.1.1 and 95 µm in the final design) to account for some of the sloping that inherently occurs due to UV light scattering during wafer exposure through the mask, 2) wafers were exposed to trichloromethylsilane (Sigma-Aldrich, St. Louis, MO) vapors for 60 minutes to silanize the surface and reduce PDMS sticking and 3) the herringbone layer SU-8 layer was spun and photopatterned directly onto the mixer channel prior to developing of the other prior two SU-8 flow layers (as described in the Supplemental Information section). This process enabled improved fabrication of herringbones along the full length of the channel. When the mixing channel layer was developed prior to spinning the groove SU-8 layer, the tall height of this channel relative to the surrounding surfaces prevented an even spin coating on the wafer that led to significant non-uniformity in groove depth along the channel as well as regions without grooves.

Additionally, in subsequent device designs the number of herringbone cycles was doubled to 20 cycles to ensure robust mixing even with some fabrication variability. This was shown to enable complete mixing in the channel as shown in Figure 3-7.
Figure 3-7: Efficient mixing in herringbone channel
3.9.2 Accurate control of lanthanide flow rates

Another critical requirement for achieving robust code generation is the ability to accurately control the flow rates of different lanthanide precursor solution. The flow rate also needs to be stable over time. These requirements along with the desire for fast switching between codes and compatibility with on-chip valves led to the use of off-chip pressure controllers to control flow rates (MFCS-FLEX, 8 channels, 0-1000 mBar range, Fluigent SA, Paris, France). On-chip valves open and close the flows for each individual lanthanide in pre-polymer solution to prevent backflows that could negatively impact subsequent codes and also to prevent any unwanted lanthanides from flowing into the mixing channel for a given code.

Adding a higher resistance input onto the lanthanide input channels relative to the mixing channel can aid in lanthanide flow control as fluctuations in downstream pressure and resistance will have less impact on flow rates from each lanthanide input. Initial designs utilized a long winding channel for each input with shorter channel height and narrower width (dark blue) than the mixing channel (light blue with pink herringbone grooves) as shown in Figure 3-8.
This design proved problematic as the resistance from these long input channels was highly variable. The polymer precursor solutions can dry out over time in the channel leading to large changes in the viscosity, impacting the hydraulic resistance. Higher pressures can also lead to PDMS deformation changing the cross-sectional area of the channel. Device to device variability in the SU-8 molds and PDMS casting led to large differences in resistance between devices as well as between input channels on a given device, making consistent performance substantially more challenging. To address these issues, the on-chip resistors were replaced with 50µm diameter PEEK tubing on each of the inputs for the final two device iterations (ver 2.3 and 2.4). The hydraulic resistance can be precisely tuned by changing the length of the tubing. The length was chosen for a resistance ~40-fold higher than the resistance through the mixing channel. This was chosen as it enabled good control over the lanthanide
flow rates while still enabling reasonably high flow rates through the system, which was limited by the max 15 psi of pressure for our high-precision pressure controller.

Even with the high resistance inputs there is coupling between all of the lanthanide input flow rates. This is particularly pronounced when one lanthanide input stream has a substantially lower flow rate than another as the difference between the applied pressure and the pressure at the mixer inlet is minimized with a low flow rate lanthanide input stream. In this case, changes in the flow rates of other lanthanides can have a significant impact on the flow rate of the lanthanide being mixed at a lower volume. In a microfluidic system the flow rate $Q$ defined by:

$$Q = \frac{\Delta P}{R_h}$$

Where $\Delta P$ is the drop in pressure across a given region and $R_h$ is the hydraulic resistance across a given region. The hydraulic resistance is dependent on the cross sectional area of the channel, the perimeter of the channel, the length of the channel and the viscosity of the fluid. The use of multiple lanthanide input streams leads to a coupling as $\Delta P$, defined as the drop in pressure between the tube containing the lanthanide precursor solution and the inlet to the mixer, will depend on the flow rates of the other lanthanides. The overall flow can be described by the following set of coupled equations (4) and (5):

$$Q_n = \frac{P_n - P_{mix}}{P_n}$$

(4)

$$Q_{tot} = \frac{P_{mix}}{R_{mix}}$$

(5)
Where $Q_n$ (for $n = 1$: number of lanthanides) is the flow rate of each lanthanide, $P_n$ is the pressure set by the pressure controller for each lanthanide, $P_{mix}$ is the difference between the pressure at the inlet to the mixer and atmospheric pressure, $R_n$ is the hydraulic resistance of each of the lanthanide input streams, which will be dependent on the length of the 50µm PEEK tubing and the viscosity of the lanthanide polymer precursor solution. $Q_{tot}$ is the total flow rate of all of the lanthanide input streams ($Q_n$). Accurate control of the input streams requires determining $R_{mix}$ and $R_n$. Once those values are determined the set of equations can be solved to calculate the applied pressures $P_n$ needed to obtain a desired ratiometric code.

These parameters are determined using a calibration routine performed at the start of generation of a new set of codes. A second reference polymer precursor solution containing a fluorescent dye can be connected to the device. When the same pressure is applied to this reference and a lanthanide input $P_n = P_{ref}$ and:

\[
\frac{Q_n}{Q_{ref}} = \frac{(P_n - P_{mix})/R_n}{(P_{ref} - P_{mix})/R_{ref}} = \frac{R_{ref}}{R_n}
\]

(5)

If the relative flow rates of the lanthanide and reference streams are known then the relative resistances can be determined from the above equation. The relative flow rates can be determined by measuring the fraction of the channel at the mixer inlet taken up by the lanthanide input stream and the fluorescently labelled reference standard as

\[
\frac{Q_n}{Q_{ref}} = \frac{W_n}{W_{ref}}
\]

(6)

An example is shown in Figure 3-9 where the resistance for the reference is much higher than the resistance for the lanthanide input.
Some lanthanide polymer precursor solutions can also often be optically distinguished in brightfield and relative resistances measured directly between these inputs as shown in Figure 3-10 where the lanthanide precursor solution on the right takes up 19% more of the channel indicating a 19% higher flow rate for the right input, corresponding to a 19% higher resistance on the left.
When only one lanthanide input is connected to the device $Q_{\text{tot}} = Q_m$ so it follows that:

$$\frac{R_n}{R_{\text{mix}}} = \frac{P_n - P_{\text{mix}}}{P_{\text{mix}}}$$

(7)

A valve is opened on a second test input and the pressure adjusted on the test input so that the flow rate is zero. When $Q_{\text{test}} = 0$ the $P_{\text{test}} = P_{\text{mix}}$. Once $P_{\text{mix}}$ is determined the relative resistance between the lanthanide input and the mixing channel can be calculated. With these two values, $R_n$ and $R_{\text{mix}}$, determined experimentally, the set of coupled equations can be solved to determine the pressure needed at each lanthanide input for each desired ratiometric code. It was determined through these calibration routines that $R_{\text{mix}}$ varied based on flow rate, so the pressures were set to ensure that $Q_{\text{tot}}$ was equal for all of the codes and equal to the $Q_{\text{tot}}$ value used during the $R_{\text{mix}}$ calibration of a device.

3.9.3 Bead Synthesizer Development History

Here the research and development process to arrive at the microfluidic device design used in publication will be summarized including the changes driven into each design iteration to meet the ultimate project aims for robust synthesis of spectrally encoded microspheres. The fabricated bead synthesizer iterations are shown below in Figure 3-11 through Figure 3-17. All device iterations were fabricated using PDMS from SU-8/AZ-50 molds formed using standard soft flow photolithography processes as described in the Supplemental Section. The device design consisted of 5 flow layers (resistor 5µm (purple), valve layer (AZ-50 45 µm rounded) (red), flow square low 45 µm (dark blue), flow square high 75µm (light blue), and staggered herringbone grooves 35µm (pink)). The control layer consisted of a single layer of square channels 25 µm (yellow). Earlier design iterations utilized a flow
square high channel of 100 µm instead of 75µm (ver 1.0-2.1), but the channel height was reduced for later designs to improve mixing performance in the staggered herringbone channel.

From the first iteration (version 1.0 (Figure 3-11)), the automated microfluidic bead synthesizer was designed to operate in two stages as previously described in Figure 3-2 and Figure 3-3. Lanthanides in prepolymer solution are flowed through a staggered herringbone mixing channel in the first stage followed by a second stage of droplet formation at a T-junction. This is conducted separately rather than in a continuous operation to enable a complete exchange of lanthanides in the mixer channel in between codes and to aid in stability of both droplet formation and spectral ratio control.

Long channels were utilized at the input of each of the lanthanides to ensure a higher resistance between the inlet and the start of the mixing channel than between the start of the mixing channel and the device exit. Similarly, high resistance channels were designed at the pre-polymer input and oil channels to ensure stable flow rates during droplet production. A wide outlet was established for the waste to minimize resistance. Similar to the original design by Rafael Gomez-Sjöberg, a large bead collection chamber was created on-chip to store beads after UV polymerization, which occurred in the light blue high channel leading up to the bead collection chamber. Two inputs immediately preceding the bead collection chamber allowed for alternating pressure to distribute the beads throughout the large bead chamber.

The length of the herringbone was determined based on the analytical equation (2) as previously discussed. Challenges in fabrication led to incomplete herringbone formation on the first iteration, so this component of the microfluidic bead synthesizer was not able to be effectively tested on the first design.

With this design, bubble formation in the large chamber (Figure 3-12) could lead to backpressure changes upstream that impacted droplet size. This was particularly problematic when
attempting to do on-chip thermal polymerization of PEG-diacrylamide droplets in this chamber. On-chip thermal polymerization also led to problems of chip delamination. PEG-diacrylamide beads were not able to be effectively polymerized utilizing on-chip UV exposure as had been utilized for PEG-diacrylate beads. These concerns led to subsequent designs where beads and droplets were immediately brought off chip for collection and thermal polymerization if needed.

The size and shape of the waste output also led to partial collapse of that region on some of the devices, leading to variations in the resistance of that portion of the device, which could impact control of lanthanide flow rates. This region was designed to be larger than the mixing channel so that multiple valves channels could be utilized for controlling the flow. Each valve needed to be smaller than the mixing channel dimensions in order to enable full closure by the PDMS control membrane at pressures less than 25 psi. Higher pressures increased the risk of PDMS delamination during operation. It was critical that this valving did not create a high resistance at the output as this could impact upstream control of lanthanides. To mitigate this impact, nineteen valves were arranged in parallel to ensure low resistance at the output relative to the mixing channel. In subsequent designs the shape of the waste output was altered to prevent collapse while still maintaining a large number of output valves arranged in parallel to control waste flow.

It was also found that utilizing prepolymer to push the mixed lanthanide prepolymer solution into the T-junction led to unstable droplet formation as the polymer precursor solution would begin to dry out in the input resistor while lanthanides were being mixed, leading to an increase in viscosity which increases hydraulic resistance, resulting in a lower flow rate for a given pressure. As prepolymer flowed through the resistor and was replaced with fresh prepolymer from the off-chip reservoir, the flow rate would gradually increase leading to an increase in droplet size over time. Subsequent designs instead used water to control this flow, which led to much more stable droplet production.
Figure 3-11: Bead synthesizer v1.0.
(The + shaped alignment marks are 400µm wide)
The next bead synthesizer design (2.0), shown in Figure 3-13, eliminated the large collection chamber and instead utilized valving to shuttle droplets between waste and bead collection. A test channel with a sieve valve was added to the bottom right corner of the device to enable on-chip testing of successful droplet polymerization after UV exposure or off-chip thermal polymerization. Additionally, EM90 in oil inputs were added to reduce the risk of droplet merging when droplets entered the wider channel. A moat consisting of a flow square high channel (light blue) not connected to the rest of the flow channels was added around this region of the chip to prevent diffusion of EM-90 to the rest of the chip. Subsequent testing found that EM-90 was more effectively added directly to the oil input for initial droplet production, rather than added downstream, so these inputs were eliminated in later design
iterations. The valves were increased from 100 x 100 µm to 200 x 200 µm to reduce the valve closing pressures to prevent device delamination (< 30 psi).

For this design, the herringbones were able to be more effectively fabricated by spinning on the flow square low, flow square high, and herringbone layers directly on top of each other after UV exposure and post-exposure bake, but without developing the layers to remove unexposed regions. Developing was done only after all three of these layers were assembled on the device. This led to substantially more robust fabrication of herringbone grooves. This fabrication technique was developed in collaboration with Camilo Diaz-Botia who assisted on device fabrication at LBNL. It was found that the base of the grooves were narrower than designed due to slanting side walls which can occur due to some light scattering during the UV exposure process. A subsequent design, chip version 2.1 was developed, which was identical to 2.0, but contained wider herringbone grooves (80µm vs. 65µm). Even with improved herringbone fabrication and the wider grooves, it was found that 10 cycles were not sufficient to achieve full mixing, so the number of cycles were doubled for subsequent design iterations starting with version 2.2.

Version 2.1.1 was similar to 2.0 and 2.1 with some changes made to the flow square high layer and flow round layers to change the shape of the Drop Out valve to prevent drops from getting caught in the taper as had occurred with the 2.0 and 2.1 device designs. The flow square high was made straight and a taper added to the flow round layer to address this problem (Figure 3-14).
Figure 3-13: Bead synthesizer v2.0
(The + shaped alignment marks are 400µm wide)
Figure 3-14: Change in valving to control shifting of droplets between waste and off-chip droplet collection. Bead synthesizer version 2.0 shown on left and version 2.1.1 shown on the right. The taper was shifted from the flow square high layer (light blue) to the rounded valve layer (red) to prevent droplets from becoming trapped at the interface.
The number of herringbone cycles was doubled from 10 to 20 for bead synthesizer version 2.2 to ensure better mixing (Figure 3-15). Greater overlap was added between the valve and the flow square high channel in the drop output to prevent droplet compression through the valve. The mix waste was moved further from the main channel to reduce risk of a connection forming between the channels due to overexposure during SU-8 photolithography. There was also a slight shape change on
Mix Waste output: a moving of posts and larger posts to reduce the roof sagging at that output. The circles were eliminated on the sieve test channel as beads were becoming trapped there during testing. A circle was added to the flow square low layer where the lanthanide input channels all meet to avoid trapping a bubble during SU-8 mold making as had periodically occurred with earlier device designs, increasing wafer yield.

For bead synthesizer 2.3 (Figure 3-16), the secondary inputs after the drop out valving were eliminated as these were not being used as it was found that adding EM90 directly to the initial oil input was more effective for preventing drop coalescence and interaction with the channel walls than adding EM90 in oil downstream of the T-junction. The valving between drop waste and drop collection was further modified to ensure similar resistances from each channel so that the outputs could be switched without creating backpressure changes that propagate upstream and disrupt droplet production or change droplet size. With the start of droplet formation the first few droplets can vary in size as the system stabilizes so it was critical that the first few droplets were discarded to the waste stream and then subsequent droplets collected for polymerization. The timing in switching from drop waste to drop collection was also critical to prevent upstream pressure fluctuations that can impact droplet formation. For the system, the droplet collection valve would be opened prior to the drop waste valve being closed and then the drop waste valve closed five seconds later after droplet formation had restabilized. Large diameter PEEK tubes were utilized on the output to ensure low resistance and similar resistance between drop collection and drop waste. Earlier experiments resulted in polymerization at the metal output pins with unpolymerized PEG-diacrylamide drops so tygon tubing with metal pins was replaced with PEEK tubing.

It had also been found in earlier device iterations that the resistance of the long PDMS channel at the lanthanide inputs was unstable due to changes in viscosity over time as polymer precursor solution remained in those channels. For this device design, the effectiveness of the original resistor
design was compared to a new design where the high resistance was shifted off-chip to 50 µm PEEK tubing. This off-chip PEEK tubing was designed to be 40-fold higher than the resistance of the mixer channel. The PEEK tubing was found to much more effectively control the lanthanide flow rates, so for subsequent device designs the on-chip input resistors for the lanthanide polymer precursor solutions were eliminated entirely and replaced with off-chip PEEK tubing. This led to bead synthesizer version 2.4 (Figure 3-17), which was used in publication. Because the publication utilized PEG-diacrylate UV polymerized on chip rather than off-chip thermal polymerized PEG-acrylamide beads the output tubing for bead collection was attached directly to the light blue flow square high channel after the T-junction and before the valving between drop collection and drop waste.
Figure 3-16: Bead synthesizer version 2.3
(The + shaped alignment marks are 400µm wide)
Figure 3-17: Bead synthesizer 2.4 (version used in publication)  
(The + shaped alignment marks are 400µm wide)

3.10 PEG-acrylamide bead development

The initial proof of concept of beads synthesized with embedded lanthanides was demonstrated by Rafael Gomez-Sjöberg prior to my beginning work on the project, with the synthesizer shown in Figure 3-4, utilizing PEG-diacrylate (PEG-DA) as the polymer. Our peptide synthesis advisor on the
project, Ron Zuckerman, suggested a switch to PEG-acrylamide beads as PEG-diacylate would not be stable upon exposure to the FMOC reagents needed for peptide synthesis.

Initial testing with PEG-diacylamide bead synthesis showed that the same polymerization conditions used with PEG-diacylate droplets, Irgacure as the photoinitiator at 6% w/v and <10 seconds of UV exposure, were not sufficient for droplet polymerization. This is likely due to the slower reaction kinetics of diacrylamide versus diacylate. Polymerization using Irgacure was possible using 6% w/v Irgacure with 330mg/mL PEG-DAM in a UV oven for 30 minutes, but this method had led to non-uniformities in bead size.

Focus was shifted to utilizing APS with thermal polymerization to generate polymer microspheres. Initial experiments were run with heating the PDMS devices for on-chip thermal polymerization, but it was found that the heat increased the risk of device delamination. Instead, droplets were brought off chip into a tube immersed in a hot water bath. Initially droplets merged in the tube prior to thermal polymerization, but it was found that filling the off-chip tube partially with oil and submerging the tygon tube outlet into the oil prevented this droplet aggregation in the tube and enabled successful thermal polymerization of PEG-acrylamide beads. Uncoded amino-PEG-acrylamide/PEG-diacylamide beads were successfully made with the microfluidic device with between 0-0.4mmol/g amine content. APS was used at a final concentration of 20mg/mL with thermal polymerization taking place overnight in a 70C water bath.

Introducing lanthanides into these beads was more problematic. The lanthanide nanoparticle solutions were found to accelerate room temperature polymerization of acrylamide with APS. 0.5 mmol/g amino-PEG-acrylamide, PEG-diacylamide, 20mg/mL APS and 1:1 LaPO4:CeTb and YVO4:Eu polymerized after 3 hours at room temp, whereas the same solution without lanthanide nanocrystals remained liquid after overnight incubation. This led to the introduction of APS on-chip rather than
premixed in the lanthanide precursor solutions. A solution of APS in water at 20mg/mL or 200mg/mL was mixed in 1:10 with the lanthanide input streams for a final APS concentration in the prepolymer mixture at 2mg/mL or 20mg/mL.

The primary challenge in utilizing PEG-acrylamide was aggregation of the lanthanide nanoparticles, which was found to occur in the presence of APS. This occurred with and without the presence of PEG-acrylamide so was not due simply to rapid polymerization of the gel precursor. Initially it was thought that this aggregation might be due to the decreased pH with APS, but adding a phosphate buffer to bring the pH from 3.95 to 7.4 led to similar levels of lanthanide aggregation in the presence of APS.

An example is shown below (Figure 3-18) for YVO4:Sm with a citrate wrapper (purchased from Sun Innovations) mixed on chip with APS in water at 200mg/mL. This leads to rapid aggregation that forms large chunks blocking the channel within seconds of mixing.

![Figure 3-18: Lanthanide nanoparticle aggregation in the presence of APS. (Channel is 200µm wide).]

Other initiators were tested for polymerization of bulk PEG-diacylamide (330mg/mL in water) in an eppendorf in a hot water bath at 70C, with results shown below in Table 3-1.
Table 3-1: Polymerization time of bulk PEG-diacrylamide in a 70°C water bath with different initiators.

These initiators were then tested with PEG-diacylamide (330mg/mL in water) droplets formed off-chip by mixing PEG-diacylamide precursor solution in a 1:4 ratio with oil (2% EM90, 0.05% Span 80, 98% mineral oil) on a shaker for 10 sec at 15hz followed by 10 sec at 17hz. This generated droplets in a range of sizes from smaller than 50µm to several fold larger. These results are shown in Table 3-2.

Lanthanides were also tested for aggregation in the presence of these initiators, and both V-50 and ACVA led to aggregation with both LaPO4:CeTb polyphosphate wrapped (synthesized by Helms lab) and YVO4:Sm citrate wrapped (Sun Innovations) lanthanide nanoparticles. These were tested by flowing initiator and lanthanide stock solution at equal flow rates into the mixing channel on the microfluidic bead synthesizer. No aggregation was seen with the negative control of water.
<table>
<thead>
<tr>
<th>Test solution</th>
<th>LaPO4:CeTb</th>
<th>YVO4:Sm</th>
</tr>
</thead>
<tbody>
<tr>
<td>milliQ water</td>
<td>no aggregation</td>
<td>no aggregation</td>
</tr>
<tr>
<td>V-50 (100mg/mL) in milliQ water (pH 6.4)</td>
<td>aggregation</td>
<td>aggregation</td>
</tr>
<tr>
<td>ACVA (2mg/mL) in milliQ water (pH 3.2)</td>
<td>aggregation</td>
<td>aggregation</td>
</tr>
<tr>
<td>ACVA (2mg/mL) in potassium phosphate buffer (pH 7.6)</td>
<td>aggregation</td>
<td>aggregation</td>
</tr>
<tr>
<td>Irgacure (4mg/mL) in milliQ water (pH 7.55)</td>
<td>aggregation</td>
<td>no aggregation</td>
</tr>
</tbody>
</table>

*Table 3-3: Lanthanide nanoparticle aggregation in the presence of initiators*

Follow up bulk testing suggested that polyacrylic acid (PAA) wrapped lanthanide nanoparticles may have less aggregation in the presence of APS than earlier citrate wrapped nanoparticle configurations. Below is an image of PEG-diacrylamide droplets produced with YVO4:Eu with a PAA acid wrapper (Figure 3-19) (0.312 mmol/g amine loading, 330mg/mL amino-PEG-acrylamide/PEG-diacrylamide, 20mg/mL APS, 50% filtered water and 50% YVO4:Eu stock solution) Some aggregation did occur ~30min after mixing the solution, but this lanthanide chemistry may be more stable with on-chip mixing of APS into a stock solution of lanthanides with amino-PEG-acrylamide/PEG-diacrylamide.

Follow up testing in a 96-well plate with YVO4:Sm and YVO4:Dy PAA wrapped lanthanides (synthesized in house as described in the supplemental section) with APS and heated to 70C for 45 minutes resulted in minimal aggregation as compared to YVO4:Eu citrate wrapped (Sun Innovations) lanthanide nanoparticles tested in parallel (Figure 3-20). More follow up testing would need to be done, but PAA wrapped lanthanide nanoparticles in PEG-acrylamide with on-chip mixed APS and off-chip thermal polymerization of droplets in a 70C water bath is a promising avenue for FMOC synthesis compatible spectrally encoded microspheres.
Figure 3-19: PEG-diacrylamide droplets with PAA wrapped YVO4:Eu
Figure 3-20: Lanthanide aggregation and emission before and after addition of 20mg/mL APS with 45 minutes incubation at 70°C. Citrate wrapped Eu shows high aggregation and significant signal reduction after treatment with APS and heat (top right) as compared to PAA wrapped BiSm and BiDy lanthanides, which had minimal aggregation and minimal signal reduction.
3.11 Publication on Programmable Microfluidic Synthesis of Spectrally Encoded Microspheres

The following section is reprinted from “Programmable Microfluidic Synthesis of Spectrally Encoded Microspheres” by Rachel E. Gerver*, Rafael Gomez-Sjöberg*, Brian C Baxter*, Kurt S. Thorn*, Polly M. Fordyce*, Camilo A. Diaz-Botia, Brett A. Helms, and Joseph L. DeRisi. Rachel Gerver’s role on the project was the development of the microfluidic bead synthesizer used to synthesize the lanthanide encoded microspheres. This included design, fabrication, and testing of devices along with the synthesis of the beads utilized in the publication. The five co-first authors co-wrote the publication with Rachel being responsible for overall editing and assembly. Brian Baxter and Brett Helms synthesized the lanthanide nanoparticles used in the publication. Rafael Gomez-Sjöberg provided overall microfluidics guidance for all of the microfluidic components of the project and developed the initial proof of concept microfluidic bead synthesizer and initial lanthanide PEG-DA beads as previously described. Kurt Thorn designed and assembled the imaging setup, imaged the beads, performed the linear unmixing to determine the codes and recommended the lanthanide ratios to be used for the initial code set of 24. Polly Fordyce developed the serpentine channel used for imaging and worked with Kurt to image the beads. Camilo Diaz-Botia assisted on device fabrication and testing for both the bead synthesizer and bead imaging microfluidic devices. Joseph DeRisi provided overall guidance and supervision of the project.
Programmable Microfluidic Synthesis of Spectrally Encoded Microspheres

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3.11.1 Abstract
Spectrally encoded fluorescent beads are an attractive platform for assay miniaturization and multiplexing in the biological sciences. Here, we synthesize hydrophilic PEG-acrylate polymer beads encoded with lanthanide nanophosphors using a fully automated microfluidic synthesis device. These beads are encoded by including varying amounts of two lanthanide nanophosphors relative to a third reference nanophosphor to generate 24 distinct ratios. These codes differ by less than 3% from their target values and can be distinguished from each other with an error rate of <0.1%. The encoded bead synthesis strategy we have used is readily extensible to larger numbers of codes, potentially up to billions, providing a new platform technology for assay multiplexing.
3.11.2 Introduction

Over the past several years, advances in biomedical research technology have driven an unprecedented explosion of genomic and proteomic data, yet the challenge of translating new biomarkers of disease into actionable diagnostics and therapeutics remains daunting. To both validate and deploy the vast numbers of recent discoveries into clinical practice requires new approaches to multiplexing and high-throughput biomarker analysis. Despite intense research, few practically available cost-effective assays for multiplexing exist, and new approaches are needed. Beyond diagnostics, advances in multiplexing may have impact on basic research and development systems, including combinatorial drug discovery.

Multiplexed assays require that individual probes be reliably identified and tracked throughout an experiment. This identification and tracking is often done using planar arrays, where the identity of each probe is encoded by its physical position. An alternative approach uses encoded beads, where each probe is attached to a separate bead that is uniquely identifiable. Bead-based assays offer faster reaction kinetics, increased assay flexibility, and improved reproducibility and reduced costs due to the ability to attach probes to multiple particles at once. However, technical challenges in bead encoding have limited their practical application to date. Existing encoding methods generally fall into two categories: spatial encoding and spectral encoding. Spatial encoding schemes create graphical patterns or bar codes in the particle material in a variety of ways. However, spatial methods face difficulties in cost-effective fabrication, often require large particles to generate large code sets, and have slower and more challenging code readout than existing spectral methods due to orientation requirements.

Spectral encoding schemes incorporate mixtures of luminescent materials such as lanthanides, quantum dots (QDs), or fluorescent dyes that emit light at different wavelengths to generate uniquely identifiable signatures. These schemes allow identification of codes in any orientation and are compatible with conventional bead synthesis procedures and standard detection optics, making them
particularly attractive. Despite the promise of spectral encoding schemes, technical challenges have limited their practical code capacity. Organic dyes have broad emission spectra, narrow Stokes shifts, and limited photostability, making it difficult to deconvolve spectral signatures from multiple dyes and reducing the usable lifetime of the codes. Quantum dots offer relatively narrow and tunable excitation spectra, and have therefore been the subject of considerable recent interest for encoding schemes. However, QDs have complicated photophysics and can undergo energy transfer and re-absorption when tightly packed together\textsuperscript{47-50}. These effects limit the number of optical codes that can be created, due to re-absorption losses at higher concentrations in the beads\textsuperscript{51,52}. As a result, the largest experimentally produced spectral code sets from organic dyes or quantum dots have fallen far short of theoretical expectations. The best known commercial system, Luminex\textsuperscript{53}, has been limited to 500 unique codes and code sets synthesized in the literature have been even smaller\textsuperscript{36,37,39-44}.

Lanthanide nanophosphors are an attractive alternative to QDs. They have narrow emission peaks due to the Laporte forbidden $f$-$f$ energy transitions within the rare earth emitter (e.g., Eu$^{3+}$)\textsuperscript{54} and are highly photostable\textsuperscript{55-57}. A common host matrix (e.g., YVO$_4$) can be used with several individual emitters, resulting in families of nanophosphors with a shared excitation profile, but with distinct emission spectra (Fig. 1A). Unlike QDs, neither re-absorption nor energy transfer is observed in lanthanide nanophosphors. They are also less prone to bleaching, chemical or oxidative damage to their emission characteristics over time. Collectively, these properties make lanthanide nanophosphors an ideal luminescent material for spectral encoding.

Here, we present a novel approach to synthesize spectrally encoded polymer beads containing multiple lanthanide nanophosphors. To generate these beads, we use a custom and fully automated microfluidic device to mix different predetermined ratios of nanophosphors suspended in monomer, followed by photopolymerization, resulting in uniquely identifiable spectral codes. Using this device, we have produced, imaged, and characterized thousands of encoded beads with 24 distinct spectral codes.
The beads are highly uniform in size and have a very tight distribution of lanthanide ratios around the programmed values, such that we can distinguish between the codes with <0.1% error. These results establish the practical feasibility of using lanthanide nanophosphors for spectral encoding, and lay the foundation for future high-throughput multiplexing of biological assays.

3.11.3 Methods

Lanthanide synthesis

The synthesis of nanophosphors is based on previously published preparations of similar compounds. A detailed synthesis is described in the Supporting Information. All chemical reagents and polymers [poly(ethylene glycol) (PEG) and poly(acrylic acid) (PAA)] for nanophosphor synthesis were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Microwave synthesis was performed using a Biotage Initiator (Biotage AB, Uppsala, Sweden). Purification of the synthesized nanophosphors was performed by ultrafiltration using Amicon Ultra-15 centrifugal filter units with a 50,000 MWCO (Millipore, Billerica, MA), resulting in suspensions with a nanophosphor concentration of ~50 mg/mL in water. Luminescence spectra were measured using a FluoroMax-3 (Horiba Scientific, Kyoto, Japan) spectrofluorometer and the nanophosphor particle size distributions were measured using a Zetasizer Nano (Malvern Instruments, Malvern, UK).

Microfluidic device production

Devices were fabricated in poly(dimethylsiloxane) (PDMS, RTV 615, Momentive Performance Materials, Albany, NY) by Multi-Layer Soft Lithography using 4" test-grade silicon wafers (University Wafer, South Boston, MA) coated with multiple layers of SU8 (Microchem Corp., Newton, MA) and AZ50 XT photoresists (Capitol Scientific, Austin, TX) patterned by standard photolithography processes.
Bead synthesis

Encoded beads were generated by varying ratios of three pre-polymer input solutions each containing different lanthanide nanoparticles. The three monomer input solutions used in the microfluidic bead synthesizer all contained purified water with 42.8% v/v 700 MW PEG-diacylate (Sigma-Aldrich), 6% v/v 2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropioiophenone (“Irgacure 2959”, a photoinitiator, Sigma-Aldrich) dissolved in methanol at 0.33 g/mL, and 5% v/v YVO₄:Eu (25 mg/mL). One of the input solutions also contained 21.3% v/v YVO₄:Dy (10 mg/mL) and one of the others contained 21.3% v/v YVO₄:Sm (10 mg/mL). Droplets were formed into a continuous flowing stream of light mineral oil (Sigma-Aldrich) that contained 2% v/v Abil EM90 (Evonik Industries, Germany) and 0.05% v/v Span 80 (Sigma-Aldrich) as surfactants to eliminate droplet merging and sticking to the PDMS walls. On device UV illumination polymerizes the droplets into beads downstream of the T-junction.

Accurately programming spectral codes requires precise flow control from each of the lanthanide inputs. This was accomplished by performing a calibration routine to directly measure relative hydraulic resistances and then solving the coupled flow equations (1) and (2) to determine the required pressure ($P_n$) from each input ($n$) to achieve the desired flow rates ($Q_n$):

(Eqn. 1): $Q_n = \frac{P_n - P_{mix}}{R_n}$

(Eqn. 2): $Q_{tot} = \frac{P_{mix}}{R_{mix}}$

Where $Q_{tot}$ is the total flow rate from all lanthanide inputs, $P_{mix}$ is the pressure at the inlet to the mixing channel where all lanthanide input streams come together, and $R_{mix}$ is the resistance of the mixing channel. The resistance of each input ($R_n$) was determined relative to a fixed reference standard, PEG-diacylate with food coloring, flowed into one of the lanthanide inputs. The pressures at these two inputs were set to the same value and the flow rate ratio ($Q_n/Q_{ref}$) was determined by measuring the
width taken up by each fluid in the channel. When the input pressures are equal, equation (1) reduces to:

$$\frac{Q_n}{Q_{ref}} = \frac{(P_n - P_{mix})/R_n}{(P_{ref} - P_{mix})/R_{ref}} = \frac{R_{ref}}{R_n}$$

(Eqn. 3)

\(R_{mix}\) was determined by flowing lanthanide in pre-polymer at one of the lanthanide inputs at a fixed pressure and then measuring the pressure \(P_{mix}\) where the inputs come together at the entrance to the mixing channel. Under these conditions \(Q_{tot} = Q_n\) so from equations (1) and (2):

\(\text{Figure 1: Characterization of the lanthanide nanophosphors synthesized for use in the automated microfluidic bead synthesizer. (A) Emission spectra for each of the nanophosphors (Dy- (green), Eu- (red), and Sm- (orange) doped Y_{0.80}Bi_{0.15}VO_4) when excited at 285 nm. Excitation spectra of all three nanophosphors (inset) are nearly identical. (B) Histogram showing particle size distribution for nanophosphors (as measured by dynamic light scattering), and a photograph of vials of nanophosphor suspensions illuminated with a UV lamp (inset).}\)
(Eqn. 4): \[
\frac{R_n}{R_{mix}} = \frac{P_n - P_{mix}}{P_{mix}}
\]

$P_{mix}$ was measured by opening the valve to a second lanthanide input and adjusting the pressure at this second input until there was no flow at this second input. Once the relative resistances for each of the lanthanide inputs ($R_n$) and the resistance downstream of the inputs ($R_{mix}$) is determined, the system of equations (1) and (2) can be solved to obtain the pressures needed for the desired flow rates of each lanthanide in monomer for accurately hitting each targeted spectral code.

**Bead imaging and data analysis**

Bead imaging was performed on a Nikon Ti microscope with a custom UV transilluminator as shown in Fig. 3. The lanthanide emission was detected through six emission filters (Fig. 4) and recorded on an Andor DU-888 camera (Andor Technology plc., Belfast, Northern Ireland). Linear unmixing was performed using least squares fitting after background subtraction and flat-field correction of the images. Beads were identified by local thresholding on the Eu channel, and the median Dy/Eu and Sm/Eu ratios for each bead were recorded. A global scaling was applied to the beads from each image to best map the measured ratios to the programmed codes. All data analysis was performed in MATLAB (The MathWorks Inc., Natick, MA).

### 3.11.4 Results

**Synthesis of lanthanide nanophosphors**

The spectral encoding scheme we describe relies critically on stable, well-characterized lanthanide nanophosphors. Here, we have synthesized nanophosphors using a polymer-assisted hydrothermal approach combined with microwave irradiation\textsuperscript{61-63}. The product is a crystalline YVO$_4$ nanoparticulate host containing one of the trivalent rare earth dopants (Eu$^{3+}$, Sm$^{3+}$, or Dy$^{3+}$) (see *Materials and Methods*), resulting in materials with unique emission spectra (Fig. 1A) when excited with
UV light (Fig. 1A, inset). The nanophosphors have a size distribution from 30-160 nm (Fig. 1B) and are coated with poly(acrylic acid) to create stable aqueous suspensions (Fig. 1B, inset, illuminated with a UV lamp). Bismuth (at a 5-15% atomic replacement of yttrium) has also been incorporated into these nanophosphors to increase their UV absorption\textsuperscript{61–64}. Henceforth, the nanophosphors are referred to simply by the rare earth dopant present (e.g., Eu). To test the reproducibility of nanophosphor production, we synthesized multiple batches of each nanophosphor and compared their emission spectra (Fig. S1). In all cases, these spectra were virtually identical, demonstrating the ability to consistently and reproducibly produce nanophosphors.

**Synthesis of ratiometrically encoded polymeric beads**

To incorporate these lanthanides into solid beads at programmed ratios, we have designed and fabricated a custom, fully-automated microfluidic device. The microfluidic bead synthesizer (Fig. 2A), operates in two stages. In stage one, the different lanthanides, suspended in poly(ethylene glycol) diacrylate, flow into the device and mix in a staggered herringbone mixer\textsuperscript{25}. During this mixing, the relative flow rates from the different lanthanide inputs determine the relative abundance of each lanthanide in a bead. In stage two, droplets are generated by flowing the lanthanide mixture into an oil stream at a T-junction\textsuperscript{65,66}, and then polymerized into beads through on-chip UV illumination\textsuperscript{67}. After producing beads with each mixture, the mixing channel is flushed with high-pressure water to clear the channel, the pressures are adjusted to pre-programmed values automatically for the next code, and the process repeats. Accurately achieving targeted spectral codes requires precise control over lanthanide flow rates, which is achieved by setting pressures based on a set of coupled flow equations and calibration parameters (see Materials and Methods). The actual device (Fig. 2C and D) is fabricated by standard Multi Layer Soft Lithography (MSL) techniques and incorporates controls for up to 8 lanthanide inputs.
To test both the feasibility of using lanthanide nanophosphors to create uniquely identifiable spectral codes and the performance of this device, we synthesized a set of ratiometrically-encoded beads including varying levels of Dy and Sm and a constant level of Eu. Codes are determined by the relative ratios of Dy/Eu and Sm/Eu, with the constant level of Eu providing an internal normalization to...
correct for spatial and temporal variations in either excitation intensity or detection efficiency. We chose to synthesize a two-dimensional grid of 24 ratiometric codes containing 6 distinct levels of both Dy and Sm. An error model based on preliminary measurements predicted Dy and Sm levels that should be separated by roughly equal numbers of standard deviations. In three hours of fully automated unattended device operation, we produced a set of 24 spectral codes with each code consisting of approximately 1500 beads.

**Imaging of spectrally encoded beads**

To measure the lanthanide luminescence ratios in these beads, we developed an additional custom microfluidic device to create an ordered linear array of ~190 beads within a narrow serpentine channel (Fig. 3A) covering a ~1 mm² area. Beads in the serpentine can be loaded and unloaded using on-chip valves in the fluidic circuit, allowing for efficient imaging of large numbers of beads. The imaging channel is flushed with buffer between batches of beads, ensuring that no bead is imaged twice. Initial images of the beads showed that they are highly monodisperse, with a mean diameter of 46.4 ± 1.0 µm. Images of these beads at commonly used fluorescence wavelengths revealed minimal luminescence of the lanthanides in the fluorescein, Cy3, and Cy5 channels, indicating that these beads are compatible with assays using these dyes for detection (Fig. S2).

Because the lanthanides are best excited in the deep UV, which conventional microscope optics do not transmit, we built a custom UV transillumination microscope to image the beads (Fig. 3B). Luminescence emission from the beads was detected in six spectral bands defined by emission filters chosen to pass the characteristic emission peaks of each lanthanide (Fig. 1A and 4A). The intensities of individual lanthanides were then determined by linear unmixing, which expresses the measured images as a sum of component images multiplied by each component’s characteristic spectrum. Here, we used as our components the three lanthanides Dy, Eu, and Sm, as well as the autofluorescence of the
microfluidic device within which the beads are held (Fig. 4B). The unmixing error (the difference between the measured images and the component images times their spectra) was <2% for a typical image set. A typical set of unmixed images is shown in Fig. 4C. To identify the lanthanide ratios in each bead, we first identified beads in the image by adaptive local thresholding of the Eu channel. For each identified bead (spanning ~90 pixels), we then calculated the Dy/Eu and Sm/Eu ratios on a pixel by pixel basis, and record the median ratios for each bead⁶⁹.

Figure 3: Bead imaging setup. (A) Photograph of microfluidic imaging device with flow channels shown in blue and control channels shown in orange. Beads are injected into a 55 mm wide serpentine channel for imaging (photograph, inset); sieve valves at the end of the channel retain beads while permitting fluid flow to facilitate channel loading. Inputs (Inj1, BdIn, Inj2) and outputs (W1, W2, BdOut) at either end of the device provide bidirectional flow. (B) Schematic of the microscopy system used for imaging beads. Light from a full-spectrum 300 W Xenon arc lamp is collected (L1), reflected off a 400 nm long pass filter (M1) to reject visible light, and passed through a shutter (S1) and an excitation filter wheel (to switch between UV and visible transillumination) before being focused (L2) into a deep UV liquid light guide. The other end of the liquid light guide is mounted on the condenser mount of a Nikon Ti microscope, where the light is collimated by a fused silica lens (L3) and projected onto the sample. Emitted light from the sample is collected by a Plan Apo 4x/0.2 NA objective (L4), with a UV blocking filter placed between the sample and the objective. Emitted light is filtered through an emission filter wheel mounted beneath the objective before being focused onto the camera.
Identification of spectral codes

To be practically useful, each code within an encoded bead set should cluster tightly around the predetermined, programmed ratios. The results of imaging 10 bead-filled serpentines containing a representative sample of 1926 beads from the 24 code set are shown in Fig. 5A. To determine cluster centroids for each code, we performed k-means clustering on the data, using the programmed values as the starting cluster centroids. The synthesized beads for each code cluster tightly, with the measured values for each code agreeing very well with the targeted values (inset Fig. 5A): the mean distance between the programmed ratios (0, 0.12, 0.27, 0.46, 0.70, and 1) and the measured ratios is only 0.014. Discounting the 0,0 code, the mean fractional error between the measured and programmed levels (distance from programmed to measured divided by distance of programmed to origin) is 2.9%. A second independently-generated code set shows similarly precise agreement with the programmed levels (Figs. S3 and S4), indicating that our synthesizer produces spectrally-encoded beads with both high accuracy and repeatability. These code sets were synthesized independently with a three-week gap between the syntheses, and both code sets were imaged several additional weeks after the syntheses, demonstrating bead and reagent stability.

Another critical requirement for a robust encoding scheme is that beads from different codes cluster tightly together and far from other codes, preventing misidentification of beads. The RMS deviation of individual beads from their code centroid, calculated as above, is 4%. To quantify how accurately we can assign beads to a code, we fit a two-dimensional Gaussian to each code cluster using a Gaussian mixture model. The three and four standard deviation ellipses around each code are shown in the grey lines in Fig. 5A. We find that 98.7% of all beads lie within three standard deviations of the cluster centroid, and only two of the 1926 beads fall more than four standard deviations away from the cluster centroid. From this model, we can also estimate the number of standard deviations at which the error ellipse of one code would begin to overlap with neighboring codes; this is the number of standard
deviations that a bead would have to fall from the cluster centroid to be misidentified. We find that this overlap occurs at about 5.5 standard deviations, corresponding to a misidentification rate of $4 \times 10^{-8}$. Consistent with this, we performed 10-fold cross validation of the Gaussian mixture model and found that no beads were misidentified. Thus, not only do the code centroids fall close to the programmed values, the individual beads are tightly distributed around these values, indicating that each bead can be assigned to a code with a <0.1% chance of misidentification. Understanding and minimizing errors is crucial to maximize the code space that can be achieved with a given encoding scheme. The distribution of measured bead ratios around their programmed values can result from both errors in bead synthesis and bead imaging. These synthesis and imaging errors, in turn, are composed of both a systematic, instrumental component as well as a statistical, shot noise (either photon or lanthanide nanoparticle number) component. To probe the relative contributions of these sources of error in our measurements, we calculated the mean error for each Dy/Eu level and each Sm/Eu level, independent of the concentration of the other lanthanide. The histograms of the Dy/Eu and Sm/Eu ratios for all beads are shown at the edges of the scatter plot in Fig. 5A, along with Gaussian fits to the data. The widths (standard deviations) from these Gaussian fits are plotted (Fig. 5B), along with the statistical measurement error (determined by repeated imaging of the same serpentine of beads). The statistical measurement error accounts for roughly one half of the total error in the Dy/Eu channel and between one half and one third of the total error in the Sm/Eu channel. While these other sources of error can be further reduced, the fact that we are within 2-3 fold of the measurement shot noise limit indicates that these other errors are relatively small.
Figure 4: Analysis procedure. (A) Raw data in each of the six luminescence channels. Data are scaled linearly. Each channel is labelled by the center wavelength / passband width (in nm) of the imaging filter. (B) Reference spectra used for unmixing. (C) Left: Linearly scaled black and white images of unmixed data from each channel (Dy, Eu, and Sm), with black set to the minimum intensity in the image and white set to the maximum intensity. Right: Bright field image of the same field of view. (D) False color overlay of Dy, Eu, and Sm luminescence, colored blue, red, and green, respectively, with scaling as in Panel C.
Discussion

Here we have demonstrated a system to precisely generate beads containing ratiometric spectral codes using a microfluidic device and luminescent lanthanide nanophosphors. We have created 24 uniquely identifiable codes containing a single reference level of Eu and 6 levels each of both Sm and Dy. Measurements of ~2,000 beads from this code set establish both that the measured ratios closely match the desired programmed ratios and that these codes are easily distinguished from one another.

Figure 5: A 24 code matrix. (A) Scatter plot of the median Dy/Eu and Sm/Eu luminescence ratios for 10 filled serpentines (1926 beads), with points false colored according to their Sm/Eu (red) and Dy/Eu (blue) ratios. Each point represents one bead. Grey ellipses around each code cluster illustrate three- and four-sigma contours derived from fitting a Gaussian mixture model to the data. Histograms of bead ratios in the Dy/Eu and Sm/Eu channels (black) and their corresponding Gaussian fits (red) are shown along each axis; these histograms group all codes together. Inset: Measured cluster centroids (red) and their corresponding programmed intensity ratios (blue); the root mean square deviation between the programmed ratios and the measured ratios is 0.014. (B) Standard deviations calculated from Gaussian fits to the bead ratio histograms in (A) as a function of ratio (filled circles, with blue for Dy and red for Sm). Square symbols illustrate the statistical standard deviation determined from replicated imaging of the same serpentine of beads.
validating the accuracy and precision of this technique. Importantly, both this scheme and the device that we have used to produce these codes can be extended to significantly larger code sets.

Previous microfluidic systems have been built for generating spectrally encoded beads using premixed solutions for each code, five fixed ratios between two colors, or continuously varying ratios between two colors. Our device is unique in incorporating automated on-chip mixing with multiple input streams while accurately achieving programmed ratiometric codes (error <3%), with low variance within a code (4%), and precise control over bead size (CV < 2.5%). The current bead synthesizer incorporates eight lanthanide inputs and is scalable with respect to both the number of lanthanide inputs and the rate of bead synthesis.

The ultimate performance of a spectral encoding scheme depends both on the number of encoding species and the number of intensity levels of each that can be reliably distinguished. The number of distinguishable intensity levels is inversely proportional to deviations from the programmed error level; therefore, minimization of synthesis and measurement errors is necessary to maximize the code space. Our results establish that we can synthesize beads with a mean deviation from the programmed ratio of 2.9%. This number is significantly smaller than deviations from programmed intensities seen for QDs indicating that lanthanide nanophosphors suffer much less from energy transfer and re-absorption between particles.

We envision a direct path to expanding our code set through both minimizing code variance and adding other lanthanides. If we assume that the errors in intensity ratios are normally distributed, and require that the midpoint between any two programmed codes is at least four standard deviations from each other (corresponding to a misidentification probability of less than 10^{-4}), then our current intra-code variance of 4% should allow the resolution of seven intensity levels for Dy/Eu and Sm/Eu. If we were able to reduce the total error to the statistical measurement error in Fig. 5, the number of
resolvable levels would increase to 12 per lanthanide while maintaining a code-calling accuracy rate greater than 99.99%.

A number of lanthanide nanophosphors with different dopants and distinct emission spectra have been synthesized, including erbium, thulium, holmium, and cerium/terbium\textsuperscript{64,71,72}. Incorporation of additional nanophosphors such as these provides an alternative approach to expanding the code space. In addition to the current downconverting (UV-excited) YVO\textsubscript{4} nanophosphors, there are also upconverting lanthanide nanophosphors that emit in the visible region upon excitation in the near-IR. Syntheses of upconverting nanoparticles with the emitting species Dy, Er, Eu, Ho, Sm, Tb, and Tm have been reported\textsuperscript{73–76}. A system combining upconverting and downconverting nanoparticles could potentially use as many as 14 orthogonal channels for encoding. As the overall coding capacity of the system scales as the number of distinguishable levels to the power of the number of dyes, extending the high precision with which we currently measure lanthanide abundance to these additional channels will allow a large increase in the coding capacity of this system, potentially up to billions of codes.

The methodology and device described here allows for simple and accurate synthesis of spectrally encoded beads using microfluidics and lanthanide nanophosphors. Given an expanded code space with additional lanthanide nanophosphors, this platform enables a multitude of diverse assays, including immuno-diagnostics, small molecule library screening, and combinatorial synthesis approaches.

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for his guidance and valuable discussion, and Colin Campbell for assistance with photographing serpentine channel devices.
3.11.6 Supplemental Materials and Methods:

Lanthanide Synthesis:5,58,59

**Preparation of YVO₄:Bi,RE Nanophosphors:** Solutions (0.1 M) of the rare-earth (RE) dopants [Sm(NO₃)₃, Dy(NO₃)₃, Eu(NO₃)₃], Y(NO₃)₃, and Na₃VO₄ were prepared beforehand. 14.2 mg of Bi(NO₃)₃ was added into 3 mL of a 10 w/w% solution of PEG (Mn ~ 2,000). This solution was then rapidly dissolved through brief sonication before being heated to 70 °C in an oil bath under magnetic stirring. A solution of Y(NO₃)₃ (800 µL) and the RE solution (e.g., Eu(NO₃)₃) (50 µL) was premixed and then added drop-wise into the stirring PEG solution. The PEG solution instantly turned white upon addition of the Y+RE mixture. This solution was stirred for 30 minutes, followed by the drop-wise addition of the Na₃VO₄ solution (950 µL). The suspension turned yellowish at this stage and the mixture was again stirred for 30 min. The suspension was transferred into a glass vial suitable for microwave synthesis and was heated to 180 °C at 15 bar for 60 min. Upon removal from the microwave, the suspension was pure white. The material was pelleted in a 15-mL disposable centrifuge tube and the PEG supernatant was removed. The pellet was then re-suspended in 3 mL of deionized H₂O, to which was added 5 mL of a 10 w/w% PAA solution (Mn ~ 1,400). This mixture was heated back up to 70 °C and stirred for 10 min. The solution was pH adjusted to 7.5 using 5 N NaOH and stirred for an additional 30 min. The suspension was then diluted 1:10 with deionized H₂O and sonicated for 18 hours. After sonication, any larger phosphor particles were pelleted under centrifugation and the remaining translucent suspension was filtered consecutively through a 1 µm and 0.45 µm PTFE filters before being added to an ultracentrifugation filter unit for concentration and the removal of excess salts and polymers. After the entire reaction volume (~100 mL) had been passed through the membrane, the retained nanophosphors were washed 4 times with 15 mL of deionized water to exchange out the remaining solution. The final
NP suspensions were white and milky in appearance and had a nanophosphor concentration of about 50 mg/mL.

**Microfluidic Device Fabrication:**

All photolithography masks were designed using AutoCAD (Autodesk, San Rafael, CA) and printed onto transparency film with a resolution of 30,000 dpi (FineLine Imaging, Colorado Springs, CO).

To improve adhesion of subsequent photoresist layers, all wafers were first coated with a 5 µm layer of SU-8 2005 negative photoresist (Microchem Corp.) according to the manufacturer’s instructions. All spin-coating steps were performed on a G3P-8 programmable spin coater (Specialty Coating Systems, Indianapolis, IN). After each coating step wafers were set on a flat surface for 10 to 20 minutes to allow photoresist to relax completely and reduce surface irregularities, except for the initial 5 µm adhesion layer of SU-8. All photoresist baking steps were done on aluminum-top hot plates (HS40A, Torrey Pines Scientific, Carlsbad CA). Mask alignment and photoresist exposure were done on a Quintel Q2001CT i-line mask aligner (Neutronix-Quintel, Morgan Hill, CA).

**Bead Synthesizer Mold Fabrication**

Bead Synthesizer control molds were fabricated using SU-8 2025 photoresist according to the manufacturer’s instructions for creating ~ 25 µm thick channels. Flow molds were constructed with five layers of photoresist, one using AZ50 XT positive photoresist (Capitol Scientific, Austin, TX), the other four using different types of SU-8. Layers 1 and 2 were developed separately, but layers 3-5 (all SU-8) were developed together, after they had all been exposed, as this was found to reduce bubble formation, improve height uniformity, and allow for significantly better staggered herringbone
fabrication. After the 5th layer, layers 3-5 were developed for 6 min in SU-8 Developer, followed by hard baking for 2 hours at 165°C, with an initial ramp from 65°C to 165°C at 120°C/hr. The five layers are:

5 µm thick SU-8 2005 layer for the high resistance push water input. Spin-coat: (1) 500rpm for 5s with 5s ramp (spread), (2) 2900rpm for 30s with 8s ramp (cast). Soft bake: 65°C 2 min/95°C 3 min/65°C 2 min. UV exposure: 7.4s at 18.4 mW/cm². Post exposure bake: 65°C 2 min/95°C 3 min/65°C 2 min. Develop: 2 min in SU-8 Developer (Microchem).

45 µm thick AZ50 XT layer to create rounded channels at valve locations. Spin-coat: (1) 200rpm for 5s with 1s ramp (spread), (2) 1400rpm for 30s with 5s ramp (cast), (3) 3400rpm for 1s with 1s ramp (edge bead removal). Soft bake: 65°C-112°C full speed ramp for 22 min. Rehydrate overnight. UV exposure: 20s x 4 with 20s pauses in between at 18.4 mW/cm². Develop: 1:3 solution of AZ Electronic Materials AZ400k developer (Capitol Scientific). Hard bake: ramp from 65°C to 190°C at 10°C/hr, remain at 190°C for 4 hrs.

45 µm thick SU8-2025 layer for the lanthanide inputs, mixer channel, and oil channels. Spin-coat: (1) 500rpm for 10s with 5s ramp (spread), (2) 1600rpm for 30s with 3.6s ramp (cast). Soft bake: 65°C 2 min/95°C 10 min/65°C 2 min. UV exposure: 13.1s at 18.4 mW/cm². Post-exposure: 65°C 2 min/95°C 9 min/65°C 2 min.

30 µm thick SU8-2025 layer on top of layer 3 in the mixer channel and downstream of the T-junction. Spin-coat: (1) 500rpm for 10s with 5s ramp (spread), (2) 3500rpm for 30s with 10s ramp (cast). Soft bake: 65°C 2 min/95°C 7 min/65°C 2 min. UV exposure: 14.3s at 18.4 mW/cm². Post-exposure bake: 65°C 3 min/95°C 6 min/65°C 2 min.

35 µm thick SU8-2025 layer on top of layer 4 on the mixing channel for the staggered herringbone grooves. Spin-coat: (1) 500rpm for 10s with 5s ramp (spread), (2) 2500rpm for 30s with 6.7s
ramp (cast). Soft bake: 65°C 2 min/95°C 7 min/65°C 2 min. UV exposure: 7s at 18.8 mW/cm². Post-exposure bake: 65°C 2 min/95°C 6 min/65°C 2 min.

Imaging Device Mold Fabrication

Imaging device control molds were fabricated using SU-8 2025 according to the manufacturer’s instructions for creating ~25 µm thick channels. Imaging device flow molds had the following layers:

~50 µm thick layer of AZ 50 XT photoresist to create rounded channels at valve locations. Spin-coat: (1) 200 rpm for 5 s with a 1 s ramp (spread), (2) 750 rpm for 30 s with a 5 s ramp (cast), and (3) 2750 rpm for 1 s with a 1 s ramp (edge bead removal). Soft bake: 25 minutes with ramp between 65°C and 112°C at full speed, and allowed to cool to room temperature. Rehydrate overnight. UV exposure: 25 s x 3 at ~18 mW/cm². Develop: 1:3 solution of AZ AZ400k developer in water. Hard bake: Ramp from 65°C to 190°C at 10°C/hour, remaining at 190°C for 4 hours.

~50 µm thick layer of SU-8 2050 to create all flow channels. This layer was fabricated largely according to the manufacturer’s instructions, although we found that soft baking set to ramp between 65°C and 95°C (rather than simply transferring wafers between hot plates set to 65°C and 95°C) helped prevent formation of bubbles within the photoresist.

Bead Synthesizer and Imaging Device Fabrication

All molds were silanized by exposure to trichloromethylsilane (Sigma-Aldrich, St. Louis, MO) vapors for 60 minutes. Each flow mold was then coated with a 4 mm thick layer of Momentive Materials RTV 615 (R.S. Hughes, Oakland, CA) mixed at a ratio of 1:5 (cross-linker:elastomer) using a Thinky AR-250 planetary centrifugal mixer (Thinky USA Inc, Laguna Hills, CA). This 4 mm thick layer was subsequently degassed in a vacuum chamber for 60 minutes. All control molds and slides for mounting
the devices were spin coated with a ~20 µm thick layer of RTV 615 mixed at a ratio of 1:20 via a 2 step spin process: (1) 500 rpm for 5 s with a 5 s ramp (spread), and (2) 1900 rpm for 60 s with a 15 s ramp (cast). Flow molds, control molds, and coated slides were baked at 80°C for 1 hour, 40 minutes, and 20 minutes, respectively. Following baking, PDMS flow layers were peeled from molds, cut to the appropriate size, punched with a drill press (Technical Innovations, Brazoria, TX) at inlet and outlet ports, and aligned to control layers (still remaining on the molds). The aligned devices were then baked for an additional hour before being cut from the molds, punched to create control access ports, and placed on the coated slides. The entire assembly was then baked at 80°C for 1-12 hours to finalize device bonding. Synthesis devices were mounted on regular microscope glass slides, while imaging devices were mounted on cyclic olefin copolymer slides (COP480R, Pure Slides LLC, Medford, MA) to minimize the fluorescence background.

**Microfluidic Device Operation:**

Valves in the microfluidic devices were actuated by 10 mm pneumatic solenoid valves (Festo Corp., Hauppauge, NY) driven by an ethernet-based, programmable fieldbus I/O system with digital output modules (750-841 Programmable Fieldbus Controller, 750-504 4-Channel Digital Output Module, Wago Corp., Germantown, WI). All fluids were injected into the microfluidic devices using pressure-driven flow from custom-made containers. Pressurized air to operate the valves and push fluids into the chips was supplied by a set of manual precision pressure regulators connected to the house air supply through a series of high efficiency filters for oil and particulate removal. A custom software platform written in MATLAB (The MathWorks Inc., Natick, MA), with a graphical user interface, allowed for real time control and script-driven automation of all aspects of the chip operation, for both the bead synthesis and imaging chips. The UV light source for droplet polymerization was a Leica EL6000
fluorescence excitation light source with a metal halide bulb and liquid light guide filtered by an Omega UV filter cube set XF02-2 (80nm band around 330nm).

**Bead Production:**

All monomer-lanthanide mixtures were injected into the chip from custom-made containers using PEEK capillary tubing with an inner diameter of 65 µm and a length of 30.5 cm to provide high input resistance relative to the resistance of the staggered herringbone channel. This helps minimize any potential flow rate errors due to inaccuracies in measuring the resistance of the staggered herringbone channel or fluctuations in set pressures. On-chip resistors optimized for stable drop production at the T-junction with input pressures near the middle of the pressure regulator range were used on the oil and push water inputs. To reduce oxygen inhibition of the PEG-diacrylamide polymerization, these containers were pressurized with nitrogen (95-99% purity) supplied by a high-precision, high-speed, computer-controlled pressure regulator with eight independent output channels (MFCS-FLEX, 8 channels, 0-1000 mBar range, Fluigent SA, Paris, France). For the same reason, the microfluidic device was surrounded by a 95-99% pure nitrogen atmosphere during operation. The high gas permeability of PDMS ensures that the interior of the microfluidic device will equilibrate with this nitrogen atmosphere.

After polymerization, the beads were always smaller than the droplets, mostly due to oxygen-driven cross-linking inhibition on the droplet surface, and this reduction in size was highly dependent on the UV dose delivered to the droplets (the lower the dose, the smaller the beads). For the experimental conditions described here, the typical diameter shrinkage was approximately 7 µm, resulting in beads of approximately 46 µm +/- 1 µm. The measured error corresponded to approximately half a pixel in the
image and thus the actual size variation of the beads is likely smaller. Sizes of beads were measured by fitting a circle to 3 user-selected points on the perimeter of the bead in a brightfield image using NIS-Elements (Nikon Instruments, Melville, NY).

**Bead imaging (microfluidics):**

A simple microfluidic device was designed to create an ordered linear array of beads within a narrow serpentine channel (Fig. 3A) that fits approximately 190 beads in one field of view of the microscope. “Sieve” valves positioned at the end of the serpentine channel permitted fluid flow while retaining beads, facilitating pressure-driven packing of beads within the channel and maximizing imaging throughput. Multiple output ports collected both buffer and bead wastes; fluid injection ports at either side of the device allowed flushing of the serpentine from either side to clear stuck particles. These devices are mounted on cyclic olefin polymer slides (COP480R; Pure Slides, LLC., Medford, MA) to minimize autofluorescence; the PDMS itself was not significantly autofluorescent. Device control lines were pressurized to 25 psi (for fully sealing valves) and 35 psi (for sieve valves).

Prior to loading, bead batches were washed ten times in a solution of 1x Phosphate Buffered Saline (PBS) with 0.5% Tween and twice in a solution of 1x PBS with 0.1% Tween before being diluted to a working concentration of 100-200 beads per µL in 1x PBS with 0.1% Tween. Bead solutions (~ 25 µL) were loaded into Tygon tubing (using a 1 mL syringe), which is connected directly to the device. Buffers were stored in pressurized vials connected to the device via Tygon tubing. During serpentine loading, both bead and buffer inputs were pressurized at 3-5 psi and excess buffer was directed to the waste port. During imaging, buffer solution pressure was reduced to ~1 psi to relax bead packing. After imaging, the output was directed to a separate bead waste port to collect imaged beads for further use.
Bead imaging (optics):

Bead imaging was performed using a Nikon Ti microscope (Nikon Instruments, Melville, NY) with a custom built illuminator. Because the microscope objectives are not transparent to the short wavelength UV illumination required to excite the lanthanides, we used a transillumination geometry as shown in Fig. 3. Light from a full-spectrum 300W Xenon arc lamp (Newport, Irvine, CA) was collected, reflected off a 400 nm long pass mirror (CVI Melles-Griot, Albequerque, NM) to reject visible light, then passed through a shutter and an excitation filter wheel (Sutter Instrument Co., Novato, CA), before being focused into a 3 mm diameter deep UV liquid light guide (Newport). The excitation wheel allowed switching between UV illumination for lanthanide excitation and visible light illumination for finding beads and imaging the device during bead loading. For UV imaging, the illumination light was filtered with a 292/27 excitation filter (Semrock, Rochester, NY) paired with UG11 absorptive glass (Newport). The illumination intensity at the sample was ~12.5 mW/cm². For visible light imaging we used the residual visible light reflected by the 400 nm long pass mirror, which was further filtered with a 409 nm long pass filter (Semrock), infra-red reflective mirror (Edmund Optics), and an OD 1.0 neutral density filter.

The other end of the liquid light guide was mounted on the condenser mount of a Nikon Ti microscope, where the light was collimated by a fused silica lens (Newport) and projected onto the sample. Emitted light from the sample was collected by a Plan Apo 4x/0.2NA (Nikon Instruments, Melville, NY) objective, with a UV blocking filter (Edmund Optics, Barrington, NJ) placed between the objective and the sample. Emitted light was filtered through an emission filter wheel mounted beneath the objective before being focused onto the camera. We collected an image stack consisting of six different images acquired through the following filters (all from Semrock): 482/35, 510/84, 543/22, 572/15, 615/20, and 630/92. The filters used here were chosen using a Monte Carlo optimization procedure to select filters which minimize the unmixing error. Typical exposure times were 5 seconds.
for the first four channels and 1 second for the last two. The camera used was an Andor DU-888 (Andor Technology, Belfast, Northern Ireland) operated in conventional readout mode at 13MHz with 2x2 binning. The microscope and camera were controlled by Micro-Manager.

Bead images for testing bead autofluorescence in conventional dye channels were acquired on a Nikon Ti microscope with a 10x / 0.3 Plan Fluor objective and a Coolsnap HQ2 CCD camera (Photometrics, Tucson, AZ). Illumination was from a Sutter XL lamp (Sutter Instrument Company, Novato, CA) and a Chroma 89000 filter set (Chroma Technology, Bellows Falls, VA) was used to define the excitation and emission channels. The lamp was operated at full power and the exposure time for each image was 1 second.

**Linear Unmixing and Image Analysis:**

All data analysis was performed with custom software written in Matlab. Reference spectra for unmixing were acquired from beads doped with a single lanthanide. These beads were spotted on quartz coverslips (to minimize background fluorescence) and an image stack was acquired as described above. The background was subtracted using a local background estimation procedure and the mean luminescence of the beads in each channel was measured. The device background spectrum was measured from a region of the microfluidic device where no beads were present. These reference spectra were then normalized so that each spectrum summed to one.

Before linear unmixing, the images of the beads in the serpentine device were corrected for camera bias and dark current by subtracting a dark image. Dark images were acquired by averaging 100 frames acquired with the same exposure times as the fluorescent images, but with the camera shutter closed. The image stack was then flat-field corrected by dividing each image by a corresponding flat-field image. Flat-field images were acquired by averaging 100 frames captured through each emission filter.
with white-light transmitted illumination and no sample present on the microscope. While our use of an internal standard corrected for variations in excitation intensity across the field of view, we were still sensitive to wavelength-dependent pixel response nonuniformity \(^80\). Linear unmixing was then performed using standard least squares analysis to fit the intensity of each pixel of the measured image stack to a sum of the reference spectra times the abundance of each lanthanide \(^{68,81,82}\). This unmixing process reduced our six-channel raw data to a four-channel image stack consisting of background fluorescence and Dy, Eu, and Sm luminescence.

We then identified beads in the unmixed image by median filtering the Eu channel and performing adaptive local thresholding. The threshold parameters were adjusted to include as many pixels as possible in each bead while maintaining separation between them. For each bead identified, we calculated the pixel by pixel ratio of Dy to Eu and Sm to Eu luminescence and recorded the median Dy/Eu and Sm/Eu luminescence ratio. To minimize the effect of wavelength-dependent pixel response nonuniformity on the CCD, we only analyzed beads within the central 300 x 300 pixels on the CCD. Because the data returned by linear unmixing were on an arbitrary scale, we used a variation of Iterative Closest Point matching to determine overall scaling factors along the Dy/Eu and Sm/Eu axes to best map the observed data to the programmed codes \(^{83,84}\). Briefly, the algorithm works as follows: an initial transformation is determined that maps the brightest bead along each axis to the highest programmed level of that lanthanide. This transformation is applied to the data and the closest programmed level to each measured bead is determined. The transformation that best matches the measured beads to their closest programmed levels is determined, and the process is iterated until convergence. To account for small systematic errors between different serpentines, we determined these scaling factors separately for each serpentine. This systematic variation was largest along the Sm/Eu dimension, and correcting it reduced the overall CV by \(~0.6\%\). This correction was statistically significant as compared to rescaling an equal number of subsets of the data without regard to which serpentines they originated from.
The Gaussian mixture model (GMM) was fit in Matlab and standard deviation ellipses and numbers of standard deviations between points and cluster centroids were determined using the Cholesky decomposition of the covariance matrix. Cross-validation was performed by splitting the bead data into ten disjoint sets, training the GMM on nine, and then testing the classification accuracy on the remaining test set. This was repeated for each of the ten test sets in turn. Measurement errors were determined by replicate imaging of two different serpentinales of beads. For each bead, the mean and standard deviation of five repeated measurements were calculated. The standard deviations were then grouped by lanthanide ratio and averaged to give the statistical error plotted in Fig. 5B.

**Supplemental Figure Captions:**

**Supplemental Figure 1:** Reproducibility of lanthanide nanophosphor synthesis. Each individual batch of nanophosphor suspensions were diluted 1:500 in DI water from the concentrated stock solutions. A luminescence emission spectrum (400-800 nm) was obtained using a FluoroMax-3 spectrofluorometer for all of these diluted stock solutions. The excitation was the same for all solutions (285 nm through a 3-nm slit width excitation monochromator) and the emission parameters were also held constant for all emitters (3-nm emission slit width, 1-nm increment steps, and 0.1 sec integration time at each step) with the exception of the Europium nanophosphors which, due to their brightness, had slit widths of 1 nm at both monochromators.

For each emitter shown, the left column shows the emission spectra of each individual batch synthesized as a stacked plot: (A) Sm, 4 batches, (B) Dy, 3 batches, and (C) Eu, 5 batches. In the right column, the normalized emission spectra for all batches are shown as an overlay for each emitter.
Typically, only one color is observed in the overlaid spectra since the high reproducibility of the batches results in several spectra that are coincident.

**Supplemental Figure 2:** Illustration of compatibility with commonly used visible fluors.

A sample of the 1/30/12 24-code beads were imaged using a Chroma Sedat quad filter set (#89000), with a Lambda XL lamp, Coolsnap HQ2 camera, 10x / 0.3 NA objective, and 1 sec exposure time for each fluorescence channel. All four channel combinations were imaged, and the corresponding images are labeled with the excitation and emission centers of the filter sets. The 402/455 (DAPI channel) image shows weak fluorescence; the other channels show negligible fluorescence with the fluorescence in the Cy5 channel being undetectable.

**Supplemental Figure 3:** Scatter plots of two different batches of synthesized beads. One batch was synthesized January 9th (Set 1, red); the other was synthesized on January 30th (Set 2, blue). Both were imaged on February 15th. The Set 1 batch of beads is missing one code due to a computer error. The Set 2 beads are the same beads analyzed in the main manuscript.

**Supplemental Figure 4:** Comparison of programmed ratios (black) and measured code centroids and three sigma error ellipses for the Set 1 (red) and Set 2 (blue) beads.
Figure 2

Brightfield

402/455  490/525
555/605  645/705
3.12 References


4.1 Prostate Cancer Diagnosis

Prostate cancer is one of the most commonly diagnosed cancers among men worldwide. The current gold standard prostate cancer diagnostic screening test measures levels of prostate specific antigen (PSA) in blood and considers patients with levels above >4ng/mL to be at risk for prostate cancer. Unfortunately this test has poor sensitivity and specificity, \(^1-^5\) with one seven year randomized prospective study\(^5\) finding sensitivity of only 20.5% and specificity of 93.8% for the typical PSA cutoff of 4.1ng/mL. Lowering the cutoff value can improve sensitivity, but at the expense of lower specificity (83.4% and 61.1% for >1.1ng/mL), leading to more unnecessary biopsies. Levels of PSA production in prostate tissue itself is similar with and without cancer and it is the disruption of the prostate lumen that leads to higher levels in the blood.\(^1\) Other conditions that lead to disruption of this membrane, such as benign prostatic hyperplasia, can result in similarly high levels of PSA.\(^1\) The current screening tool leads to unnecessary invasive procedures such as tissue biopsies and to the aggressive treatment of mild slow-growing forms of prostate cancer that might pose no long term risk to the patient, while failing to catch a large percentage of prostate cancer. As a result, the use of total blood PSA quantitation as part of regular medical screening for asymptomatic men has increasingly generated controversy in the medical community.\(^6\)

Other tools that can more directly and specifically detect biological changes that occur in the presence of prostate cancer (and other cancers) are critically needed. Improved diagnostic tools are needed to more effectively detect early stages of prostate cancer, prior to lumen disruption, distinguish between prostate cancer and other non-threatening medical conditions that lead to elevated PSA in the
blood, and lastly better predict cancer virulence so that doctors and patients can make more informed decisions as to how aggressively to treat prostate cancer.

One potential approach for improved prostate cancer diagnosis is detection of PSA glycan structures. Glycans are carbohydrate structures post-translationally attached to proteins. These structures play a role in protein folding, transport, signaling pathways, and cell-cell interactions both within an organisms and between organisms. Aberrant glycosylation patterns are associated with a number of cancers, and numerous studies have found differences in the glycosylation pattern of PSA from prostate cancer versus healthy tissue. These studies typically use small numbers of patients or pool patient samples together to generate large enough sample volumes for testing. Better tools are needed to enable large-scale screening of individual patient samples to determine the capability of particular glycan specific PSA isoforms to accurately predict the presence of prostate cancer and/or the virulence of prostate cancer to enable doctors and patients to make more informed treatment decisions. These tests should ideally require low sample volumes and be amenable to multiplexing and automation.

4.2 PSA Isoforms and Detection Methods

A number of existing research methods have been utilized for detection of glycosylation patterns including enzyme linked lectin assays (ELLA), binding columns followed by measurement of PSA concentration, mass spectrometry, lectin microarrays, and slab-gel western blot, isoelectric focusing (IEF), or 2D electrophoresis followed by lectin probing. Requirements for large sample volumes, interactions between lectins and the glycans on antibodies, low binding affinity of lectins (10$^6$ to 10$^7$ vs. 10$^8$ to 10$^{12}$ for antibodies), glycosylation patterns on background proteins, and in some cases high background and/or long assay times, have made detection on large numbers of clinical
samples challenging. A microfluidic platform allowing for rapid detection of glycosylation patterns of PSA in complex samples after IEF separation could address many of these limitations.

Microfluidics can offer advantages for patient sample screening due to the need for low sample volumes and capabilities for high levels of multiplexing to enable screening of larger numbers of patient samples against large numbers of targets. Prostate cancer biopsies are typically obtained using a needle, leading to limited sample mass. Including a separation step, based on pl or molecular weight, can improve sensitivity and specificity of the assay as lectins may bind to many proteins within a sample containing similar glycan structures.

ProPSA is a glycoprotein produced primarily by prostate ductal and acinar epithelium and cleaved into its active form PSA through the removal of seven amino acids. PSA is secreted by the epithelial cells of the prostate gland into the lumen where it cleaves semenogelin I and II. Other proPSA cleavage forms such as [-5] [-4] and [-2] proPSA exist due to partial degradation as well as cleavage forms of PSA. A portion of the PSA will be complexed with protease inhibitors. In healthy men, levels in the blood are low, but disruptions to the prostate wall as can occur with prostate cancer or other factors such as BPH can lead to elevated levels (> 4 ng/mL) in blood. Studies have shown statistically significant differences in rates of these cleavage isoforms and percent complexed between healthy and prostate cancer patients, but nothing has proven definitive in diagnosis or prognosis of prostate cancer.

PSA isoforms are in the isoelectric point (pl) range of 6.2-7.5 and contain ~8.3% carbohydrate with a single N-linked oligosaccharide side chain at Asn-45. Differences in sialic acid content of this glycan will appear as differences in PSA pl. Different PSA glycan isoforms have MW too similar to be resolved with a size based separation, but differences can be resolved by pl due to different charges on the glycan structure. Based on both prior 2DE studies of PSA from sera as well as general research
on the impact of sialic acid on pI, we can expect that PSA should shift by \(~0.2\) pI per sialic acid residue. The ends of N-linked glycans can each be capped with a sialic acid residue, and since N-linked glycans can exist in bi, tri or tetraantenary forms this would correspond to a theoretical maximum of up to 4 sialic acid residues per PSA. Prior HPLC studies of PSA from sera have shown PSA glycan structures with up to 3 sialic acid residues. Each pI point can contain diverse glycan structures of the same total sialic acid content, so combining lectin probe with pI provides further specificity on the differences between prostate cancer and non-prostate-cancer PSA as compared to either IEF or lectin probe alone.

2DE studies have found some statistically significant differences in fraction of PSA at each pI between serum from prostate cancer and non-prostate-cancer sources and other studies have shown statistically significant differences in glycan structures via HPLC or lectin probe of PSA from prostate cancer and non-prostate-cancer sources. Increases in core fucosylation and \(\alpha2-3\) linkage of sialic acid were found to be the most significant changes between PSA in 17 BPH and 34 prostate cancer sera as measured by HPLC and exoglycosidase digestions by Saldova and colleagues. A mass spec study by Kyselova et al found more than 50 unique glycan structures with 12 forms showing a statistically significant difference between healthy and prostate cancer patients.

Previous studies have identified a number of lectins, which preferentially bind to either PSA from prostate cancer cells or PSA from healthy tissue (Figure 4-1). Lectins are proteins which bind to carbohydrate structures and each lectin will have a specificity for a particular carbohydrate structure or family of structures. Figure 4-1 shows some of these lectins, the carbohydrate structure recognized by the lectin, and whether previous studies have identified binding with healthy PSA or PSA from prostate cancer cells. The lectins TJA-II, WFA (116kDa), UEA-1 (63kDa), and SSA have shown evidence of differences in binding of PSA between PCa and non-PCa sources.
Figure 4-1: Lectins identified in the literature as binding to PSA in healthy seminal fluid and/or prostate cancer patient serum or cancer cell lines. A. Cummings et al.\textsuperscript{31}, B. Fukushima et al.\textsuperscript{21}, C. Kuno et. al.\textsuperscript{32}

4.3 Anticipated Study Outcomes

The goal of the project was to detect PSA glycan isoforms using microfluidic IEF and probing with fluorescently labelled lectins. Initial studies were conducted using tissue slice culture media from both healthy and cancerous tissue provided by the Peehl lab at Stanford. Tissue slice culture of prostate cancer biopsy tissue is a technique pioneered by the Peehl lab\textsuperscript{33–35} and enables researchers to study behavior of prostate tissue ex-vivo. Patient biopsy slices are cultured in a petri dish and cells will continue to interact with surrounding tissue and produce PSA and other proteins for several days after
biopsy. Studying these samples offers the opportunity to study the production of PSA from healthy cells and cancerous cells from the same individual.

Isoform patterns were to be compared to find isoforms that may be most effective at distinguishing healthy from cancerous tissue as well as potentially correlating with Gleason score. Quantitative output of the assay would be ratio of lectin probe signal at each isoelectric point to total anti-PSA signal to approximate fraction of PSA with a particular glycosylation pattern. After obtaining data from the TSC media, the assay would then be applied to banked patient blood serum samples with elevated PSA with and without prostate cancer to determine the assay’s ability to accurately distinguish cancer state and severity from PSA glycan isoform identification.

Based on a study by Zhu et al., most of the PSA in the TSC media (~96.8%) is uncomplexed\(^{36}\) so could be tested directly with microfluidic IEF and lectin probe. In contrast, in blood serum the vast majority of PSA is complexed with \(\alpha_1\)-antichymotrypsin so would need to be pretreated to release the PSA, such as by adding 1M ethanolamine\(^{37}\) prior to IEF and lectin probe.

Pairwise comparisons of culture media from prostate tissue slices of the same patient from sections with or without cancer would provide more statistical power for a given sample size than comparing different patients. Another advantage of doing this pairwise comparison is that age has also been shown to influence glycosylation and so some prior studies have done glycan normalization for age, which would be difficult to do with the limited samples available.\(^{12}\)

For our analysis, the lectin probe would be quantified at each pI and normalized to the total PSA as measured by anti-PSA signal in IEF in a parallel channel. Some lectins have been shown to bind to the glycan structures on antibodies,\(^{22}\) so lectin probes and antibody probes would have to be conducted in separate lanes or antibodies would need to be deglycosylated prior to use. For any pl's containing a signal for a particular lectin we would compare the ratio of \((\text{lectin y signal at pl x.x})/(\text{total PSA signal})\)
between the healthy and cancer tissue as measured by lectin probe signal and anti-PSA probe signal of the IEF. For cancer and non-cancer tissue from the same individual this could be done using a paired t test if the differences between healthy and cancer tissue are all normally distributed or alternatively a Wilcoxon signed-rank test for non-parametric results.

For lectin signals ((lectin y signal at pl x.x)/(total PSA signal)) showing a statistically significant difference between healthy and cancerous prostate tissue we would conduct receiver-operator curve analysis to see with what sensitivity and specificity each of the lectin probe signals (and combination of signals) can distinguish between healthy and cancerous tissue.

Following these pairwise comparisons, we would compare this same metric ((lectin y signal at pl x.x)/(total PSA signal)) between cancer tissues containing different Gleason scores, if available, and calculate the Spearman Rank correlation coefficient to see if any of the lectin probes correlate well with cancer severity.

Further information could be obtained on glycan structures via IEF by introducing various sialidases (some of which will only target particular sialic acid structures and not others) to the sample prior to IEF and then seeing how the anti-PSA signal intensity shifts between isoelectric points. Previous studies in this area (from the same lab) seem to have conflicting results as to whether some of the PSA pIs are due solely to sialic acid content or from a combination of sialic acid content and other unspecified sources such as deamidation. This information could be combined with lectin probe signal for potentially improved prognostic and diagnostic performance beyond lectin probe alone.

4.4 Microfluidic IEF

A microfluidic platform for performing IEF on biological samples followed by immunoprobing with fluorescently labelled antibodies against targets of interest was previously developed in the Herr lab by Hughes et al. This platform was modified here for probing of PSA in tissue slice culture media
utilizing fluorescently labelled lectins. Further developments to the microfluidic platform include higher sample concentrations through higher electric field strengths (800V/cm vs 300V/cm), a new loading protocol to enhance sample concentration, longer incubation times with lectins, and development of assays to rapidly screen lectins and other reagents for performance and compatibility in the Herr lab BPMA and polyacrylamide-gel-based microfluidic systems.

IEF enables the separation of proteins by isoelectric point (pI). A pH gradient is established across a scaffold, such as an acrylamide gel matrix utilizing ampholytes, molecules containing an acidic and basic group, of varying isoelectric point. When a voltage is applied, proteins will migrate towards the pH at which the charge on the protein is zero. At lower pH the positive charge will lead the protein to electromigrate towards the negative electrode and at higher pH the negative charge will lead the protein to electrophoresis towards the positive electrode. This will lead all proteins in a sample to focus in a tight band around their isoelectric point as proteins that drift from that point will be exposed to a restoring force from the electric field. The local concentration of that band will depend on the diffusion rate of the sample and the restoring force from the electric field, which depends on both the pH gradient and electric field strength.

In a microfluidic platform this process occurs in a smaller channel over a shorter distance, enabling faster focusing. Higher electric fields are also theoretically possible, as the high surface area to volume enables better heat dissipation. The microfluidic device consists of two wells 2mm in diameter connected by 3 channels that are 1cm long by 10µm deep and 70µm wide as pictured in Figure 4-2 below. Chips were designed in the Herr lab and fabricated using standard wet glass etching at a foundry (PerkinElmer).
A polyacrylamide gel matrix containing ampholytes is polymerized in the channel and cross-linked to the channel walls. To enable cross-linking to the channel walls, glass channels are flushed with 1M NaOH and then silanized to functionalize the walls with acrylate monomers as previously described. Gel precursor solution consisted of 4%T, 2.6%C acrylamide/bis (Sigma Aldrich A3699), 2% Pharmalytes (3-10 (GE Healthcare 17-0456091) or 5-8 (GE Healthcare (17-0453091)) buffered to pH 9.7 with NaOH (~25% w/v), 2.25mM N-[3-[(4-benzoylphenyl)formamido]propyl]-methacrylamide (BPMA) (purchased from PharmAgra labs as a custom synthesis product), 10% Sorbitol (Sigma-Aldrich S1876), 3% CHAPS (SigmaAldrich S1876), and 200mM (NDSB) 3-(Benzyldimethylammonio)propanesulfonate in DI water with 0.08% APS and 0.08% TEMED as initiators for polymerization. The acrylamide/bis, DI H₂O, Pharmalytes, and BPMA are mixed together and then degassed for 5min on a sonicator. The remaining components are then mixed in and the solution loaded into a glass microfluidic chip for polymerization. After 15min, the gel precursor in the wells is washed out and replaced with IEF buffer solution consisting of 10% Sorbitol, 3% CHAPS, 200mM NDSB, and 2% Pharmalytes in DI water. The chip is then stored in the dark at 4C until use.

In a protocol similar to that previously published sample is first loaded into both wells in a loading buffer consisting of 2% w/v Pharmalytes (buffered to pH 9.7), with 3% CHAPS and 200mM NDSB in DI water (Figure 4-3 (1)). An electric field is then applied to load sample into the channel (typically 50-
100V/cm). The sample is then removed from the wells and replaced with anolyte and catholyte buffers. A voltage ramp is then applied to focus the sample. After focusing, the chip is exposed to UV light to photocapture the proteins to the BPMA (Figure 4-3 (2)), typically leading to capture efficiencies of around 2% for unlabeled GFP, notably lower than the capture efficiencies achieved with the μWestern blotting platform.40,41 The pH gradient is then mobilized and unbound sample washed out through the introduction of a buffer consisting of 15mM Glycine NaOH pH 10.4 and an applied electric field. After washout of unbound samples, the fluorescently labelled probe (antibody or lectin) is then electrophoresed through the channel to probe the target of interest (Figure 4-3 (3)). Utilizing this method, highly repeatable and linear pH gradients were established as shown in Figure 4-4.

*Figure 4-3: Microfluidic IEF protocol.*
4.5 Assay Development for On Chip IEF Lectin Probe

Detecting isoforms with lectins is particularly challenging due to the high Kd and promiscuity compared to antibodies. Many lectins will recognize multiple glycan structures and a number of proteins in a sample may have similar glycan structures and bind to the same lectins. Probing in gel can have advantages for binders with high Kd as the gel prevents rapid diffusion of the binder from the target as can occur with surface based systems. The ability to image during the lectin washout step also enables rapid signal quantification within seconds of unbound lectin washing out from the target region of the gel, which would be difficult to do with traditional benchtop wash steps consisting of membranes on shakers. Timelapse imaging of the microfluidic gel system also enables identification of dissociation timescales allowing for assay optimization for lectins with a range of koff values.

Initial work was done probing purified PSA with the lectin SNA (140kDa) as previous research has shown high binding to PSA and SNA has a low enough koff that it had been successfully used in lectin arrays and enzyme linked lectin assays with multiple wash steps.\textsuperscript{13,17} SNA had been expected to
be one of the easier lectins with which to detect PSA binding, although prior sources vary as to whether SNA has statistically significant differences in binding to PSA from prostate cancer and non-prostate cancer sources. After proof of concept demonstration with SNA, the microfluidic IEF assay would then be expanded to a range of other lectins including WFA (116kDa), UEA-1 (63kDa), and TJA-II (65kDa) which some prior studies have found to bind more strongly to PSA from prostate cancer patients. Assay results would then be validated against a current gold standard such as slab gel IEF with lectin probe and/or lectin column chromatography along with assaying for total PSA.

4.5.1 Lectin Compatibility Testing with Microfluidic IEF

Experiments with purified PSA and microfluidic IEF followed by lectin probe showed no strong signal with LCA or SNA (Figure 4-5) lectins despite previous literature indications that PSA from healthy seminal fluid should bind to both LCA and SNA lectins. In the microfluidic IEF, LCA showed low signal across the channel and SNA showed high signal across the channel. LCA also did not bind to PSA above background with a nitrocellulose dot blot, whereas SNA demonstrated PSA binding in an off-chip dot blot on a nitrocellulose membrane (Figure 4-6 E,F,G). Non-specific binding to the nitrocellulose membrane was notably higher with LCA than SNA.
Figure 4-5: Microfluidic IEF with LCA and SNA lectin probe. Neither LCA nor SNA have a distinct signal at the expected pIs of PSA (6.2 – 7.5). LCA has low signal across the channel and SNA has high signal across the channel due to high non-specific interaction between SNA and BPMA that has been UV photoactivated. Lectin probe signal in green. UV markers in blue.

Figure 4-6: Comparison of SNA and LCA dotblots against PSA from healthy seminal fluid. A-C: no PSA (negative control), D: 0.1uM lectin (positive control), E: 1uM PSA, F: 0.5uM PSA, G: 2uM PSA, H 0.5uM lectin (positive control). Images were taken using automated slide scan in Metamorph on an inverted epifluorescence microscope imaged at 10x for 100ms. Lectins contained a FITC label. Membranes were incubated in Synbock blocking agent (AbD Serotec BUF034A) prior to lectin probe. Binding to PSA only seen with SNA dot blot.
To determine the source of non-specific SNA interaction in the microfluidic IEF, a series of experiments were run to assess different microfluidic IEF components. Four conditions were compared: (1) GFP, PSA, and UV markers with UV exposure, (2) GFP & PSA with UV exposure, (3) no sample with UV exposure, and a (4) gel focused, but with no UV exposure. All gels were 4%T and focused with the same IEF buffer, anolyte, and catholyte. Results (Figure 4-7) indicated that UV exposure was required for high SNA non-specific adsorption in the channel, but not UV markers, PSA, or GFP. This suggested that interaction was due to the UV activated BPMA or one of the IEF buffer components.

Figure 4-7: SNA background in microfluidic IEF gels under the following four conditions 1) PSA, UV markers, and GFP with UV exposure, 2) PSA and GFP with UV exposure, 3) no sample with UV exposure, and 4) no sample and no UV exposure. All conditions show high SNA background except for the case of no UV exposure.

On-chip dot blots were developed to rapidly assess probe performance of lectins against PSA in a microfluidic device and compare interactions with UV activated BPMA versus target PSA. Building on
the protocol developed by Lin et al.\textsuperscript{43} for the lateral e-flow assay, laser cut steel masks (Figure 4-8) were used to illuminate a small region of the channel for protein photopatterning for an on-chip dot blot assay (Figure 4-9). First, proteins were loaded into the channel by applying a field of 200V/cm. Then the electric field was turned off and a steel mask placed over the microfluidic channel. A light guide attached to a Hamamatsu Light Curing system was then placed against the mask (100% for 10 sec) to photocapture proteins in a small region of the channel. Unbound proteins in the channel were then washed out. Multiple patterned regions can be established in series using this method. Fluorescently labelled lectins or antibodies can then be electrophoresed through the channel to both rapidly assess binding with PSA and compatibility with the in-gel photocapture system.

SNA was shown to have high non-specific interaction with UV photoactivated BPMA (Figure 4-10), consistent with the high background seen in the microfluidic IEF assay. SNA signal against UV activated BPMA was higher than against photocaptured PSA. This result suggests that SNA interaction is with BPMA, rather than any of the IEF buffer components as the dot blot is conducted in 1x Tris-glycine buffer without any IEF buffer components. This prevents use of SNA as a probe in the current microfluidic IEF system, which relies on UV photoactivated BPMA for blotting.

LCA demonstrated some potential binding to PSA in an on chip dot blot (\textasciitilde 3 fold higher than signal from UV activated BPMA). With such a weak signal even with 1.5\mu M immobilized PSA, LCA sensitivity may not be high enough for utilization in a microfluidic IEF system with biological samples. While previous studies demonstrated binding of purified PSA from healthy seminal fluid to LCA in lectin arrays,\textsuperscript{32} the binding has been demonstrated to be lower with than with SNA.\textsuperscript{32} It is also possible that differences may exist in the purified PSA utilized here compared to previous studies leading to lower lectin binding (e.g., glycan degradation during sample processing or storage). As expected, antiPSA showed high binding to PSA compared to the UV photoactivated region of the gel with no immobilized protein (Figure 4-10).
LCA lectin had high background on a nitrocellulose membrane versus SNA (Figure 4-6), but low background in a UV photoactivated BPMA gel, whereas SNA had low background on nitrocellulose, but high background on UV photoactivated BPMA, suggesting that different assay formats may yield better performance depending on the lectin used and its non-specific interaction with the matrix material.

Figure 4-8: Laser cut steel mask (fabricated by Rob Lin) used for UV photopatterning. Small slit in center used for patterning and larger squares are for alignment on the wells.
4.5.2 Biological Sample Testing Results

The tissue slice culture (TSC) media provided by the Peehl lab at Stanford was separated in a microfluidic IEF device followed by probing with anti-PSA and WFA lectin. No clear PSA isoforms were identified in a microfluidic IEF anti-PSA probe of the tissue slice culture media as shown in Figure 4-11,
suggesting concentrations below the lower limit of detection of the assay or significant degradation of PSA. Follow up studies utilizing nitrocellulose dot blots to test for antibody binding to tissue slice culture media also yielded no detectable signal (estimated dot blot LLOD of 50pM, data not shown). Further testing would need to be done to quantify the PSA concentration in the TSC media, using a more sensitive detection method such as ELISA, and determine if it is within the achievable limits of detection of the microfluidic IEF assay. Alternatively, prior to microfluidic IEF, the TSC media samples could be further preconcentrated to detectable levels using spin columns or with direct magnetic bead purification of PSA as has been used previously for glycan characterization with lectin arrays.42 Another approach is to modify the microfluidic IEF protocols to enable higher levels of sample loading onto the microfluidic chip, as will be described in the next dissertation section.

Figure 4-11: anti-PSA probe of tissue slice culture media after microfluidic IEF. No detectable PSA isoforms are seen in either media, suggesting PSA concentration is below the limits of detection of the assay or is degraded and not focusing at the expected isoforms.
WFA signal was observed for both healthy and prostate cancer TSC media (Figure 4-12), but this may represent binding to another higher abundance protein in the sample given the lack of clear antiPSA signal in either media sample.

![Fluorescence micrographs of IEF with WFA lectin probe of TSC media cancer and TSC media normal. WFA lectin probe signal observed in both cases, but at different pIs.](image)

Purified PSA, blood serum from a prostate cancer patient, and healthy pooled blood serum were tested with anti-PSA and WFA lectin probe with results shown in Figure 4-13. AntiPSA signal was seen with prostate cancer blood serum and purified PSA from healthy seminal fluid, but not with healthy pooled blood serum, as expected. No WFA signal was seen with purified PSA and signal was seen in blood serum from prostate cancer patients at around the same pl as PSA, as expected from the literature. A slight, much lower signal (SNR ~3), was seen with WFA in healthy blood serum. More follow up studies would need to be conducted to determine if WFA is binding to PSA or other proteins in
the sample. This could be accomplished by utilizing magnetic beads with antiPSA to separate PSA from the other blood serum or TSC media components and separately running IEF and WFA lectin plus antiPSA probe on the purified PSA and blood/TSC media with PSA removed.

UEA-1 lectin, which a Fukushima et al. study utilizing column affinity chromatography had shown preferential binding to PSA from prostate cancer,\textsuperscript{21} was also tested against purified PSA from healthy seminal fluid and blood serum from prostate cancer patients and no detectable signal was seen in either case (data not shown). TJA-II another lectin that the same study\textsuperscript{21} indicated should bind to PSA from prostate cancer, but not healthy patients, is no longer available commercially, so was not tested.
Figure 4-13: Comparison of antiPSA and WFA lectin binding to microfluidic IEF of blood serum from a prostate cancer patient, healthy pooled blood serum, and purified PSA from healthy seminal fluid.

4.5.3 Increased On-Chip Sample Concentration for Improved Detection of Low Concentration Targets

Previously published microfluidic IEF protocols from the Herr lab involve a sample loading phase for five minutes with sample diluted in a 9.9 pH buffer followed by replacing the loading buffer with anolyte and catholyte and then focusing. While sufficient to resolve targets at higher concentrations, this limits the mass of loaded sample based on channel length as with longer loading times the sample will electromigrate to the opposite well and be removed when the loading buffer is replaced with anolyte and catholyte (Figure 4-14).
An alternative approach was investigated that utilizes a combined loading and focusing approach with sample added directly to anolyte and/or catholyte during focusing (Figure 4-14). This method enables higher levels of sample concentration, critical to the detection of low starting concentration targets. With this method, a larger amount of sample can be loaded onto the chip as sample will continually load and each protein will focus at its pI rather than electromigrate to the opposite well to be washed out, as occurs with a uniform pH across the channel during loading.

It should be noted that both loading methods can lead to sample loading bias. In the case of a traditional slab-gel IEF, the sample is loaded into a well and the full sample is loaded and focused, but due to the design of the microfluidic chip, only a small fraction of the total sample from the well will be loaded into the channel in either case. The charge of the protein and the MW of the protein will impact the mobility of the protein into the channel and the rate of loading (i.e., if two proteins have the same charge at a given pH the smaller protein will migrate faster in the gel; if two proteins have the same MW, the protein with a higher charge will migrate faster in the gel). The use of a 4%T gel, which has large pore sizes, minimizes loading variability due to MW as compared to a higher %T gel.
Figure 4-14: Schematic comparing loading sample prior to focusing and loading sample during focusing. (A) In the case of loading sample prior to focusing, the sample is added to a buffer at pH 9.7 and loaded by applying an electric field. Sample will migrate from negative electrode to positive electrode. After loading, the wells are replaced with anolyte and catholyte and any sample that migrated into the opposite well is removed, limiting the maximum loaded sample mass. An electric field is then applied to focus the sample. (B) In the case of loading sample during focusing, sample is added to anolyte and catholyte buffers and an electric field applied. Sample will load during focusing and each protein will begin to focus and concentrate at its pI. This will lead to a continual increase in protein concentration over time that can exceed the maximum possible loaded sample mass when proteins are loaded prior to focusing.

This method was shown to work well with purified protein eGFP loaded from the catholyte well as shown in Figure 4-15, leading to a substantial increase in eGFP concentration over time and stable IEF with little cathodic drift once the voltage is increased to 800V/cm.
Figure 4-15: Increase in eGFP concentration over time with continuous loading during focusing from the catholyte well.

Figure 4-16: Timelapse image plots of eGFP signal with loading during focusing at 500V/cm. Green line is first time point, red lines are taken at 2min intervals (6th interval is missing) and the black dashed line is the final time point.
While some cathodic drift was present during the 17 minutes of focusing at 500V/cm as shown in Figure 4-16. The voltage was increased to 800V/cm and no cathodic drift was observed for the subsequent 25 minutes of loading/focusing as indicated by a fixed position for the eGFP as shown in Figure 4-17. During this time, eGFP concentration continues to increase while remaining in a fixed position.

![Graphs showing intensity vs position](image1)

*Figure 4-17: Timelapse image plots of eGFP signal with loading during focusing at 800V/cm. Green line is first time point and black dashed line is final timepoint. Red lines are taken at 2 minute intervals.*

Initial testing with TSC media of loading during focusing lead to instability and high current flows when using the same voltage ramp used for purified proteins (Figure 4-18). TSC media is mixed 1:2 with anolyte and catholyte. This is hypothesized to be due to the higher salt and protein content in the TSC media. After initial focusing, the current remains higher with the TSC media, both normal and cancer. After the voltage ramp is restarted a second time from 50-300V, the TSC media and cancer begin to
focus and eGFP concentration increases over time (Figure 4-19). Focusing and eGFP concentration increase begins sooner with purified proteins (Figure 4-19).

![Figure 4-18: Current during focusing for unconditioned media, TSC media cancer, TSC media normal, and purified proteins. The most stable focusing is observed with purified proteins. Current spikes and unstable focusing are observed with the TSC media. After a second voltage ramp up, the TSC media samples start to focus as indicated by a rapidly declining current at fixed voltage.](image-url)
Initial experiments using a much slower ramp up, 4 min per voltage level (Figure 4-20) instead of 2 min (Figure 4-18), seem to produce more stable loading during focusing with TSC media (Figure 4-20) added 1:2 into anolyte and catholyte, but more extensive experiments would need to be done. Loading from just the catholyte well, rather than both wells, led to instability in this initial experiment, but has not been replicated. This method was shown to lead to an increase in eGFP signal over time in the TSC media (Figure 4-21) (the lower signal seen with eGFP in unconditioned media is because the eGFP band initially focused off the gel in the free solution region, leading to high levels of diffusion back into the well.)

Figure 4-19: Increase in eGFP concentration over time for eGFP & PSA spiked into unconditioned media, TSC media normal, TSC media cancer, and for purified eGFP & PSA directly into anolyte and catholyte. (Gap in the center is when the voltage ramp up was restarted to improve focusing in the TSC media channels.)
Figure 4-20: Current during focusing, showing high current spike in lane with loading only from the catholyte, but stable focusing from all other lanes with and without TSC media.
Preliminary testing with this method demonstrated the probing of 40nM PSA spiked into unconditioned TSC media as shown in Figure 4-22. The spiked unconditioned TSC media was then added 1:2 with anolyte and catholyte for loading during focusing. Further development would need to be conducted to determine the increase in sample concentration achievable with this method as compared to loading prior to focusing.
The primary limitation in length of time for loading during focusing and the obtainable sample concentration increase with TSC media is currently the cathodic drift that occurs during focusing with the TSC media samples (Figure 4-23). Eventually the cathodic drift results in the target proteins drifting off the end of the gel and into the well. Further development would need to be done to try to minimize this to enable higher levels of sample concentration in TSC media. Possibilities include sample prep to remove salts from the TSC media buffer prior to running IEF. While techniques exist to form a permanent pH gradient such as the use of immobilines immobilized to the gel, which should eliminate cathodic drift, the need to subsequently load antibodies into the channel makes their use difficult in an on-chip enclosed isoelectric focusing platform. An inability to remove the pH gradient after IEF focusing...
and photocapture will prevent electrophoresis of antibodies and lectins across the full gel. Even in the
case of the mobile ampholytes currently used, the small amount photocaptured to the gel during BPMA
UV photoactivation leads to a residual pH gradient that leads to variability in antibody transport across
the gel as originally demonstrated by Tia et al.44

![Graph](Figure 4-23: Cathodic drift with loading during focusing.)

4.6 Free-Standing Gel IEF

To enable greater sample loading into a gel and eliminate the need for electrophoretic antibody
and lectin probing, a free-standing IEF system was briefly investigated, building on the work of
Duncombe et al.45 for utilizing photopatterning to create thin free-standing polyacrylamide gel
structures for protein separation. Gel fabrication was conducted using a protocol similar to that
published in Duncombe et al.,45 utilizing photopatterned acrylamide gel on gelbond. Gel precursor
consisted of 7.2% T, 3.3%C acrylamide/bis (from 40%T, 3.3% stock solution Sigma-Aldrich A7802) in DI
water with 2% VA-086 photoinitiator. After fabrication the gel was soaked in the same IEF buffer used for in-chip microfluidic IEF (as previously described).

The photopatterned polyacrylamide gel structures (Figure 4-24) contain a small well for loading sample as well as wider regions on either end for interfacing with the cylindrical graphite electrodes used to apply an electric field within an enclosed red plastic environmental chamber (Figure 4-25) (mask originally designed by Todd Duncombe for size-based protein separations). The red environmental chamber, designed by Todd Duncombe, reduces water evaporation from the gel and allows easier interfacing with the graphite electrodes.

Figure 4-24: Photograph of photopatterned polyacrylamide free-standing gel for use with IEF.
Initial testing with an 8% T free-standing gel enabled focusing of GFP as shown in Figure 4-26 utilizing a voltage ramp from 100V-500V/cm. UV pl markers were not visible due to autofluorescence from the gelbond material. The curved bands are hypothesized to be due to variations in electric field from the presence of a photopatterned well for loading and the drying out of the well and gel over time. While individual isoforms can be identified in the current system, further development such as the use of molds instead of photopatterning to generate cleaner and more repeatable well interfaces would likely enable improved isoelectric focusing performance. Covering the gel with glycerol or other non-conducting material could also reduce drying out of the system as has been demonstrating by Yuchen Pan and Todd Duncombe for size-based protein separations in free-standing gels.

A free-standing gel IEF system combined with the BPMA photocapture gel utilized in glass chip microfluidics can enable direct probing in gel without the need to electrophoresis antibodies through a variable pH gel or the addition of a transfer step as is utilized in traditional bench top IEF assays. The
form factor can also enable higher levels of multiplexing and easier integration with automated liquid handling systems such as a Tecan for high throughput testing of large numbers of samples.

![Fluorescent microscope image of Free-standing gel IEF. Focusing of GFP (3 isoforms) shown.](image)

4.7 Glycan Isoform Detection Barcode Assay

An alternative microfluidic approach was investigated for rapid multiplexed detection of glycan isoform identification utilizing a similar approach as described for the on-chip dot blot assay (Figure 4-9), based on the work from Lin et al\(^{13}\) for an electrophoretic lateral flow assay. Various lectins would be photocaptured to the BPMA gel using steel masks and UV light as previously described. Sample would then be electrophoretically loaded through the channel and proteins with glycan structures recognized by each lectin would be captured by the lectin band. A fluorescently labelled deglycosylated antibody against the target protein of interest would then be electrophoresed over the channel to detect the quantity of a particular protein bound to the lectin.

An initial proof of concept was tested utilizing fluorescently labelled purified PSA from healthy pooled seminal fluid electrophoresed over UEA-1, Con A, and SNA. Strong PSA signal is seen with SNA,
but not with either of the other lectins. UEA-1 is only expected to bind to PSA from prostate cancer patients, but Con A is expected to bind to PSA from both sources based on previous studies. While providing less information due to the lack of a pI separation step, this format enables SNA to be used in a the microfluidic format by eliminating the non-specific interaction between SNA and photoactivated BPMA by immobilizing the lectin directly to the gel.

200nM each of FITC labelled lectins (ConA, WFA, and SNA) was immobilized to the polyacrylamide gel matrix using the fabrication protocol shown in Figure 4-9. 200nM of Alex Fluor 488 labelled PSA in 1X Tris Glycine was electrophoresed at 200V/cm across the barcode for 10 minutes. The image below shows the signal after 150 seconds of washout with 1X Tris Glycine at 200V/cm.

Figure 4-27: Barcode lectin assay against purified healthy PSA.

The amount of bound PSA decreases over time based on the dissociation rate of SNA and PSA, but signal is still detectable 25 minutes after binding (13 min wait and 12.5 min of applied electric field at 200V/cm), indicating likely assay compatibility with a secondary detection antibody. The top red line in Figure 4-28 is the initial signal after 2 min of washing at 200V/cm. The second red line is after 150 seconds, and the black lines are taken at 30sec intervals after a 13 min wait period with no applied
electric field. Characterization of the kon/koff of lectin-PSA binding can enable improved design optimization of the barcode and IEF lectin probe assays to maximize SNR. Utilizing higher concentrations of immobilized lectin to the polyacrylamide gel matrix can further increase the amount of PSA bound in the barcode assay.

Figure 4-28: Purified PSA signal (peak at SNA) over time with washing at 200V/cm. Timepoints are taken at 30sec intervals. Red lines are at 120 and 150 seconds of washing and black lines begin after 13min of no applied voltage and are taken at 30 sec intervals for a total of 10 additional minutes of washing at 200V.

4.8 Conclusions

Further development is still needed with the microfluidic systems to obtain reliable lectin binding data with biological samples for publishable results on differences in PSA glycan isoforms between prostate cancer and healthy patients. Approaches such as the lectin barcode assay can be applied for lectins that non-specifically bind to photoactivated BPMA, including SNA, to enable their inclusion in studies, but this lacks the additional information from an IEF separation. The low capture efficiency in the microfluidic IEF system, combined with the high Kds of lectins versus antibodies, and
the low mass of sample that is loaded in a microfluidic channel versus a benchtop assay, makes this a particularly challenging application for microfluidics. Further sample prep of the TSC media including sample concentration and potentially off-chip purification of PSA from other sample components with the use of magnetic beads can enable higher levels of PSA concentration for improved microfluidic IEF detection with lectins. Separating PSA from buffer salts and other TSC media components would likely also enable much higher levels of sample concentration with loading during focusing on the microfluidic IEF device due to the reduced cathodic drift seen in purified protein samples as compared to TSC media. These advancements may enable the use of microfluidic IEF with lectin probe for the screening of large numbers of TSC media samples for an improved understanding of how PSA glycan isoforms can be used as biomarkers for better prognosis and diagnosis of prostate cancer in the clinic.
4.9 References


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