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Microchamber integration unifies distinct separation modes for two-dimensional electrophoresis

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Abstract

By combining isoelectric focusing (IEF) with subsequent gel electrophoresis, two-dimensional electrophoresis (2DE) affords more specific characterization of proteins than each constituent unit separation. In a new approach to integrating the two assay dimensions in a microscope slide-sized glass device, we introduce microfluidic 2DE using photopatterned polyacrylamide (PA) gel elements housed in a millimeter-scale, 20 micron-deep chamber. The microchamber minimizes information loss inherent to channel network architectures commonly used for microfluidic 2DE. To define the IEF axis along a ‘lane’ at the top of the chamber, we used free solution carrier ampholytes and immobilized acrylamido buffers in the PA gels. This approach yielded high resolution (0.1 *pH* units) and rapid (<20 min) IEF. Next, protein transfer to the second dimension was accomplished using chemical mobilization perpendicular to the IEF axis. Mobilization drove focused proteins off the IEF lane and into a region for protein gel electrophoresis. Using fluorescently labeled proteins, we observed transfer induced band broadening factors ~7.5-fold lower than those observed in microchannel networks. Both native polyacrylamide gel electrophoresis (PAGE) and pore-limit electrophoresis (PLE) were studied as the second assay dimension and completed in <15 min. PLE yields protein molecular mass information without the need for ionic surfactant or reducing agents, simplifying device design and operation. Microchamber-based 2DE unifies two independent separation dimensions in a single device with minimal transfer-associated information losses. Peak capacities for the total assay ranged from 256 to 35 with <1 hr assay duration. The rapid microchamber 2DE assay has the potential to bridge an existing gap in targeted proteomics for protein biomarker validation and systems biology that may complement recent innovation in mass spectrometry.

Keywords

proteomics; microfluidics; isoelectric focusing; polyacrylamide photopatterning

INTRODUCTION

While genomics has benefited from tremendous analytical technology advances, similar technology innovation for proteomics has lagged. 2DE is a prime example of a slow-to-advance bench-top proteomic technology. 2DE integrates two serial assays to separate proteins by isoelectric point (*pI*) and molecular mass (*M_r*) [1]. By measuring not one but two physicochemical properties, 2DE offers enhanced resolution of complex samples and, thus, is a workhorse for protein identification and characterization [2]. The total assay peak capacity of a multi-dimensional separation is equal to the product of the peak capacity of

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each individual dimension [3]: $n_{2D} = n_1 * n_2$, where the peak capacity for each separation is defined as the separation axis length L over the band width 4σ : $n = L/4\sigma$. While information rich, the labor and time intensive nature of the workflow presents a major limitation of conventional slab gel 2DE, severely limiting throughput. A single assay can take over a day to complete with significant manual intervention [1]. Long duration buffer exchange for transition between the distinct chemical conditions needed for each separation (i.e., pH gradient disruption, addition of electrophoresis buffers and surfactants) increases assay duration and confounds quantitation (e.g. biased protein losses). Consequently, 2DE remains underutilized in discovery and targeted proteomics. This important line of targeted inquiry has seen significant advances through mass spectrometry, while complementary bench-top assays lag.

Microfluidic design has been explored as one means to expedite and automate 2DE assays. Owing to favorable transport scaling with miniaturization, the capacity to realize complex fluidic networks, and fine control of sample manipulation, such systems are well-suited as multi-dimensional assay platforms. A canonical 2DE microfluidic integration strategy employs one microchannel (for IEF) intersected by an array of channels (for electrophoresis) [4-7]. Nevertheless, as is true with all multi-dimensional assays, ideally no material or information should be lost during transfer between the dimensions. While miniaturization has certainly expedited 2DE, transfer induced losses have been estimated at ~10-fold. With the intersecting microchannels architecture, such losses are inherent and arise directly from discretization of the continuous first dimension separation by sampling to the second dimension array. Under-sampling reduces 2DE peak capacity n'_{2D} [8, 9] and can be quantified by:

$$n'_{2D} = n_{2D} * \frac{\sigma_{IEF}}{\sigma_{transfer}} \quad (1)$$

where the correction term $\sigma_{IEF}/\sigma_{transfer}$ is the effective peak broadening due to under-sampling of the first dimension. This under-sampling results in loss of information from the first dimension, as separation resolution R_s also depends on the effective band width: $R_s = \Delta x/4\sigma$ [10]. Using channel networks for microfluidic 2DE gives band broadening factors determined by the sampling rate [4], the channel spacing [5-7], and transfer channel width leading to broadening factors $\sigma_{IEF}/\sigma_{transfer}$ of ~10. These considerations are irrelevant to macro-scale slab-gel 2DE assay design [8-10].

With an interest in designing rapid, targeted protein assays, we introduce a new design strategy for microfluidic 2DE, that combines the benefits of microfluidics (speed, automation, low sample needs) with those of slab-gels (lossless transfer). Inspired by early work on compact 2DE [11, 12], we introduce a microchamber patterned with distinct but contiguous PA gel regions. The continuous gel elements do not discretize the first stage and minimize transfer losses by harnessing fine microfluidic transport control without valves [13-18]. Further, we integrate gradient PLE gels to achieve size-based separations in the second dimension without reagent addition (e.g., surfactants, reducing agents) during transfer. The device and assay design are explored as means to reduce the complexity of 2DE assays with an eye towards scale up necessary to advance targeted proteomics.

MATERIALS AND METHODS

Reagents and Materials

UV photoinitiator VA-086 was purchased from Wako Chemicals (Richmond, VA). Acrylamide (99%, electrophoresis grade), *N,N'*-methylenebisacrylamide (>99%), CHAPS (98%, electrophoresis grade), sodium hydroxide (97.0%, ACS grade), Polybuffer® 74,

Polybuffer® 96, ovalbumin (OVA, chicken, 98%, electrophoresis grade), transferrin (Trans, human, 98%), and fluorescent IEF markers were acquired from Sigma Aldrich (St. Louis, MO). Immobiline II pK 3.6 and Immobiline II pK 9.3 were obtained from GE Healthcare Biosciences (Pittsburgh, PA). Anode Buffer (10×) and Tris/Glycine Buffer (10×) were acquired from Bio-Rad (Hercules, CA). Recombinant wild-type GFP from *Aequorea Victoria* (wtGFP) was sourced from Clontech (Mountain View, CA). ZyMAX™ anti-human immunoglobulin G (H+L)–FITC (αIgG, goat), and Alexa Fluor® 488 conjugated proteins parvalbumin, trypsin inhibitor, OVA, bovine serum albumin (BSA) were purchased from Invitrogen Life Technologies (Carlsbad, CA). CE Dye 540 labeling kit was purchased from Active Motif (Carlsbad, CA).

Device Fabrication

Glass (soda lime) chips were designed, fabricated, functionalized with acrylate-terminated monolayers, and filled with photopatterned PA gels, as previously described [13-16,19,20] (see SI for details). For the IEF dimension, all gel precursor buffers contained 0.2% (w/v) VA-086 photoinitiator and 4% (w/v) CHAPS. Gels used to buffer at basic pH (~10.1) contained 14.4 mM of Immobiline II pK 9.3 and 5.6 mM of Immobiline II pK 3.6. Gels used to buffer at acidic pH (~3.3) contained 6.4 mM of Immobiline II pK 9.3 and 13.6 mM of Immobiline II pK 3.6. Acrylamide and bisacrylamide concentrations of 4%T, 3.3%C were used unless specified otherwise. Focusing regions contained Polybuffer 74 and Polybuffer 96 in 1:5 (v/v) dilutions as the carrier ampholytes.

Photomasking during gel photopatterning employed an AutoCAD-designed chrome-glass photomask (Photo Sciences, Inc., Torrance, CA). A Hamamatsu LightningCure LC5 (Hamamatsu City, Japan) with intensity control with its UV beam directed through the light path of a Nikon Diaphot 200 (Tokyo, Japan) inverted microscope with a UV-transmission objective lens (UPLANS-APO 4×, Olympus, Melville, NY) and a Prizmatix silver-LED (355 nm) with intensity control fiber-coupled to a 1 inch beam collimator (Givat Shmuel, Israel) were used for gel photopolymerization. UV intensities ranging from 6-12 mW cm⁻² measured with a pocket UV light meter (Lutron, Taipei, Taiwan) and 4-8 min exposure times were used for all steps as specified.

Glass chips were recycled by removal of the gel using an overnight incubation with a 2:1 perchloric acid and hydrogen peroxide solution heated to 75°C with appropriate safety precautions, as previously described [19].

Samples and Experimental Protocols

Ovalbumin and transferrin were labeled with CE540 according to manufacturer instructions, and 37 μg ml⁻¹ of each protein was loaded onto the devices. All sample solutions contained 4% CHAPS, 100 μg ml⁻¹ of wtGFP, and 30× dilutions of fluorescent pI markers. 3 μl sample volumes were titrated with 1 M sodium hydroxide to pH >9 immediately prior to loading. All other wells other than the sample loading well contained focusing buffer (4% CHAPS, 1:5 v/v PB 74, and 1:5 v/v PB 96) during the loading step. After electrophoretic sample loading, electric field application was stopped and the solutions in the focusing terminal wells were replaced with catholyte (40 mM sodium hydroxide) and anolyte (7 mM phosphoric acid) buffers. Pipette tips were used as 5-10 μl buffer reservoirs. Electric field was then reapplied for the focusing step. Continuous monitoring and control of electrical voltage and currents was achieved using a custom built, eight-channel high voltage power supply (see SI for the voltage protocols used during each step).

Imaging and Image Processing

Chip imaging was conducted using an Olympus IX71 inverted fluorescence microscope equipped with an EMCCD camera iXon3 885 (Andor, Belfast, Northern Ireland), a motorized stage (Applied Scientific Instrumentation, Eugene, OR), an automated filter cube turret controlled by MetaMorph software (Molecular Devices, Sunnydale, CA), and an X-Cite Exakte mercury arc lamp illumination source coupled to an automated shutter and attenuation system (Lumen Dynamics, Mississauga, ON, Canada).

Fluorescent *pI* markers were imaged using a custom UV-longpass filter cube (XF1001, excitation 300-380 nm; XF3097, emission >410 nm). Green channel and red channel fluorescence imaging was done using filter cubes optimized for GFP (XF100-3, excitation 445-495 nm, emission 508-583 nm), and DsRed2 (XF111-2, excitation 525-555 nm, emission >575 nm) (Omega Optical, Brattleboro, VT), respectively. Images were captured using exposure times of 100-200 ms and 4×4 binning with 500-1000 ms intervals between successive images with a 4× magnification objective lens (Olympus UPlanFI, NA 0.13) unless specified as 10× (Olympus UPlanFI, NA 0.3). Whole device imaging at 4× magnification was conducted via stitching of adjacent overlapping images in ImageJ software (NIH, Bethesda, MD), as previously described [21]. MATLAB scripts written in house were used to determine analyte band positions and widths (MathWorks, Natick, MA).

RESULTS AND DISCUSSION

Design of a polymer-patterned microchamber for microfluidic 2DE

Two overarching design constraints are relevant to development of 2DE assays, namely: (1) implementing two distinct, uncoupled separation modes and (2) minimizing information loss during transition between the two assay dimensions. We address both constraints by developing a microchamber wherein IEF is performed along a horizontal “lane” across the top of the chamber and PAGE is conducted vertically in a region occupying the rest of the chamber (Figure 1). To satisfy the first design criterion, confinement of IEF to a lane at the top of the chamber allows definition of the PAGE separation axis in a physically distinct area. After IEF equilibrates, chemical mobilization in the direction perpendicular to the IEF axis drives focused protein zones off the IEF separation axis. Importantly, mobilization is applied orthogonally to the IEF separation axis causing the focused species to migrate down the length of the chamber, towards the bottom wall. The perpendicular mobilization direction contrasts with typical 1D chemical mobilization approaches that drive focused species along the IEF separation axis [22, 23]. To address the second design constraint, mobilization off the IEF axis is controlled by the directionality of applied chemical boundary and electric field conditions. As the species migrate down the chamber during mobilization, the separation mechanism switches from *pH* gradient supported IEF to molecular sieving *via* electrophoresis through a PA gel.

To fabricate the IEF and PAGE separation axes, contiguous PA gels having distinct properties were photopatterned in the microchamber and flanking microchannels (see SI for detailed fabrication). To generate the IEF separation axis, we utilized a large pore-size PA gel housing liquid phase carrier ampholytes. Especially critical to IEF are the low and high *pH* boundaries at each terminus of the IEF axis. To achieve the stationary boundary conditions, we covalently incorporated acrylamido buffers (immobilines) into the acrylamide matrix at the edges of the IEF separation axis during the photopatterning process. Thus, the immobilines define *pH* boundary conditions at the edges of the microchamber (as opposed to at the distant fluid reservoirs), with the non-immobilized carrier ampholytes defining the *pH* gradient itself along the separation axis. For the second dimension, we explored two modes of PAGE: native PAGE and PLE. Native PAGE separates proteins by charge-to-mass ratio whereas PLE separates according to M_r .

Importantly, PLE is capable of reporting M_r while obviating the need for surfactant addition, sample handling/manipulation, and the dispersion associated with such processes.

IEF: Defining a 1D separation axis in a 2D microchamber

As the first dimension of the 2DE assay, IEF in a microchamber requires: (1) lateral confinement of the IEF pH gradient to within the microchamber (i.e., not in the channel networks flanking the microchamber) and (2) longitudinal confinement of the IEF focusing region to a lane along the top portion of the microchamber. By satisfying both requirements, the entire pH range can be transferred to the second dimension of the assay. Consequently, spatial confinement of IEF is required in both the x - and y -dimensions of the microchamber. We discuss each aspect of 2D IEF confinement here.

Confinement of IEF to the microchamber along the x-axis—Confinement of the IEF pH gradient to the microchamber (x -axis confinement) is necessary so all focused species are subsequently mobilized into the PAGE region. If the IEF boundary conditions were applied at the terminal wells, a large portion of the pH gradient would be generated outside of the microchamber, thus permitting mobilization of only a portion of the IEF separation axis into the PAGE region. (Note that each edge of the 3 mm wide microchamber is 6 mm from the nearest reservoir. The 6 mm control channels ensure uniform electric fields in the microchamber and, in turn, minimize dispersion during transport in both dimensions [14, 17, 18]). To confine the pH gradient to the microchamber, we covalently incorporated immobilines into PA gels at the edges of the microchamber to limit the extent of the resulting pH gradient. While typically used for the formation of immobilized pH gradients [24, 25], immobilines have been employed in conjunction with carrier ampholytes [26, 27]. Combinations of immobilines buffering at basic ($pH \sim 10.1$) and acidic ($pH \sim 3.3$) pH formed the boundary conditions while the non-immobilized carrier ampholytes comprised of broad range polybuffers (pH 4-9) formed the pH gradient.

We sought to compare the IEF performance of pH gradients laterally confined to the microchamber (termed “confined”) to gradients that spanned the microchamber and flanking microchannels (termed “unconfined”). Imaging of UV fluorescent pI markers during IEF allowed us to measure the extent, linearity, and stability of the pH gradients (Figure 2). Both confined and unconfined pH gradients supported a linear gradient (confined $R^2 = 0.99$; unconfined $R^2 = 0.89$). The confined gradient spanned the microchamber width with a 1.7 pH/mm slope, compared to the shallower 0.5 pH/mm unconfined pH gradient. Peak capacities of $n_{\text{confined}} \sim 8$ and $n_{\text{unconfined}} \sim 26$ were measured using average pI marker band widths. Variabilities in the extent of the pH gradient and band width were calculated from replicate measurements to be 12.7% and 13.1%, respectively, and represent the uncertainty of our determination of L and σ . We observed 67% narrower average peak widths and a geometrically determined five-fold loss in L for the confined gradient compared to the unconfined case. These empirical observations are consistent with our understanding that steeper gradients yield narrower focused zones according to:

$$\sigma_{IEF} = \sqrt{DE^{-1} \left(\frac{dpH}{dx} \right)^{-1} \left(-\frac{du}{dpH} \right)^{-1}} \quad (2)$$

where peak width is given by $4\sigma_{IEF}$. The sharper bands resulting from the confined IEF do not make up for the loss in peak capacity arising from the shortened separation axis L . As a further consequence of the shorter separation length is an expected increase in the minimum resolvable pI difference $\Delta pI = 3\sigma_{IEF} \left(\frac{dpH}{dx} \right)$ with increasing pH gradient steepness [28]. Focusing time, however, favorably scales linearly with reduction L for a given pH range. Here, we observed focusing times of $t_{\text{confined}} \sim 3$ min and $t_{\text{unconfined}} \sim 7$ min. We observed

substantially lower cathodic drift velocities for the confined IEF ($10.2 \mu\text{m min}^{-1}$) as compared to unconfined or microchannel-based IEF ($73.8 \mu\text{m min}^{-1}$, Figure S3) [21]. We attribute the seven-fold reduction in observed drift to either confinement of the gradient to the five-fold smaller length or to use of immobilized (versus non-immobilized) buffers as the boundary conditions; further study is underway. If considering IEF as a single dimension assay, some aspects of performance (n , ΔpI) improve with increasing microchamber size in the IEF dimension. Nevertheless, design of IEF as the first dimension of a 2DE assay must integrate with PAGE in a manner that limits information loss from the 1st dimension.

Confinement of IEF to a quasi-1D lane in the microchamber—Confinement of IEF to a lane in the microchamber was designed to yield a distinct IEF region proximal to the PAGE region. To meet this design goal, we controlled the y -span of the IEF focusing region by controlling sample loading (Figure 3). Here, prior to focusing, we loaded sample through a single side channel and not through the entire side channel array (well 1 contained sample while wells 2 and 8 contained only IEF buffer). With this loading approach, $wGFP$ focused 7 min after entering the chamber. pI -marker based characterization showed a linear pH gradient spanning the entire 4 mm chamber width (4–9 pH units, 1.28 pH/mm , $R^2 = 0.99$). While successful for constraining IEF to a ‘virtual’ lane (i.e., no walls), such confinement generates only a quasi-1D axis. Namely, y -axis diffusion is not countered by a focusing electrophoretic force. The y -axis band broadening can thus be described as: $\sigma_{y,\text{total}}^2 = \sigma_{y,D}^2 + \sigma_{y,\text{Inj}}^2 + \sigma_{y,\text{Other}}^2$ with broadening sources including diffusion in the y -axis ($\sigma_{y,D}^2$), the process of injecting sample into the chamber ($\sigma_{y,\text{Inj}}^2$), and any other sources ($\sigma_{y,\text{Other}}^2$).

First, we sought to measure the y -axis band broadening during confined IEF. After reaching its pI , $wGFP$ was observed to broaden in the y -direction (Figure 3b) according to

$\sigma_{y,D} \sim \sqrt{2Dt}$, where D is the diffusivity of $wGFP$ in 4%T PA (determined as $2.51 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$) [29], thus suggesting that y -axis broadening is free diffusion as hypothesized. This y -axis diffusional broadening depends on analyte diffusivity and duration of focusing in the chamber. On this latter point, given the short assay duration, the $wGFP$ isoforms were remained confined to a $<0.75 \text{ mm}$ lane, thus reserving a contiguous region of the microchamber for subsequent PAGE. We next sought to characterize the y -axis dispersion arising from sample injection, $\sigma_{y,\text{Inj}}$. Analysis of $wGFP$ injection into the chamber showed that protein injected from a $65 \mu\text{m}$ wide loading channel broadened to a $\sim 600 \mu\text{m}$ wide lane ($4\sigma_{y,\text{Inj}}$) upon entering the microchamber. Broadening from injection can be reduced by optimization of device design and voltage shaping protocols [17, 18]. Lastly, we introduce the metric $\sigma_{x,\text{effective}}/\sigma_{x,\text{center}}$ to quantify x -axis broadening arising from pH gradient non-uniformities along the IEF separation axis. With sample loading from the entire side channel array, we compared the width of the pI -marker bands averaged across the height of the microchamber ($\sigma_{x,\text{effective}}$) to the width of the pI -marker bands averaged along only the center of the microchamber ($\sigma_{x,\text{center}}$). This band-broadening factor $\sigma_{x,\text{effective}}/\sigma_{x,\text{center}}$ is minimized and equal to 1 for perfectly uniform conditions along the y -axis for each point on the IEF axis, as determined for IEF in a single channel. The $\sigma_{x,\text{effective}}/\sigma_{x,\text{center}}$ was 1.33 for the confined pH gradient (Figure 2b). Next, we measured $\sigma_{x,\text{effective}}/\sigma_{x,\text{center}}$ for $wGFP$ in the quasi-ID case in Figure 3. $\sigma_{x,\text{center}}$ was empirically determined at the intensity mean in the y -direction μ_y of the focused protein lane. $\sigma_{x,\text{effective}}$ was determined by averaging the intensity between $+2\sigma_{y,\text{Total}}$ and $-2\sigma_{y,\text{Total}}$ from μ_y . $\sigma_{x,\text{effective}}/\sigma_{x,\text{center}}$ was calculated to be 1.09, indicating that x -axis broadening due to pH gradient nonuniformities was minimized by 22.2% by IEF confinement to the quasi-ID lane.

IEF resolution of $wGFP$ isoforms suggests that this approach can resolve single charge differences and ΔpI is $<0.1 \text{ pH units}$ [29, 30]. The IEF enrichment was calculated as $\sim 38.5\times$ from geometric estimates. The bulk of protein material loaded onto the 11.24 mm long by 65

μm wide loading channel was focused to a $\sim 50 \mu\text{m}$ ($2\sigma_x$) by $\sim 380 \mu\text{m}$ ($2\sigma_y$) zone. In terms of peak capacity, the microchamber confined IEF yielded a value of ~ 52 , calculated using average $\sigma_{x,\text{effective}}$ for the *w*GFP isoforms and the 4 mm chamber width as L . The confined IEF performance metrics compare well to results obtained in single microchannels (Figure S4) given the decreased separation lengths used in order to provide compatibility with second dimension PAGE [21].

Transfer between dimensions: Microchamber integration of IEF with PAGE

Low dispersion electrophoretic transfer of proteins from IEF to a second dimension assay is particularly challenging given that the focused proteins are at their pI after IEF and, thus, have zero net charge. In capillaries, chemical mobilization has been reported to mobilize IEF focused proteins along the IEF separation axis. Application of an electric field follows disruption of the chemical boundary conditions, achieved by replacing catholyte with anolyte (or vice versa) or by introducing zwitterions or salts to the terminal wells [22, 23]. The introduction of an ionic species changes the local hydronium and hydroxide levels to satisfy electroneutrality. Consequently, the local pH is altered resulting in focused species that acquire charge and can, thus, electromigrate.

To integrate IEF with PAGE in a microchamber, we mobilized focused species by adapting chemical mobilization and electrophoretic control, now for mobilization in the direction perpendicular to focusing (Figure 4). To study the transfer of proteins from IEF into a second dimension, we first considered transfer to a uniform pore-size PAGE gel. To drive proteins into the PAGE region, we controlled the chemical and electrical boundary conditions in two terminal wells, located at the top and bottom of the microchamber (wells 3 and 7). Here we used a mobilization approach in which both wells contained catholyte, termed the “catholyte-catholyte” mobilization scheme.

After IEF completion, the electric field was switched to the y -direction by applying a potential difference of 400 V between two catholyte-filled wells. Protein transfer was rapid, taking <2 min to complete. Owing to rapid mobilization from IEF to PAGE, diffusional band broadening along the x -axis was minimal. Broadening was attributed to an initial upwards drift of the bands at transfer initiation. As may be relevant, we note that when used to mobilize proteins parallel to the IEF axis, the catholyte-catholyte mobilization scheme can lead to non-uniformities in transfer across the pH range as well as zone broadening [23]. We also anticipated possible non-uniformities in transfer across the pH range when using the catholyte-catholyte scheme to mobilize proteins perpendicular to IEF. Analysis of the catholyte-catholyte mobilization scheme resulted in band broadening in the x -axis (quantified through the ratio $\sigma_{\text{transfer}}/\sigma_{\text{IEF}}$) of <1.3 . Drift in the relative position of the bands during transfer was $\sim 1.5\%$ of the total separation axis length ($60 \mu\text{m}$). Traditional slab gel 2DE typically results in 1st dimension broadening factors of 1.4 due to protein diffusion that occurs during the ~ 2 hour buffer exchange step [31]. Comparison to microchannel array approaches showed that our microchamber assay yielded a ~ 7.5 -fold reduction ($1.3\times$ vs. $10\times$) in losses during transfer by avoiding undersampling [4, 5, 6]. Thus, the 2DE microchamber system offers improved preservation of IEF separation information during transfer to PAGE.

The catholyte-catholyte transfer scheme led to a time-dependent increase in system conductivity during transfer. We observed the mobilization current increasing from 5 to 20 WA during the transfer process. These high currents placed an upper limit on the applied voltage, as the PA gels have been observed to break down at $\sim 300 \text{ V cm}^{-1}$ [20]. Lower conductivity buffers such as Tris-glycine were tested, but the catholyte-catholyte transfer scheme resulted in a more uniform transfer (data not shown). Optimization of the transfer conditions to minimize distortion and improve speed are currently under study.

Use of native PAGE as a second dimension allowed assessment of protein drift and dispersion as species were mobilized off the IEF axis and into the uniform pore-size PA gel. As species migrated into the PAGE region, the proteins separated based on different relative electrophoretic mobilities. Ovalbumin and transferrin achieved a constant velocity of 32 and $9 \mu\text{m s}^{-1}$, respectively, 20 s after transfer. We interpreted the constant mobility as indication of a switch in mechanism from transfer to PAGE. Despite the rapid transfer, the two ladder species studied, ovalbumin (45 kDa) and transferrin (80 kDa), were not resolved by PAGE under uniform pore size PA gel conditions. The inability of PAGE to resolve these two species was not unexpected due to the absence of a stacking gel, discontinuous buffers, and surfactants.

PAGE: PLE as the second assay dimension

PLE is attractive as a potentially robust second dimension separation that reports protein size without the need for detergents or reducing agents [32]. PLE is conducted in gradient PA gels, where protein mobility decreases until a pore-limit is reached and the species become pseudo-immobilized [33–35]. PLE affords the desirable opportunity for detergent-free sizing, thus potentially reducing transfer- and handling-associated dispersion, yet providing the gold standard second dimension assay output: protein M_r .

To integrate PLE subsequent to IEF, we first developed a fabrication process to realize PLE gels in a defined region of the microchamber. Previously, microfluidic PLE gradient gels have been formed in single channels using molecular diffusion of acrylamide monomer between reservoirs at two different concentrations [20, 35–37]. In this way, a linear concentration profile of acrylamide monomer is established at equilibrium and photopolymerized in situ. In contrast, here the microchamber reservoirs are located at terminal wells of the flanking microchannel arrays. Thus, if diffusion between the reservoirs was used to establish the acrylamide monomer concentration distribution, just a fraction of the gradient will be located within the microchamber. Similar to previous discussion regarding confining the pH gradient to the chamber, constraining just a portion of the PLE gradient to the microchamber would severely limit the M_r range of the assay. To constrain the PLE gel to the microchamber, we developed a fabrication protocol using photopatterning of a scaffolding gel structure to mitigate hydrodynamic flow during acrylamide monomer diffusion intervals (Figure 5). After formation of a linear acrylamide monomer concentration gradient in the scaffold gel (Figure S2), the region was exposed to UV thus forming a cross-linked PLE gel within the scaffold gel.

By measuring the position of each species at the pore-limit, M_r can be assessed assuming that migration distance is proportional to $\log(M_r)$ [13, 33, 34, 38–41]. We characterized surfactant-free sizing through analysis of a fluorescently labeled wide range (12–150 kDa) protein ladder in a PLE gradient gel formed using Tris-glycine buffer (Figure 6a) in the microchamber. Protein size was available in <1 min in <1.2 mm separation distances. Proteins were enriched by a factor of 4-fold, from a $400 \mu\text{m}$ wide starting zone into $100 \mu\text{m}$ wide bands. The PLE bands were uniform in the x -direction, with an effective band broadening factor $\sigma_{y,\text{effective}}/\sigma_{y,\text{center}}$ (in the PLE direction) equal to 1.21.

Microchamber 2DE: Performance of IEF-PLE for targeted proteomics

We completed a 2DE assay with IEF followed by catholyte-catholyte mobilization of pI resolved species to the PLE dimension (Figure 6b). The central question regarding PLE as the second dimension centered on the capability to resolve peaks not resolvable by native PAGE. Here, ovalbumin and transferrin were indeed resolved with R_s of 1.14 within 15 min. Transfer and PLE separation times were longer than the 2 min times observed with a uniform PA gel (Figure 4), as would be expected (Figure S5). Band broadening during

transfer was quantified using the $\sigma_{\text{transfer}}/\sigma_{\text{IEF}}$ metric, which yielded a value of 2.8 as compared to the 1.3 in uniform native PAGE gels. The higher value of $\sigma_{\text{transfer}}/\sigma_{\text{IEF}}$ is consistent with the longer transfer and, hence, longer diffusion times. Additionally, x -axis drifts of $\sim 10\%$ of pH axis length added to x -axis broadening as proteins accumulated at their pore-limit [42]. Nevertheless, the overall transfer-associated band broadening remains substantially less than the $10\times$ values observed in microchannel network 2DE architectures. The microchamber-integrated 2DE assay supported an estimated maximum peak capacity n_{2D} of between 256 and 35 with an effective n_{2D}' including the transfer associated losses of between 90 and 12. The range of peak capacity values was calculated using the range of protein standard band widths resolved in our system in each dimension. The 3 mm chamber width and the 2.2 mm portion of the chamber containing the gradient gel were used 1st and 2nd dimension axis lengths L , respectively.

CONCLUSIONS

Targeted proteomic assays typically seek to identify a handful of predetermined proteins across large numbers of samples. While substantial effort has been focused on introducing targeted mass spectrometry based protein assays, we see the performance of this microchamber 2DE assay as a feasible format for fast, targeted protein measurement at the bench. In particular, 2DE profiling of targeted proteins from single cells, whole microscopic organisms, and biopsies where protein mass is limited and material losses cannot be tolerated. Achieving peak capacities of 10,000 is possible in high resolution slab gels. These values might not be realizable in our devices given that n is fundamentally limited by separation axis length, but by utilizing larger microchambers, resolving power and peak capacity in both dimensions can be improved while still retaining total assay times to ~ 1 hour (several hours to days for slab gels) [4, 5, 31]. As implemented here, our platform also has the additional benefits of low reagent consumption ($<1 \mu\text{l}$ of sample) and capability for automation and parallel operation. In addition, PLE as a second dimension simplifies the integrated assay workflow while providing protein size. The IEF-PLE assay is also suitable for unlabeled samples, as at PLE completion proteins are pseudo-immobilized in the PLE gel at their pore limit locations, allowing introduction of staining reagents. Such post-2DE staining is not possible in reported 2DE microsystems. Further, as IEF and PLE are both enriching processes, potential arises for low concentration sample detection [35, 43]. Future work will investigate refinement of the transfer approach including study of different mobilization buffers containing zwitterions which should lead to lower conductivity and therefore faster protein transfer. Additionally, zwitterion containing mobilization buffers have been demonstrated to lead to uniform mobilization of proteins across broad pH ranges [28]. We see the present contributions and future advances as forming a new design paradigm for automated multi-dimension assays with relevance to bench-top targeted proteomic analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Jung K, Reiche J, Boehme A, Stephan C, Loening SA, Schnorr D, Hoesel P, Sinha W. Clin. Chem. 2004; 50(12):2292–2301. [PubMed: 15472031]
2. O'Farrell PH. J. Biol. Chem. 1975; 250(10):4007–4021. [PubMed: 236308]
3. Giddings JC. Anal. Chem. 1984; 56(12):1258A–1270A.
4. Herr AE, Molho JJ, Drouvalakis KA, Mikkelsen JC, Utz PJ, Santiago JG, T. Kenny W. Anal. Chem. 2003; 75(5):1180–1187. [PubMed: 12641239]
5. Yang S, Liu J, Lee CS, Devoe DL. Lab Chip. 2009; 9(4):592–599. [PubMed: 19190795]
6. Liu J, Chen CF, Yang S, Chang CC, Devoe DL. Lab Chip. 2010; 10(16):2122–2129. [PubMed: 20556309]
7. Das C, Zhang J, Denslow ND, Fan ZH. Lab Chip. 2007; 7(12):1806–1812. [PubMed: 18030404]
8. Seeley JV. J. Chromatogr. A. 2002; 962(1-2):21–27. [PubMed: 12198965]
9. Davis JM, Stoll DR, Carr PW. Anal. Chem. 2002; 80(2):461–473. [PubMed: 18076145]
10. Li X, Stoll DR, Carr PW. Anal. Chem. 2009; 81(2):845–850. [PubMed: 19053226]
11. Kenrick KG, Margolis J. Anal. Biochem. 1970; 33(1):204–207. [PubMed: 5413240]
12. Ruchel R. J. Chromatogr. 1977; 132(3):451–468. [PubMed: 885944]
13. He M, Herr AE. Anal. Chem. 2009; 81(19):8177–8184. [PubMed: 19731927]
14. He M, Herr AE. J. Am. Chem. Soc. 2010; 132(8):2512–2513. [PubMed: 20131779]
15. Tia SQ, He M, Kim D, Herr AE. Anal. Chem. 2011; 83(9):3581–3588. [PubMed: 21456518]
16. Kim D, Karns K, Tia S, He M, Herr AE. Anal. Chem. 2010; 84(5):2533–2540. [PubMed: 22304398]
17. Lerch MA, Jacobson SC. Anal. Chem. 2007; 79(19):7485–7491. [PubMed: 17718538]
18. Lerch MA, Hoffman MD, Jacobson SC. Lab Chip. 2008; 8(2):316–322. [PubMed: 18231672]
19. He M, Herr AE. Nat. Protoc. 2010; 5:1844–1856. [PubMed: 21030959]
20. Hou C, Herr AE. Anal. Chem. 2010; 82(8):3343–3351. [PubMed: 20334346]
21. Hughes AJ, Lin RK, Peehl DM, Herr AE. Proc. Natl. Acad. Sci. U. S. A. 2012; 109(16):5972–5977. [PubMed: 22474344]
22. Rodriguez-Diaz R, Zhu M, Wehr T. J. Chromatogr. A. 1997; 772:145–160.
23. Zhu MD, Rodriguez R, Wehr T. J. Chromatogr. 1991; 559(1-2):479–488.
24. Righetti PG, Gelfi C. J. Biochem. Biophys. Methods. 1984; 9(2):103–119. [PubMed: 6736557]
25. Sommer GJ, Singh AK, Hatch AV. Anal. Chem. 2008; 80(9):3327–3333. [PubMed: 18341355]
26. Wen JA, Albrecht JW, Jensen KF. Electrophoresis. 2010; 31(10):1606–1614. [PubMed: 20419703]
27. Wen J, Wilker EW, Yaffe MB, Jensen KF. Anal. Chem. 2010; 82(4):1253–1260. [PubMed: 20092256]
28. Rilbe H. Ann. N. Y. Acad. Sci. 1973; 209:11–22. [PubMed: 4577168]
29. Hughes AJ, Tentori AM, Herr AE. J. Am. Chem. Soc. 2012; 134(42):17582–17591. [PubMed: 23017083]
30. Tsien RY. Annu. Rev. Biochem. 1998; 67:509–544. [PubMed: 9759496]
31. O'Farrell PH. J. Biol. Chem. 1975; 250(10):4007–4021. [PubMed: 236308]
32. Margolis J, Kenrick KG. Nature. 1969; 221(5185):1056–1057. [PubMed: 5774398]
33. Retamal C, Babul J. Anal. Biochem. 1988; 175(2):544–547. [PubMed: 3239780]
34. Margolis J, Kenrick KG. Nature. 1967; 214(5095):1334–1336. [PubMed: 6056848]
35. Sommer GJ, Singh AK, Hatch AV. Lab Chip. 2009; 9(18):2729–2737. [PubMed: 19704990]
36. Lo CT, Throckmorton DJ, Singh AK, Herr AE. Lab Chip. 2008; 8(8):1273–1279. [PubMed: 18651068]
37. Hughes AJ, Herr AE. Anal. Chem. 2010; 82(9):3803–3811. [PubMed: 20353191]
38. Andersson L, Borg H, Mikaelsson M. FEBS Lett. 1972; 20(2):199–202. [PubMed: 11946415]

39. Johnson G. *Biochem. Genet.* 1979; 17(5-6):499–516. [PubMed: 117795]
40. Ferguson KA. *Metabolism.* 1964; 13:985–1002. [PubMed: 14228777]
41. Rodbard D, Chrambach A. *Proc. Natl. Acad. Sci. U. S. A.* 1970; 65(4):970–977. [PubMed: 4191703]
42. Margolis J, Wrigley CW. *J. Chromatogr. A.* 1975; 106(1):204–209.
43. Graner M, Raymond A, Akporiaye E, Katsanis E. *Cancer Immunol. Immunother.* 2000; 49(9):476–484. [PubMed: 11092614]

Figure 1.

Gel photopatterning enables microchamber 2DE. (a) Etched glass microdevice features a 3×3 mm² microchamber flanked by channels for sample loading and electric field control. (b) The device houses contiguous PA gel regions with distinct chemicophysical properties to define the IEF and PAGE separation axes. (c) After sample loading to the microchamber, IEF is conducted across a line in the top portion of the chamber. After IEF, chemical mobilization is applied in the direction perpendicular to IEF to disrupt the pH gradient and transfer focused protein zones off the IEF separation axis and into the PAGE region.

Figure 2.

pH gradient confinement in $3 \times 3 \text{ mm}^2$ microchamber. Inverted micrograph scan montages of fluorescent pI markers during IEF (a) Catholyte and anolyte buffers are used at the terminal wells to form the pH gradient using carrier ampholytes. (b) Immobilines are incorporated into the gel flanking the microchamber to constrain the pH gradient created by the (non-immobilized) carrier ampholytes within the chamber.

Figure 3.

IEF within a quasi-1D lane in microchamber. (a) Inverted fluorescence micrograph of *wtGFP* focusing in the 4x4 mm² microchamber showcases proteins focusing in 1D lane. Time = 0 s denoted at 505 s after the loading step when the characteristic 3 isoform pattern of *wtGFP* becomes visible. Intensity profiles along the vertical axis are plotted for increasing times. Broadening of bands in the direction perpendicular to focusing occurs due to diffusion. (b) 10x magnification image and profile plot of GFP isoforms.

Figure 4.

Microchamber integration enables transfer for subsequent native PAGE. (a) Inverted fluorescence micrographs of CE540 labeled proteins focusing in 1D lane in 4×4 mm² microchamber and subsequent transfer. Upon switching electric field direction (time = 0 s), the proteins migrate downward through the sieving matrix. (b) Offset intensity profile plots of transferrin and ovalbumin transfer to PAGE. (c) Band positions in both the *x* and the *y* directions as a function of time. Bars denote band widths σ_x and σ_y .

Figure 5.

A multi-step PA photopolymerization protocol allows definition of spatially heterogeneous gels within the microchamber for microchamber 2DE. Steps 1-2: PA gels containing immobilines imparting a basic and acidic pH were photopolymerized at the edges of the microchamber. Step 3: a low density gel was polymerized at the desired location of the PLE gel. Step 4: low and high concentration acrylamide precursor solutions were continuously flowed adjacent to the scaffold gel. Step 5-6: The scaffold gel prevented the flowing acrylamide precursor solutions from generating hydrodynamic flow in the PLE region, but allowed formation of a linear acrylamide monomer concentration gradient in the scaffold gel by diffusion, which is then photopolymerized. Step 7-8: unpolymerized solutions are replaced with 4%T acrylamide precursor solutions and photopolymerized.

Figure 6.

Gradient gel integration improves size-based separation performance. (A) Inverted fluorescence micrographs of fluorescently-labeled ladder loaded and separated in gradient gel (4%T 3.3%C to 40%T 12%C) containing Tris-glycine buffer in $1.5 \times 1.9 \text{ mm}^2$ microchamber. The linear relationship between $\log(M_r)$ and migration distance confirms ability to measure M_r in gradient gels in 2D geometries. (B) Inverted fluorescence micrographs of CE540 labeled transferrin and ovalbumin focusing in confined 1D lane in $3 \times 3 \text{ mm}^2$ microchamber and subsequent transfer onto gradient gel (4%T 3.3%C to 40%T 12%C).