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Protein and Proteome Measurements with Microfluidic Chips

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Major scientific discoveries in the last two decades have been enabled by the development of high-throughput sequencing and mass spectrometry (MS) technologies. Omics analysis, at the genome, transcriptome, proteome, or metabolome levels, has paved the way for the elucidation of many molecular mechanisms that underlie disease, biomarker and drug-target discovery, and diagnostics and precision medicine applications. With the realization that the biological interpretation of results generated from biological tissues or cell-derived samples is highly impacted by cell heterogeneity, many efforts have been redirected toward the omics characterization of single cells. As miniaturized devices represent ideal platforms for the analysis of small sample amounts, omics analysis has found a flourishing ground in the microfluidics instrumentation development arena. This Review aims at capturing the latest protein and proteome analysis technologies that have been developed and implemented on microfluidic platforms, with a focus on developments that have been documented in the past two years. Emphasis was placed on challenging aspects that relate to sensitivity and handling sample complexity, as well as on applications that address demanding biological and biomedical problems.

MICROFLUIDIC CHIPS AND MASS SPECTROMETRY DETECTION

The vast applications of proteomic experiments involve the use of mass spectrometry detection. The ability of MS to accurately identify protein sequences and their isoforms, mutated counterparts, and posttranslational modifications (PTMs) rendered MS a superior detection choice for the miniature platforms that facilitate fast sample processing by capitalizing on integration, multiplexing, and high-throughput capabilities. Recent advances in MS instrumentation, in terms of both hardware and software, have led to remarkable accomplishments. For example, improvements in ion source design, transfer ion optics, and resolution at high data acquisition speeds (40 Hz for MS/MS) enabled the identification of

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10 000–15 000 peptides (~800 peptides per min) in only 15 min of nanoliquid chromatography (LC)-MS/MS gradient time from only ~100 ng of sample.¹ These results represent roughly an order of magnitude improvement in speed of analysis, detection limits, and sensitivity when compared to what was achievable only a few years ago. To interface microfluidic platforms to mass spectrometers and capitalize on the unique benefits of the two technologies, the design of the microchip device must support efficient sample ionization and delivery to the MS ion source. For proteomic samples, two main ionization principles have been developed, electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI), the first being commonly used for interfacing sample separation platforms to MS instrumentation (e.g., HPLC or capillary electrophoresis-CE^{2–5}), while the second for enabling, MS detection from samples prepared in a microarray format.⁶ Microchip devices that enable fluidic manipulations integrate additional functional elements for fluid delivery, sample cleanup and preconcentration, derivatization, and separation.^{7,8}

The development of microfluidic chips with MS detection has been captured over the years in numerous review articles that describe in detail both functional components and operational characteristics.^{9–12} The overall performance of these devices was typically benchmarked against that of the best nano-LC-ESI/MS systems that rely on the use of nanospray fused silica capillary emitters for enabling electrospray ionization of the sample. The continued efforts for developing novel chip-MS interfaces or alternative approaches of detection were motivated by the limitations of the existing platforms. For example, for ESI-MS, a variety of micro-fabricated nebulizers, monolith frits, nano-ESI capillary emitters, and liquid sheath or liquid junction interfaces have been developed.^{9,11} However, shortcomings related to fabricating sharp nozzles or emitters, to integrating the ESI electrodes, or to generating sufficient flow of ESI-compatible buffer systems continue to exist. The ability to bring ions from the liquid to the gas phase as effectively as with a nanospray emitter (5 μm i.d., <20 μm o.d.) is difficult to achieve. In practice, the use of chip-inserted capillary emitters is troublesome; machined flat or cut-glass surfaces do not generate strong-enough electrical fields for efficient ESI, while spray nozzles that are typically fabricated from silicon through deep reactive ion etching suffer from electrical breakdown at high voltages. In the case of polymeric microchip platforms, widespread sample losses due to protein adsorption and contamination from polymer bleeding create additional concerns.¹³ For MALDI-MS from a chip, the MS interface designs evolved from simple deposition on a target plate to progressively more complex and challenging rotating-ball interfaces and piezo-actuated flow-through dispensers, and ultimately to centrifugal CDs that encompass multiple functional elements.⁶ Performing liquid phase separations on these devices is, however, possible only after dispensing the sample on a target, with the caveat that separation efficiency may be lost. Overall, the development of ESI vs MALDI chip-MS ion sources has dominated the field, mainly due to the power of tandem MS in elucidating the structure of unknown compounds, a capability that works at its best with ESI-MS. Work on improving the chip-MS interfaces and the sample ionization process has continued over the years, resulting in novel strategies that use surface acoustic wave nebulizers,¹⁴ electro-sonic flow focusing ionization,¹⁵ voltage-assisted liquid desorption,¹⁶ or multichannel configurations for simultaneous electrospraying and sample extraction.¹⁷ Most efforts in the past two years, however, focused on expanding the applicability of the microchip technology

to solving various analytical or biological problems. In addition, alternative detection techniques that enable protein identification and quantitation have been developed. Relevant examples are described below.

In terms of technological developments, the miniaturization of not only benchtop sample analysis systems but also complex mass spectrometers, as well as the interfacing of the two, have been pursued.¹⁸ A demonstration was performed with a microchip fabricated from glass that incorporated a CE separation channel, an electroosmotic flow (EOF) channel/pump for directing the CE eluent to the chip corner to electrospray the sample, and a high pressure (HP)MS miniature cylindrical ion trap. The optimization of the system focused mainly on the inlet and operational parameters of the trap, but ultimately, the setup enabled the detection of peptides from 5 μ M samples (~ 7 fmol) at CE separation efficiencies of 445 000 theoretical plates.¹⁹ Comparisons with a commercial mass spectrometer showed roughly six times better S/N ratios with the benchtop MS, but the miniaturized chip-trap system has the potential to advance targeted detection with much smaller and cheaper platforms than conventional MS can offer for a number of screening and field applications.

In an interesting development, to facilitate MALDI-MS detection from microfluidic chips, a commercial ring-shaped piezoelectric acoustic atomizer was integrated into a PDMS channel device to support spray generation from the channel, followed by sample deposition on a MALDI-MS target plate (Figure 1).²⁰ In contrast to typical sample volumes deposited on MALDI targets (i.e., ~ 1 μ L), the piezo-ring pulsatile spraying system enabled the detection of model peptides from only 40 nL samples of $\sim 10^{-6}$ M concentration with ~ 20 -fold improved MALDI-MS detection limits. The benefits of starting with small sample volumes were also demonstrated by an automated droplet microfluidic system capable of generating nanoliter-size aqueous droplets in oil that were used to deposit peptides and protein digests on stainless steel target plates for MALDI-MS. The droplet deposition approach resulted in 30-fold enhanced MS signal and the ability to detect peptides from subnanomolar concentrations at detection limits in the low attomole range. The improved results were attributed to a focusing effect of peptides on the target plate arising from the presence of oil and the slow delivery of the analyte to the plate.²¹

Further, to benefit the investigation of continuous flow reactions in microfluidic chips, Schulze et al. have implemented a laser-induced liquid beam ionization/desorption chip-MS interface (LILBID-MS).²² The technique relies on generating high-velocity liquid microjets from nozzles on the chip and irradiating the jets with an IR laser to induce sample desorption and ionization. The authors have investigated only fast organic chemical reactions with the device; however, the ionization technique has been previously shown to be suitable for studying noncovalent interactions of macromolecules and appears to be promising for analyzing protein complexes.²³ This is particularly relevant to biological research due to the important role that protein assemblies play in driving the biochemical processes in a cell, and with the advance of ultrahigh resolution mass spectrometers, the efforts invested in analyzing intact protein complexes have grown correspondingly.

Albeit not demonstrated yet for the analysis of proteins, alternative arrangements have been demonstrated for advancing ESI from droplets as well. A microfluidic device that enabled

high-throughput handling of droplets of 65 pL to 1.2 nL, at 10 droplets/s, with a linear concentration-based response, sample carryover of <3%, and stable ESI operation for 2.5 h was devised. The device consisted of a PDMS chip that accommodated an inserted ESI capillary to sample the droplets generated in the microchip channels by mixing sample streams with a fluorosurfactant carrier phase. The authors envisioned the applicability of the device to monitor biological fluids such as cell secretions.²⁴

ANALYSIS OF PROTEINS AND PROTEOMES

Methodologies for analyzing various levels of protein sample complexity, from single protein assays to complex proteome profiling, have been attempted with either home-designed or commercial microarray or microfluidic platforms, with the aim of reducing analysis times or improving separation efficiencies, detection limits, sensitivity, reaction kinetics, and throughput. Targeted or exploratory applications, ranging from protein structure characterization to proteomic profiling of various biological samples or processes, have been demonstrated over the years. While mass spectrometry has evolved into the de facto detection technology for proteome analysis, due to the very small amounts of starting material that can be handled by microfluidic chips, large-scale proteome profiling with comparable results to conventional instrumentation has been difficult to accomplish. Nevertheless, notable accomplishments have been reported by using either alternative detection technologies such as fluorescence microscopy visualization of targeted antibody-antigen interactions or technologies that enhanced the MS capabilities for detecting low abundance proteins. With the advent of high mass accuracy and resolution instruments, the trends in proteomics research have shifted toward the characterization of PTMs, protein structure, and protein complexes, as well as toward addressing biological problems of various levels of complexity. This was immediately followed by pursuing similar goals with the miniaturized platforms.

Posttranslational Modifications.

Protein glycosylation is a posttranslational modification that alters protein folding, structure, stability and function, aberrant glycosylation patterns being associated with a number of devastating diseases, including cancer. The main challenges associated with characterizing complex glycoforms revolve around identifying the glycosylation site, deciphering glycan structure, and characterizing glycoform microheterogeneity. To address some of these issues, a ZipChip (908 Devices) microfluidic device that capitalized on the power of capillary electrophoresis separations was used for the analysis of released glycans, glycopeptides, and monosaccharides.²⁵ ESI was generated from the chip corner via an integrated sprayer that enabled easy interfacing to the mass spectrometer. TMT-labeling of released glycans/monosaccharides and sialic acid derivatization were used for reducing the analyte adsorption on the microchip channel surfaces and peak broadening, and for improving the electrophoretic migration of analytes. The CE mechanism of separation enabled improved separation performance of glycoforms in comparison to chromatography-based methods. The CE method was demonstrated for the separation of neutral pauci-/high-mannose and complex asialo N-glycans, of sialylated glycans, and of glycopeptides released from human transferrin and AGP within a time window of 10–60 min. The method represents an

attractive approach that can be integrated with off-line chromatographic fractionation prior to MS detection.

To capitalize on lectin-glycan binding affinity and specificity, an integrated microfluidic lectin barcode system was developed for optimizing the binding kinetics, the speed of analysis on lectin microarrays, and the differential profiling of tissue-specific glycosylation changes in targeted biomarkers.²⁶ The microchip consisted of a lectin array-patterned glass substrate and two PDMS layers, one for handling sample fluidics and one for pneumatic control. For increasing throughput, the prototype platform incorporated 8 units, each comprising an assay chamber and a three-valve pump. The assay chambers were aligned with the lectin array patterned glass (a barcode of 16 lectins per chamber). The samples were pumped through the assay chambers, and captured glycan isoforms were visualized and quantified in a sandwich assay by using biotinylated primary antibodies and fluorescent DyLight 488-conjugated streptavidin. The system was demonstrated for the analysis of standard proteins, glycoproteins, and cancer biomarkers. Tissue-dependent glycosylation changes of the human CA125 cancer biomarker (7500 U/mL) from three ovarian cancer cell lines, adenocarcinoma tissue, and ascetic fluids could be characterized with the aid of 16 lectins in 4 channels (3 sample channels and 1 control). Overall, this simple microfluidic sandwich antibody-lectin assay proved to perform as well or better than the commercial CA125 kits, with sensitive detection and low sample consumption (20 μ L), demonstrating the suitability for rapid screening of glycomic fingerprints specific to disease.

Protein phosphorylation is a PTM with essential roles in cellular signal transduction, and aberrant phosphorylation patterns are involved in a variety of pathogeneses. To support the advance of technologies that enable large-scale discovery, Tyr autophosphorylation of cell membrane receptor tyrosine kinases (RTKs) in a high-throughput format was monitored with an integrated microfluidic-protein array platform (Figure 2).²⁷ The method was coined Integrated Microfluidics for Autophosphorylation Discovery (IMAD) and enabled the measurement of Tyr signals from 882 proteins on the chip. Sensitivity and specificity were estimated to be 70% and 80%, respectively. The platform consisted of a microarray spotted with a His/Myc-double tagged cDNA library and a two-layer PDMS device for flow and control. The design also incorporated channels and pneumatic valves for capturing the cDNA and proteins in individual chambers, and enabled the parallel expression of thousands of proteins for biochemical assaying. Target proteins that were expressed in the DNA chambers, after incubation in a reticulocyte mammalian cell lysate, diffused to and were immobilized in the protein chambers with their His tag. It was hypothesized that in vitro transcription and translation products that had intrinsic phosphorylation activity will be subjected to autophosphorylation during the protein expression process in the presence of the reticulocyte lysate, and that the levels of p-Tyr phosphorylation will be quantifiable by immunofluorescence. Cy3-coupled anti-Myc antibodies were used for quantifying the captured proteins, and Cy5-coupled anti-p-Tyr antibodies for quantifying p-Tyr phosphorylation. In terms of reagent consumption, 10 μ L reagent volumes were sufficient for covering the chambers and completing a step of the process. In addition to throughput, one major advantage of the method is that it circumvented the limitations associated with the MS quantification of phosphorylation when the abundance of protein is low, the caveat being

that the method is limited only to the study of Tyr phosphorylation due to the poor specificity of anti-p-Ser/Thr antibodies.

In a simpler approach that involved MS analysis of protein phosphorylation, a glass microfluidic device that incorporated two microreactors packed with C18 and TiO₂ particles and an inserted nano-ESI emitter for MS detection was developed for the targeted analysis of phosphopeptides from ~10 μ L whole cell digests (1 μ g/ μ L).²⁸ The chip enabled phosphopeptide enrichment on TiO₂ and sample cleanup on C18 particles, as well as targeted multiple reaction monitoring (MRM)-MS of phosphopeptides involved in ERBB2/MAPK signaling in SKBR3/HER2+ breast cancer cells. The ability to quantify peptides representative of key MAPK signaling components in the low 1–10 nM range was demonstrated. No prior fractionation of the cell extracts or bioaffinity enrichment was necessary, and the microfluidic process could be completed within much shorter time frames (1–2 h) than with benchtop instrumentation (~8–10 h). As the quantified peptides were involved in early biological signaling pathways, the platform presents the potential for exploring the biological stimulatory or inhibitory role that agents such as drugs or endogenous growth factors have on cell proliferation or arrest.

Protein Structure and Protein-Protein Interactions.

The analysis of protein structure and protein-ligand interactions is often pursued by hydrogen/deuterium exchange (HDX)-MS. HDX relies on measuring the rate of exchange of hydrogens from the protein backbone amide groups with deuterium from solution to provide information about the structure and conformation of proteins. The H/D exchange rate reflects the solvent accessibility to the protein and, therefore, can be used for inferring structure. However, transient interactions and conformations that develop within a protein or complex pose a challenge to measuring fast exchanging amide protons. To address this problem, a microfluidic chip that enabled rapid on-chip labeling of proteins and reaction quenching was developed (Figure 3).²⁹

The fastHDX chip prepared from thiol-ene had a simple design that consisted of a labeling channel in which two monolith plug junctions facilitated first the mixing of the protein/deuterium labeling buffers and then the protein/quenching buffers. The chip was connected to an LC pump and two valves. The spatially confined monoliths supported efficient mixing at the channel junctions, enabling highly reproducible HDX within 0.14–1.1 s. HDX reactions performed for 1.1 s confirmed the crystal structure of the hemoglobin tetramer, and when the flow rates were controlled, protein labeling times from milliseconds to seconds could be achieved in a reproducible manner. The robustness and ease of use of the HDX chip make the platform suitable for fast analysis of weak ligand binding interactions and transient protein structures.

On a higher level of cellular control, protein-protein interactions (PPI) and their networks regulate cellular function, and their dynamics and altered state play critical roles in the development of disease. Affinity enrichment, epitope tagging, or proximity labeling strategies have been used for studying such interactions (Figure 4).³⁰ To support the identification of PPIs from low-input sample amounts, an on-chip affinity purification mass spectrometry (AP-MS) method has been developed for purifying tagged baits and studying

the interaction partners of native cellular proteins.³⁰ The analysis was completed from only 4 μg of HeLa cell lysates ($\sim 12 \times 10^3$ cells), representing a 50–100-fold downscaling of microcentrifuge-based protocols, conditions under which the detection of interaction partners from such small sample amounts is lost. The microfluidic device consisted of a Fluidigm PDMS prototype that was composed of 24–48 reactors for loading frit- and anti-GFP nanobody beads. The chip enabled sample and reagent loading, distribution, and valving for completing the on-bead pull downs, as well as pressure control and thermocycling. After the pull down, the protein complexes were digested on the bead with trypsin and eluted for LC-MS/MS analysis and label-free IBAQ (intensity-based absolute quantitation). Human Cohesin, Mediator, and CCC protein complexes containing over 10–12 components involved in cell division, transcription, and copper trafficking were purified and quantified with this platform. Limitations in terms of detecting low abundance substoichiometric and dynamic interactions between the complex subunits were encountered, however.

Targeted Protein Analysis.

Sadlowski et al. developed a digital proteomics technique, Click-A+Chip technology, for the rapid profiling of azido-nor-leucine (ANL) labeled proteins, to minimize analysis time, amount of starting material, and false positive/negative detection as previously observed with either mass spectrometry or antibody array detection schemes for such samples.³¹ The chip was used for the analysis of systemic proteins that have age-specific effects on tissue health and repair, in an experiment that involved the surgical joining of two mice, one old and one young (parabionts). The identification of target systemic proteins was enabled by the prior development of a selective proteome labeling technique, coined BONCAT (bio-orthogonal noncanonical amino acid tagging), that relies on the metabolic labeling of proteins during de novo synthesis by incorporating a Met substitute-ANL in cells that express a mutant methionyl-tRNA synthetase (MetRS). Labeled proteins produced by the MetRS transgenic mouse can then be identified by MS. In contrast to MS detection, however, the Click-A+Chip is a biosensor with a graphene channel that is functionalized with a synthetic molecule that selectively binds ANL-labeled proteins via “click chemistry”, the binding events resulting in the alteration of graphene conductivity and electrical parameters that can be detected. The identification of bound proteins is enabled by using primary antibodies. The technique enabled the detection of two ANL-labeled proteins with different levels in young vs old parabionts, Lif- and leptin, that contributed to the rejuvenation of the old tissues. The click-able graphene surface detection strategy can be implemented in many biosensing applications, with the potential to replace more expensive optical and mass spectrometry-based assays. Applications for biomarker detection, diagnostics, and vaccine development were further envisioned.

Microfluidic Immunoassay Devices for Point-of-Care Diagnostics.

Microfluidic immunoassay devices (MFIDs) have already been used for chemical and biological research, but one of the areas in which they are expected to have a major contribution in the future is the field of diagnostic testing of protein biomarkers. The expectations for MFIDs used at point-of-care (POC) for diagnostic testing have been recently discussed by Barbosa and Reis.³² The paper examined the status of the development of the different MFID components that included capture-antibody immobilization and

surface-area-to-volume ratios, sample preparation, fluid handling modalities, and signal detection systems. The cost of manufacturing and affordability of MFIDs intended for POC applications have also been discussed. The authors concluded that MFIDs are promising tools for POC diagnostic testing but also indicated that further developments are needed for delivering affordable tools capable of providing reliable analytical results.

During the past couple of years, several papers have been published describing the novel aspects that could be potentially incorporated into MFIDs intended for POC diagnostics. For example, a 3D-printed microfluidic platform was used for testing protein biomarkers in multiple samples in an immunoassay format.^{33,34} The 3D-printed sample/reagent array was coupled to a pyrolytic graphite sheet that contained detection microwells in which single-wall carbon nanotube (SWCNT) forests with attached capture antibodies (Ab1) were deposited. The detection antibodies (Ab2) were coupled to RuBPY silica nanoparticles functionalized for electrochemiluminescence detection. The device was tested with human serum samples, and the detection limits for prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) in calf serum were 150 and 230 fg/mL, respectively.³³ A dynamic range of 250 fg/mL to 5 ng/mL and reproducibility with 8% RSD were demonstrated. The capability to detect femtogram levels of target proteins in the presence of thousands of other much more abundant counterparts, with concentrations in the mg/mL range, represents a very encouraging result. Simple and low cost fabrication as well as rapid, sensitive, and reliable detection make the technology suitable for implementation in the clinical setting.

Digital microfluidics (DMF) is a technology for transporting very small, nanoliter-sized liquid droplets that are loaded with reagents or biological materials such as cells, proteins, or DNA. The technology has gained traction due to the ability to accurately control the fluidic manipulation of droplets (merging, mixing, splitting, dispensing, or storing) and perform biochemical reactions more efficiently in an automated manner. In a recent development, a digital microfluidics immunoassay was implemented as a POC system for serological testing of children (9–59 months) and caregivers for measles and rubella IgG in at-risk populations in remote areas.³⁵ The POC system was compact, portable, and fielddeployable and enabled the analysis from a few microliters of blood at sensitivities and specificities above 80–90%. The device consisted of top and bottom plates that encased the droplets of sample and reagents. Measles and rubella IgGs were captured on paramagnetic particles coated with viral antigens, and after a series of nine sequential steps for moving the droplets and particles for sample processing, detection was accomplished via chemiluminescence, with the readout being proportional to the bound IgG. While DMF devices have been used in the past for performing immunoassays and sampling biological fluids, this work represents the first use outside the laboratory, demonstrating great potential for field testing of disease specific antibodies and future disease protein biomarkers.

DMF devices for performing label-free immunoassays were also demonstrated with alternative detection schemes such as on-chip acoustic wave detectors. Thin-film bulk acoustic wave sensors are small and have high sensitivity.^{36,37} In one such DMF device, the capture antibodies were immobilized on the surface of the device, exposed to serum sample droplets, and then washed with water droplets. The captured biomarker molecules generated

frequency shifts that were detected by the acoustic wave sensor. The digital microfluidic device with on-chip acoustic wave detector was compared to conventional ELISA using 0, 5, and 20 $\mu\text{g/mL}$ PSA solutions, and it was concluded that it could generate comparable results. Further improvements were planned for complete automation of the device.³⁷

In a broader context, Wang et al. explored and published the design of disposable MFIDs.³⁸ They carefully considered the geometry of the channels (spiral and serpentine designs), the material (glass and different polymeric materials), the micro-fabrication procedures of the chip (laser ablation and hot embossing), automation for reagent delivery (ten different reagents), immobilization of the capture antibodies (functionalized channels), and the detection approach (chemiluminescence in a conventional plate reader). Prototype chips were built from various polymeric materials and comprised spiral sample flowing channels of various dimensions and shape. For optimized designs, the detection limits for interleukin 6 (IL-6) and glial fibrillary acidic protein (GFAP) in human serum were 10 and 50 pg/mL , respectively. The device offered shorter analysis times (~ 1 h) than conventional ELISA, but further optimizations were envisioned to reduce the relatively high variability of the results due to variations in channel properties (dimension, roughness, sealing). Overall, significant advances have been made in the development of microfluidic devices that are capable of measuring disease biomarkers based on the affinity interactions that develop between capture antibodies and antigen proteins present in biological fluids. With improvements in affordable chip fabrication methods and stand-alone operation, antibody immobilization, reduction of biological matrix interferences, simplified sample preparation processes, and the implementation of novel and sensitive detection and read-out techniques, it is expected that the technology will advance to levels where it is suitable for detecting not just one but comprehensive panels of proteins indicative of disease.

SAMPLE PREPARATION

As the majority of proteomic experiments involve bottom-up analysis approaches (i.e., peptide-level detection of proteins), the continued interest in developing miniaturized enzymatic reactors and experimental conditions for fast proteolytic digestion led to the development of simple but effective protocols for handling the samples. For example, Kecskemeti et al. developed an immobilized enzymatic reactor (IMER) by physical adsorption of trypsin on the porous surface of a serpentine channel built within a PDMS/glass chip (Figure 5).³⁹ The IMER was successfully demonstrated for the digestion of human tear proteins within ~ 1 min, and CE and LC-MS/MS analyses of the digestion products confirmed that a similar number of protein IDs, but with somewhat higher sequence coverage, could be generated with the microreactor than with the in-solution protocols. The device could be multiplexed (8 serpentine channels), and the trypsin layer could be regenerated through subsequent immobilization. The authors anticipated that the device could find applicability for the processing of clinical specimens. Improvements in the overall sample analysis and MS detection protocols to enable the identification of a larger, more representative panel of tear proteins (>1500) is, however, necessary. Accelerated digestion times were also demonstrated for IMERs that contained trypsin immobilized on polymer monolithic materials, with reproducible proteolytic digestion within 5 min from dried blood spots and ability to deliver ~ 150 protein IDs per measurement.⁴⁰ Aside from

using proteolytic enzymes, an interesting electrochemical cleavage strategy for proteomics studies was also reported.⁴¹ The procedure relies on using a microfluidic electrochemical cell with integrated boron doped diamond working and counter electrodes that are used to cleave proteins at the C-terminal of Tyr and Trp and then at the disulfide bonds formed by Cys residues. It was anticipated that this instrumental alternative to enzymatic or chemical protein cleavage methods will provide better control, faster analysis, and reduced sample handling for proteomic studies. Nevertheless, a demonstration of the performance of the approach for cleaving proteins in complex cell extracts is further needed.

In terms of sample preparation, it is well-known that the composition of the sample matrix has a large impact on the ability to ionize the sample and generate quality MS data. To address the deleterious ion suppression effects induced by high concentrations of nonvolatile salts in biological samples, a silica isoporous membrane-SIM (90 nm thick, 2.3 nm pore size, 4×10^{12} pores/cm²) with a polyethylene terephthalate support was integrated into a PMMA chip (Figure 6).⁴² The desalting capability of the membrane was tested with proteins (5–10 μ M) dissolved in NaCl/KCl (~100–150 mM) solutions forced through the device at various flow rates. At 1 μ L/min, the desalting efficiency was as high as 99%, while maintaining protein losses at <5%. Much improved quality mass spectra, with substantially reduced Na⁺/K⁺ protein clusters and tailing peaks, especially after two passages through the device, could be obtained. At 5 μ L/min, the desalting efficiency continued to be above 90%. In comparison to the established methods such as solid phase extraction or gel filtration, the membrane approach showed comparable desalting efficiency but fared favorably in terms of costs and sample losses. The applicability of the device to the purification biological macromolecules from complex matrices such as blood or urine was anticipated.

MICROFLUIDIC CHIPS FOR THE ANALYSIS OF CELLS AND VESICLES

Extracellular Vesicles and Circulating Tumor Cells (CTCs).

Extracellular vesicles (EVs) are cell-derived particles that consist of a lipid bilayer with an enclosed cargo or nucleic acids and proteins.^{43,44} EVs can be found in body fluids, and the recent interest in their characterization emerged from the observation that their cargo is specific to the cells of origin and is delivered to target cells as a means of (immune) communication in various cellular processes. As carriers of cell-specific RNAs, surface proteins, or lipids, their potential as biomarkers has been recognized. According to the cellular origin, EVs can be classified into exosomes (30–100 nm), microvesicles (100–1000 nm), and apoptotic bodies (800–5000 nm).⁴³ The classical protocols that have been used for their isolation include ultracentrifugation, precipitation, filtration, and immunocapture on antibody-decorated magnetic beads.⁴⁴

The use of microfluidics for the isolation and purification of EVs evolved due to the ability to handle the EVs in their native environment, with two major methods dominating the field, i.e., static methods based on physico/chemical surface binding or filtration processes, and dynamic methods based on electrokinetic or hydrodynamic equilibrium effects.⁴⁴ There is, however, no standardized method for the isolation of exosomes that could provide consistent and reproducible results. A notable accomplishment in this field includes the microfluidic

isolation of exosomes from cell culture media and high-grade serous ovarian carcinoma (HGSOC) samples with mass spectrometry characterization of their protein content.⁴⁵

The device was created using a multilayer photolithography process, using PDMS to create molds of SU-8 patterns. Exosomes from cell-conditioned media and ovarian patient serum were captured on the PDMS device with herringbone pattern channels functionalized with antibodies for cell membrane-specific cancer markers (CD63-cluster of differentiation 63 and EpCAM-epithelial cell adhesion molecule). MS analysis of the isolated exosomes resulted in the identification of 355–870 proteins in the cell line derived exosomes. A very diversified proteome profile of HGSOC cancer cells (OVCAR-8) vs cells derived from the site of disease origin was observed (i.e., vs precursor epithelial cells that originated from normal fallopian tube secretory epithelial cells-FTSEC and ovarian surface epithelial cells-OSE). The upregulated pathways in cancer cells included integrin, PI3K/AKT, and HGF signaling, while the downregulated ones included HIPPO and PTEN, with HGF, STAT3, and IL6 emerging as candidate biomarkers that were identifiable in patient serum as well. Over 20 exosomal proteins were characterized as “high priority candidate protein biomarkers”. The findings are relevant because HGSOC has a poor prognosis due to the lack of specific, sensitive, and early detection biomarkers, and because the results of the analysis revealed the HGF/STAT3 axis as a potential therapeutic target. The microfluidic device was also compared to a commercial exosome isolation kit, and it was found that it delivered higher yield, specificity, and faster processing times at much reduced costs.

The analysis of circulating tumor cells (CTCs) has gained interest because it has been shown that their presence in blood is correlated to cancer metastatic processes with poor response to treatment and overall prognosis. CTCs differ from other cells in peripheral blood due to their physical characteristics (size, density, deformability, hardness, and dielectric/optical/acoustical properties), cell-surface antigen presentation, gene expression, and mutations.^{46–48} CTCs are present in low concentration in blood (i.e., 1–10 CTCs/mL) and are dwarfed by the presence of white ($\sim 10^6$ /mL) and red (10^9 /mL) blood cells.⁴⁷ Integration and enhanced reaction speeds have promoted again the use of microfluidic chips for CTC capture, enrichment, single-cell release, analysis, and heterogeneity studies. The CTCs are larger (15–25 μm) than the normal surrounding cells, and the development of devices that enabled their capture based on physical properties (trapping, hydrodynamic isolation, dielectrophoresis (DEP), or acoustic cell sorting) and immunoaffinity methods (immunomagnetic or immunocapture on microstructures), as well as controlled-release from the capture surface (chemical or field-triggered), was particularly pursued.⁴⁷ Recently, a microfluidic single-cell resolution Western blot device and method capable of capturing a panel of 12 proteins from ER+ and HER2+ circulating breast cancer cells was reported by Sinkala et al.⁴⁹ The microfluidic workflow included CTC enrichment from 2 to 4 mL of blood using a commercial size and deformability-selective microfluidic device (Vortex Biosciences), Hoechst/nuclear staining for visualizing the putative CTCs, single-cell CTC deposition into a microwell (50 μm diameter), and CTC lysis in the well, followed by PAGE separation (within seconds), covalent immobilization of proteins to the gel, and Western immunostaining of the panel of 12 proteins. The panel comprised proteins that enabled cancer cell identification (EpCAM, panCK, CK), cancer subtype classification (HER2, EGFR, ER), mammalian cell identification (GAPDH, tubulin β), and white blood cell

identification (CD45). With the level of microfluidic integration and the ability to perform targeted CTC protein profiling, the approach is expected to receive broad acceptance for exploring the CTCs proteome, biology, and clinical utility.

By using an alternative approach, Zhu et al. demonstrated the isolation of spiked tumor cells in the blood by immunodensity enrichment, laser capture microdissection (LCM), and nanodroplet sample processing followed by nano-LC/MS proteomic profiling.⁵⁰ LNCaP cells were spiked into 7 mL of blood (~7000 cells/mL), and after a benchtop enrichment in cancer cells, LCM was used to select the fluorescently stained CTC mimics and transfer them in wall-less nanowells. Processing by the nanoPOTS platform (nanodroplet processing in one pot for trace samples⁵¹) included cell lysis, protein extraction, reduction/alkylation, and proteolytic digestion within a small ~200 nL volume droplet. By minimizing protein losses during sample preparation in the wall-less droplet, protein expression could be monitored at a level of ~160 protein groups for triplicate analysis of one single cell and ~600 for five LNCaP cells. Cancer-specific epithelial and cytokeratin markers could be detected. Label-free proteomic quantitation by using the IBAQ method enabled reproducible protein abundance measurements that allowed for the differentiation between 1 and 5 LNCaP cells. The method showed good proteomic results for single-cell analysis and, due to wall-less cell manipulation capabilities, is particularly promising for the identification of less abundant proteins in a cell.

In a further effort to test the ability to perform proteomic profiling of rare CTCs (100–1000 cells in a model clinical specimen), a microfluidic chip that performed cell sorting was used to select immunostained THP-1 human monocytic live cells and subject them to MS analysis via a regular bench workflow. The authors demonstrated the ability to detect ~300–900 proteins from 1000 cells and up to ~500 proteins from 100 cells (Figure 7).⁵² The report represents the first microproteome profile of THP-1 cells, which are proxies for monocytes that have important roles in modulating immune responses and secreting cytokines. The simple sorting approach for selecting the cells for downstream MS analysis provides a quick means for exploring the function of monocytes and the role in disease.

Cells and Bacteria.

A variety of microfluidic platforms that incorporate microchambers, microtrap arrays for cell isolation, valves, pneumatic microstructures, and gradient mixers that enable microscale control of fluid delivery to cells, handling of cells, and mimicking the cell microenvironment have been developed for pharmaceutical assays, precision medicine, and nanomedicine applications. These devices were demonstrated for the study of cell behavior, cell-cell interactions, cytotoxicity, and high-throughput drug screening/discovery and delivery.^{53–56} Microfluidic handling of cells has gained popularity with the advent of biochemical assays that were implemented on miniaturized platforms. From simple immunoassays such as ELISA to complex DNA/RNA sequencing and PCR reactions, the portfolio of processes that were incorporated on microfluidic platforms has witnessed a continuous growth. Confined microfluidic architectures and advanced integration led to capabilities that enabled cell culture, transport, capture, sorting, separation, lysis, and analysis.^{57–59} The analysis of intact cells, cell content, and cell secretions has been pursued with techniques ranging from

cytometry to imaging, microscopy, spectroscopy, and electrochemical detection. With improvements in mass spectrometric sensitivity and detection limits, promising opportunities for the future development of associated proteomic screening assays exist as well.

Microfluidic arrayed chips are often used to monitor the cellular response to a perturbation. In a recent work, Bui et al. devised a PDMS/glass chip with an array of 600 microchannels (5 μm width $\times$ 12 μm depth) that was used for generating a 3D physical confinement of aggressive brain cancer glioblastoma cells (G55) to assess their migration.⁶⁰ The position of the cells was visualized with nuclear staining fluorescent dyes (Hoechst), and after removal from the device, Western blotting was used to quantify differences in protein expression between migrating and proliferating cells. Migrating cells expressed ~2.5-fold higher levels of vimentin and decreased levels of β 3-tubulin. Through its simple design, the multiplexed microchannel device represents a very practical and effective platform for exploring the behavior of cancer cells and for providing insights into the characteristics that are indicative of invasion and metastasis.

Secreted proteins play an important role in intra- and intercellular communication and represent a rich source of diagnostic and therapeutic drug targets. Hormones, growth factors, cytokines, or chemokines are just a few among the important signaling molecules that have been targeted by a number of detection methods. To facilitate the dynamic quantification of cell secretion, a highly integrated and automated platform that can measure cytokine secretion and transcription factor activation from stimulated cells has been developed.⁶¹ The chip contained patterns of antibodies deposited in spot chambers on glass, and a PDMS layer with channels for delivering the fluids and enabling valve switching. By exposing the cells to time-controlled inputs of a stimulant, the chip could quantify the cell secretion response via micrometer-sized immunoassays. The device comprised 16 units, each consisting of a cell chamber, culture media transfer pump, and a chamber with 10 antibody capture spots that were sequentially used per time-point measurement (Figure 8).

Upon stimulation, secreted proteins were transported to the chamber that contained the antibody spots. The device was demonstrated for tracking and quantifying NF-KB activity and TNF (tumor necrosis factor) secretion in RAW macrophage p65^{-/-} cells in response to various concentrations of LPS (lipopolysaccharide) stimulant. A similar device was also used for performing nanoliter sandwich immunoassays from single cells captured in 1.35 nL PDMS chambers, but with antibody functionalized beads instead of antibody capture spots.⁶² The highly integrated and automated assay with the capability to quantify the dynamic response of cells to stimuli and secretion of cytokines is expected to play an important role in identifying the molecular factors involved in cell communication and in biomedical research focused on studying infection, immune response, and immunotherapy.

Cell secretions could also be monitored with a PDMS chip that incorporated a cell chamber, valves for automating the cell perfusate solution, a serpentine injection loop with the functionality of a 6-port valve for automated fraction collection, and a packed solid phase extraction (SPE) bed and ESI capillary.⁶³ While the system was demonstrated for the analysis of secreted metabolites only (nonesterified fatty acids, 1.4 μM detection limits), at

30 min intervals, a similar arrangement could be used for exploring the nature of other types of cell-secreted components such as peptides, proteins, growth factors, or signaling molecules. In another development, to illustrate the ability to analyze cell-secreted proteins in nanoliter droplet arrays by using fluorescence microscopy and MALDI-MS, droplet microfluidic approaches were used to encapsulate yeast cells in nanoliter volumes and deposit them in spots on a glass slide. After cell incubation to produce phytase (a phosphatase enzyme), MALDI-MS could be used to monitor the hydrolysis of the phytase substrate, phytic acid, in 7 nL droplets containing ~50–100 cells.⁶⁴ Moreover, to advance the overall miniaturization of the cell culture environment sampling process, previously described micro-fluidic chips with ESI generated from the chip corner, comprising CE separation or infusion channels, were interfaced to miniaturized ion trap mass spectrometers.⁶⁵ The platforms were used for the analysis of cell culture media to monitor cell growth via the consumption of amino acids and, due to short analysis times under 5 min, present promising opportunities for bioreactor monitoring applications.

In a recent development, DMF devices (DropBot DB3–120, Sci-Bots, Inc.) that enable sample preparation for bottom-up LC-MS proteomics, from as little as 100 mammalian cells, were developed.⁶⁶ The processing steps that were included on the chip included cell loading in droplets, lysis in buffer/detergent systems compatible with downstream processing, protein extraction, reduction/alkylation, and proteolytic digestion. Sample cleanup and removal of the detergent was enabled by using SPE magnetic beads that could be immobilized on the chip with magnets. Processed samples were aspirated from the outlet chip reservoir by GELoader tips and analyzed by LC-MS/MS. The analysis of ~100 and ~500 cells led to the identification of 1200 and 2500 proteins, respectively, and stable isotope-based labeling reactions for quantitative proteomics were further developed. The approach represents the first proteomic sample preparation workflow on a DMF device with performance comparable to commonly used benchtop LC-MS/MS platforms.

In the pursuit of a strategy that enables single-cell proteomics, Li et al. built a multilayer miniaturized assembly that enabled the isolation of 100, 50, or single HeLa cells in a small droplet microreactor (~550 nL) that could be used for sample pretreatment (cell lysis, reduction/alkylation, enzymatic digestion).⁶⁷ The assembly was built from four layers of polymeric (PDMS, polypropylene) and glass plates, with the top cover enabling the formation of an oil layer that prevented the evaporation of the liquid from the droplet. Access to the droplet for reagent delivery was achieved with a capillary probe, and MS analysis was enabled by inserting the LC capillary separation column directly into the droplet. Up to ~50 and ~1300 proteins could be detected from 1 or 100 cells, respectively. As a proof of principle, the method appears to be promising for proteomic sample preparation from a small number of cells, by using means that are easily accessible in a research laboratory.

In an alternative approach, to enable proteomic profiling of time-sensitive cellular responses, a glass microfluidic chip that enabled the capture of cells in a chamber, uniform fluid delivery for simultaneous stimulation of all cells, and fast cell recovery from the chip for quick sonic lysis and content delivery to downstream MS analysis, was developed (Figure 9).⁶⁸ The delivery of the stimulant solution to the cell chambers ($500\ \mu\text{m} \times 50\ \mu\text{m} \times 10\ \text{mm}$)

was accomplished with a syringe pump in an axial or transversal fashion (through hundreds of shallow $\sim 2\ \mu\text{m}$ deep channels) and was evaluated in terms of the ability to quickly create homogeneous stimulation conditions for all cells. Cells were taken off chip for sonic lysis because complete lysis of the entire cell population on the chip was difficult to accomplish within 1–2 min. The platform was designed for enabling (near) real-time sampling and proteomic profiling of cellular responses to a perturbation that were induced as soon as 1–2 min after cell stimulation started. About ~ 1000 high confidence proteins could be identified from an estimated batch of 2000 SKBR3/HER2+ breast cancer cells. The identified proteins represented biological processes related to proliferation, metabolism, adhesion, transport, and pathways representative of DNA damage repair and signaling (ERBB2, MAPK, Wnt, NF-KB, p53), and confirmed the ability to sample quickly and effectively the cellular proteome of the stimulated cells. With improvements in MS detection sensitivity, the ability to capture the biological processes that are critically responsive to a perturbation, from a much smaller population of cells, is expected to improve.

Bacteria that develop antimicrobial resistance represent a major concern to public health. To address this threat, a microfluidic chip that contained bacterial cell culture wells and enabled the generation of antibiotic gradients via a “Christmas tree” mixer was designed.⁶⁹ After observation of morphological changes upon exposure to antibiotics, the cells were harvested and lysed, and the mutations that evolved in protein expression in drug-resistant strains were analyzed by MALDI-TOF/MS and HPLC-MS/MS. Four drug-resistant bacteria were studied (CR-KP, ESBL-EC, CR-PA, VP-61), and eight protein markers were found to be associated with morphological changes in CR-KP, of which 3 were confirmed at the transcriptome level. By virtue of its simple design, the microfluidic device presents new opportunities for exploring the behavior of bacteria and accelerating the discovery of effective drugs against resistant strains.

Single-Cell Analysis.

The need for advancing single-cell analysis technologies evolved from the observation that cells that are part of a population can behave differently and present a heterogeneous response to a perturbation. Bulk measurements from cell populations average out the individual behavior of cells, failing to provide an accurate account of the underlying (epi)genomic, transcriptomic, proteomic, or metabolomic profiles that lead to functionally distinct cell behaviors.^{70–73} Single-cell sequencing technologies have enabled, for example, the study of genetic heterogeneity, revealing insights into the molecular basis of diseases such as cancer, cell communication, and the response to therapy. Despite efforts, however, due to the very limited amount of proteins that are present in a cell, single-cell proteomic analysis, especially by mass spectrometry, has lagged behind the accomplishments of genomic/transcriptomic efforts. Single-cell proteomic assays have been generally pursued by alternative technologies including mass cytometry, immunoassaying, barcoding with miniaturized antibody arrays, electrophoresis/Western blotting, and droplet microfluidics.^{74–77} Nevertheless, while beyond the purpose of this Review, it is worth mentioning that a variety of elaborate approaches that allow for the large-scale measurement of protein expression in single cells have been devised, but the advances relied on either the sophistication of genetic tools for incorporating reporters into genes that generate

fluorescently tagged proteins^{78,79} or the use of single-cell barcode chips that have been developed in earlier years.^{80,81}

Several notable accomplishments that involved the use of microarray or microfluidic platforms revolved around detecting and/or quantifying the expression of one protein or a limited number of proteins, by using, again, rather simple antibody capture assays. For example, Magness et al. devised a microfluidic antibody capture (MAC) chip that measured the expression of p53, a tumor suppressor protein, and its phosphorylation at Ser15.⁸² The PDMS/glass chip enabled live cancer cell selection from a tumor population of cells loaded in a reservoir (based on the surface marker EpCAM), transport in analysis microchannels via an optical trap, lysis with a high energy laser pulse, and capture of the p53 protein on antibody spots. Detection occurred via total internal reflection (TIRF) single molecule imaging of the p53 antibody fluorescence spots.

In another development, immunoassays of single cells that were compartmentalized in tens of thousands of 40 pL droplets immobilized in a 2D array enabled the screening for the dynamics of IgG secretion from cells in mice immunized with tetanus toxoid.⁸³ The system is expected to provide a novel platform for monitoring immune response and optimization of the vaccination protocols. Moreover, a microfluidic droplet platform was demonstrated for the analysis of cytokines (VEGF and IL-8) secreted by single cells.⁸⁴ Water-in-oil droplets contained individual cells and four kinds of particles (antibody-conjugated and magnetic beads, each conjugated with a different antibody for the two cytokines). The two types of particles enabled a sandwich immunoassay to occur when the cytokines were secreted, which in turn activated SERS (surface enhanced Raman scattering) detection. The cytokine limit of detection in one droplet was estimated to be 1 fg/mL. Last, quantification of proteins in single cells could also be achieved with a rather simple microfluidic flow cytometer setup that comprised a constriction channel with a small cross-sectional area (smaller than the cell cross section).⁸⁵ Absolute protein quantification was achieved by combining the quantitative fluorescence intensity data of antibody-labeled cells that were squeezed through the constriction channel with data from calibration curves. A demonstration for the measurement of copy numbers of β -actin in single tumor cells was provided. While still limited to detecting only a few numbers of proteins, all these microfluidic platforms capitalize on the ability to isolate and manipulate cells within very small volumes, often droplets, that act as ideal microreactors for containing the cells, delivering the reagents to the cells, and facilitating sensitive detection. Also, the detection is frequently based on antibody-antigen recognition mechanisms. Nevertheless, the value of obtaining single-cell data for accurately assessing cell behavior as well as the outcome of a heterogeneous cell population response cannot be overstated. It is only a matter of time until technologies such as mass spectrometry will develop capabilities for what appears to be the ultimate frontier in proteomics.

CONCLUSIONS AND OUTLOOK

The work that was captured in this Review covers many developments and innovations that span the different subfields of microfluidics and highlights clear progress in the efforts focused on identifying or quantifying proteins in biological fluids and profiling whole cell

proteomes. The work on developing novel microfluidic-MS interfaces, coupling micro-chips to miniaturized mass spectrometers, or improving the sample preparation methods has continued. However, there has been a much larger emphasis placed on the development of platforms that enable the detection of target proteins or disease biomarkers, and the characterization of protein PTMs, structure, and interacting partners. The interest in developing immunoassay devices with various detection techniques, for protein biomarker identification and POC diagnostics, has grown as well. Nonetheless, of particular relevance is the work that led to the development of microfluidic platforms for cell culture, treatment, and analysis. By combining microarray with microfluidic technologies, genetic manipulations with (immuno)biochemical assays, and by using a variety of detection technologies, protein or proteome profiling evolved from the identification/quantification stage to a level where it can be used for exploring long-sought, real-world biological questions. With the increased focus on single-cell whole-content profiling, the most challenging problem continues to reside in the ability to detect low abundance proteins in single cells. Recent developments in high resolution/high mass-accuracy mass spectrometry instrumentation enable the detection of over 5000 proteins from <100 ng protein extracts, within only ~15 min of LC-MS analysis time.¹ This, however, is accomplishable only after completing an extensive and lengthy number of sample preparation and fractionation steps. The raw protein content of a mammalian cell is only ~120–480 pg, i.e., ~2–3 orders of magnitude below the levels that generate comprehensive profiles of cell extracts.^{68,86} To enable, therefore, a similar performance for microfluidic proteomic profiling of either single cells or of comparably small amounts of proteins, improvements in mass spectrometry detection limits, scanning speeds, and intelligent data acquisition strategies, as well as in microfluidic sample processing and integration, will be necessary. With the ongoing accumulation of high-quality data sets in proteomic repositories, it is foreseeable that MS detection strategies that make use of existing data will be the most likely candidates for achieving the needed performance for proteomic profiling, in a targeted fashion, of single cells.

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Biographies

Iulia M. Lazar earned her Ph.D. in Chemistry from Brigham Young University in 1997 under the supervision of Professor Milton L. Lee. Following two postdoctoral appointments at Sensor Larson-Davis (1997–1998) and Oak Ridge National Laboratory (1998–2000) and a Principal Research Scientist position at The Barnett Institute/Northeastern University (2000–2003), she joined The Virginia Tech Bioinformatics Institute in 2003 as an Assistant Professor. Presently, she is a Professor in the Department of Biological Sciences and holds additional appointments in Health Sciences and at the Carilion School of Medicine at Virginia Tech. At present, her research evolves at the interface between technology development and biology, with a focus on exploring the molecular mechanisms of breast cancer cell cycle regulation by using mass spectrometry-based systems biology approaches.

Her laboratory develops microfluidic and proteomic technologies for investigating the pathways that enable cancer cells to bypass tightly regulated molecular checkpoints, proliferate in an unrestrained manner, metastasize, and hijack normal biological function.

Nicholas S. Gulakowski is a senior student majoring in Systems Biology, with a minor in Chemistry, at Virginia Tech. He is interested in using mass spectrometry data for developing mathematical models of biological pathways that lead to aberrant proliferation of cancer cells.

Alexandru C. Lazar received his Ph.D. in Analytical Chemistry from Brigham Young University in 1998, after which he completed his postdoctoral training at Oak Ridge National Laboratory. He worked as a Senior Scientist at biopharmaceutical companies (Zycos and EMD Pharmaceuticals) until 2004, when he joined ImmunoGen, Inc. As Senior Director of the Analytical and Pharmaceutical Development department, he was responsible for the development of analytical methods for the analysis of immunoconjugates and their components (antibodies, linkers, and cytotoxic agents) and the development of formulations for antibodies and immunoconjugates. He has recently joined Abzena in Bristol, PA, as Senior Director of Analytical Method Development and QC, where he is involved in the development of antibody-drug conjugates and their components.

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Figure 1. Schematic representation (a) and experimental setup (b) of a piezo-ring-on-chip microfluidic device for depositing sample on a MALDI-MS target plate in small 40 nL volumes. Reprinted with permission from Tsao, C.-W.; Lei, I. C.; Chen, P.-Y.; Yang, Y.-L. *Analyst* **2018**, *143*, 981–988 (ref 20). Copyright 2018 Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry.

Figure 2.

Integrated platform composed of a microfluidic device and a His/Myc-double-tagged ORF library spotted on glass for monitoring the parallel expressions and phosphorylation of thousands of proteins. One assay unit contains DNA and protein chambers isolated by valves. (a) The microfluidic device. (b) Following incubation with a mammalian reticulocyte lysate, the target proteins are expressed in the DNA chambers, diffused, and immobilized in the protein chambers via a His tag. (c) Proteins expressed in mammalian cell lysates; the enzymes that have autophosphorylation activity are expected to be functional and undergo autophosphorylation during expression. (d, e) The proteins and the p-Tyr proteins are both quantified in situ using Cy3-coupled anti-Myc antibodies and Cy5-coupled antiphosphorylated-Tyr antibodies, respectively. Adapted with permission from Nevenzal, H.; Noach-Hirsh, M.; Skornik-Bustan, O.; Brio, L.; Barbiro-Michaely, E.; Glick, Y.; Avrahami, D.; Lahmi, R.; Tzur, A.; Gerber, D. *Commun. Biol.* 2019, 2, 42 (ref 27). Copyright 2019 granted under Creative Commons CC BY license (<https://creativecommons.org/licenses/by/4.0/>).

Figure 3. Schematic representation of a microfluidic chip for performing HDX of proteins on subsecond time scales. Reprinted from Svejidal, R. R.; Dickinson, E. R.; Sticker, D.; Kutter, J. P.; Rand, K. D. *Anal. Chem.* **2019**, *91*, 1309–1317 (ref 29). Copyright 2018 American Chemical Society.

Figure 4.

Schematic representation of a workflow for on-chip protein affinity purification and identification of interaction partners. Cells expressing a GFP-tagged form of a protein of interest are loaded on the chip and lysed to obtain whole cell extracts. The chip is preloaded with all buffers and reagents required during immunoprecipitation and preparation for LC-MS/MS analysis. During the run, the GFP-nanobody beads are packed in single reactors and washed before loading the protein samples from the inlet onto the beads. Tagged macromolecular assemblies from the cell lysate are retained on the GFP-nanobody beads and digested into peptides for downstream MS analysis. Adapted with permission from Furlan, C.; Dirks, R. A. M.; Thomas, P. C.; Jones, R. C.; Wang, J.; Lynch, M.; Marks, H.; Vermeulen, M. *Nat. Commun.* **2019**, *10*, 1525 (ref 30). Copyright granted under Creative Commons CC BY license (<https://creativecommons.org/licenses/by/4.0/>).

Figure 5. Microfluidic IMER chip for proteolytic digestion with trypsin adsorbed on the porous surface of PDMS channels. (a) Microfluidic chip. (b) Multiplexed design of the IMER channels. (c) Serpentine IMER channels. Reproduced with permission from Kecskemeti, A.; Nagy, C.; Csoosz, E.; Kallo, G.; Gaspar, A. *Prot. Clin. Appl.* **2017**, *11*, 11–12, 1700055 (ref 39). Copyright 2017 John Wiley and Sons.

Figure 7.

Workflow for selecting THP-1 cells with a microfluidic cell sorter device to test the ability to perform proteomic profiling of rare CTCs. Cell collection was performed in tubes with various coatings to enable different processing procedures prior to LC-MS/MS analysis: Group 1, 1000 cells in a BSA-coated tube digested with medium; Group 2, 1000 cells in a BSA-coated tube with the medium removed; Group 3, 1000 cells in a hydrophilic-coated tube with the medium removed; Group 4, 100 cells in a BSA-coated tube digested with medium; Group 5, 100 cells in a BSA-coated tube with the medium removed. Reprinted with permission from Kasuga, K.; Katoh, Y.; Nagase, K.; Igarashi, K. *Proteomics* **2017**, *17*, 1600420 (ref 52). Copyright 2017 John Wiley and Sons.

Figure 8.

Schematic diagram of a multiplexed immunoassay microfluidic chip for analyzing cell-secreted cytokines. The chip contains 16 units, each comprising a cell culture chamber, transfer pump for cell culture media, and a chamber with 10 antibody capture spots for analyzing the secreted cytokines in the cell supernatant. The control layer (red) modulates the fluid flow (blue) in the cell/immunoassay layer. Reprinted with permission from Kaestli, A. J.; Junkin, M.; Tay, S. *Lab Chip* **2017**, *17*, 4124–4133 (ref 61). Copyright 2017 The Royal Society of Chemistry.

Figure 9.

Microfluidic chip incorporating a reactor that enables cell culture, stimulation, and cell recovery for monitoring proteome-level fast cellular responses by mass spectrometry (a). COMSOL simulation of transversal delivery of a stimulant solution to the cell chamber (b–d). Reprinted with permission from Lazar, I. M.; Deng, J.; Stremler, M. A.; Ahuja, S. *Microsystems & Nanoengineering* **2019**, 5, 7 (ref 68). Copyright granted under Creative Commons CC BY license (<https://creativecommons.org/licenses/by/4.0/>).