Review

Recent methodological advances towards single-cell proteomics

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Abstract: Studying the central dogma at the single-cell level has gained increasing attention to reveal hidden cell lineages and functions that cannot be studied using traditional bulk analyses. Nonetheless, most single-cell studies exploiting genomic and transcriptomic levels fail to address information on proteins that are central to many important biological processes. Single-cell proteomics enables understanding of the functional status of individual cells and is particularly crucial when the specimen is composed of heterogeneous entities of cells. With the growing importance of this field, significant methodological advancements have emerged recently. These include miniaturized and automated sample preparation, multi-omics analyses, and combined analyses of multiple techniques such as mass spectrometry and microscopy. Moreover, artificial intelligence and single-molecule detection technologies have advanced throughput and improved sensitivity limitations, respectively, over conventional methods. In this review, we summarize cutting-edge methodologies for single-cell proteomics and relevant emerging technologies that have been reported in the last 5 years, and provide an outlook on this research field.

Keywords: single-cell analysis, single-cell omics, proteomics, single-molecule microscopy

1. Introduction

Complex biological processes involve dynamic interactions between individual cells often spanning from multiple cell types and cell states. Over the past decades, traditional bulk analysis was the preferred choice to study omics, however, this method only provides an average measurement across cell populations. As a result, researchers have started to delve into single-cell analysis allowing further characterization of cell-to-cell heterogeneity and revealing their unique biological characteristics.

Most single-cell studies have so far focused on nucleic acids, specifically the transcriptomes, which represent all expressed genes in the cell. However, transcriptomics measurement alone has been re-

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Non-standard abbreviation list: 2D-GE: 2D gel electrophoresis; AI: artificial intelligence; ATAC-seq: assay for transposase accessible chromatin sequencing; CITE-seq: cellular indexing of transcriptomes and epitopes by sequencing; CODEX: co-detection by indexing; COVID-19: coronavirus disease 2019; CRISPR: clustered regularly interspaced short palindromic repeats; cryo-EM: cryo-electron microscopy; DART: direct analysis in real time; DDA: data-dependent acquisition; DIA: data-independent acquisition; DNN: deep neural network; DO-MS: data-driven optimization of MS; ES: embryonic stem; FFPE: formalin fixed paraffin embedded; FISH: fluorescence *in situ* hybridization; FP: fluorescent protein; FSC: forward scatter; FUCCI: fluorescent, ubiquitination-based cell cycle indicator; G13F-FraC: G13F-Fragaceatoxin C; GFP: green fluorescent protein; LC: liquid chromatography; LO: labeling occupancy; ML: machine learning; mPOP: minimal proteomic sample preparation; MS: mass spectrometry; MS/MS: tandem mass spectrometry; Nb: nanobody; NGS: next generation sequencing; NHS: N-hydroxysuccinimide; nPOP: nano-proteomic droplet sample preparation; PC: photocleavable; PDT: phage-derived tag; PEA: proximity extension assay; PTM: post-translational modification; RFP: red fluorescent protein; ROI: region of interest; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; SCoPE-MS: single-cell proteomics by mass spectrometry; SCoPE2: second generation of SCoPE-MS; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPARC: single-cell protein and RNA co-profiling; SSC: side scatter; TIRF: total internal reflection fluorescence; TMT: tandem mass tag; UV: ultraviolet.

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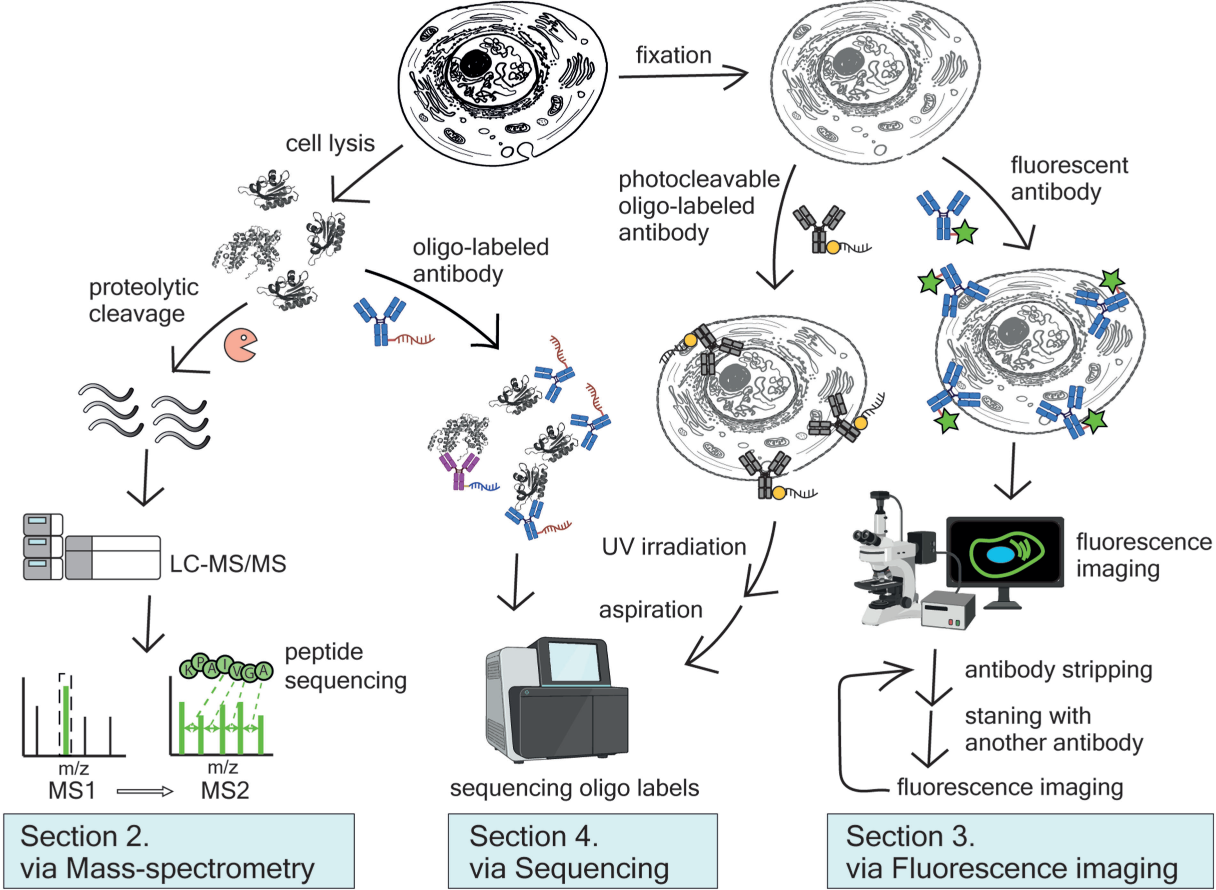


Fig. 1. Overview of representative workflows of single-cell proteomics. It is noteworthy that detecting protein abundance and localization with live cells is also possible with fluorescence imaging of fluorescent protein tags, as discussed in detail in sections 3 and 5.

ported as a poor indicator of protein abundances and failed to replace direct measurements of the proteome.[1)–3)] The concentration of each protein species is the result of dynamic synthesis and degradation depending on the circumstances and cellular status. Furthermore, proteins being the workhorses in living organisms often reside in a specific intracellular organelle or translocate dynamically. Thus, not only the identification and abundance but also subcellular localization is important to understand biological phenomena especially at single-cell resolution.[4),5)]

Yet, single-cell proteome analysis poses a great challenge because the amount of protein from a single-cell sample is infinitesimal, and each protein species exhibits a wide dynamic range. The total number of protein species and protein molecules in one mammalian cell are in the order of 10^4 and 10^9, respectively, while the abundance per protein species ranges from 10^2 to 10^7 copies per cell.[2),6)] Furthermore, protein molecules cannot be amplified like nucleic acids, implying the importance of sensitivity in the methodologies for single-cell proteomics.

To date, analyses with mass spectrometry (MS) and antibody-based methods are the most common paths to examine the proteome in single cells. MS remains a standard technique in proteomics as it can identify and quantify thousands of proteins including post-translational modifications (PTMs). Antibodies are used to analyze the amounts and types of proteins through imaging and sequencing. This review summarizes three major methods for single-cell proteomics: MS, fluorescence imaging, and sequencing (Fig. 1). Next, recent attempts that combine more than two methodologies, incorporate modern technologies, such as artificial intelligence (AI), single-molecule peptide sequencing, and exploit advanced single-molecule microscopy, will be introduced. Finally, our approach to comprehend single-cell pro-

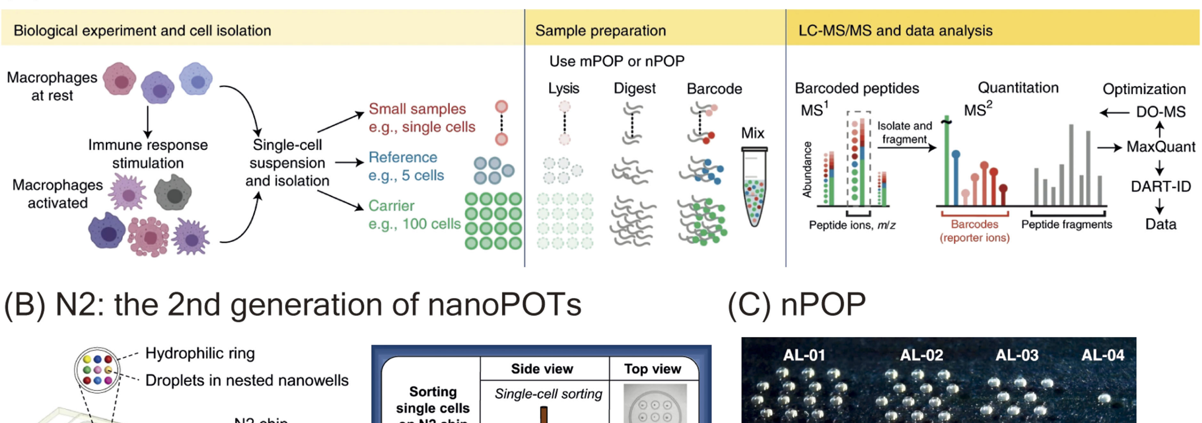
teomes using single-molecule fluorescence microscopy and perspectives on this field will be suggested.

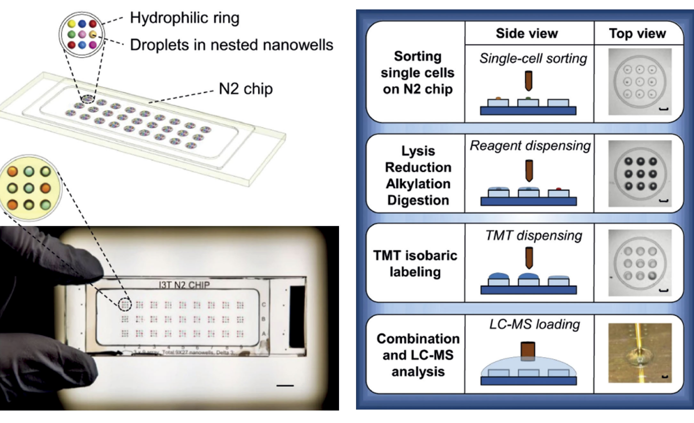
2. Single-cell proteomics via MS

MS is a benchmark method in modern proteomics due to its capability of protein identification, characterization, and quantification with high multiplexity.[7] There are two approaches of MS-based proteomics depending on the type of the applied sample: bottom-up and top-down. Bottom-up proteomics uses proteins digested into small peptide fragments using enzymes such as trypsin. These peptides are separated and analyzed using techniques such as liquid chromatography (LC) and tandem mass spectrometry (MS/MS). The resulting peptide fragments are identified and sequenced from their mass spectra, allowing for the identification of proteins by matching the identified sequence with the proteome database. Meanwhile, top-down proteomics uses intact proteins and protein complexes without prior digestion.[8] Mass spectra of intact proteins allow for the identification of protein isoforms and post-translational modifications. In single-cell proteomics, bottom-up MS has been used due to its 100-fold higher sensitivity and larger proteome coverage (Fig. 1). Higher sensitivity of bottom-up MS originates from the exponential decay in the signal-to-noise ratio with increasing mass and the reduced sample loss due to adsorptive property of proteins.[9] The principles and conventional applications of both MS techniques have been well summarized in previous reviews,[10)–13] thus we focused on recent single-cell application in this review.

In order to investigate single-cell proteome profiles by MS, there are several problems to overcome that are not typically issues with bulk samples.[14] First, sample loss must be minimized when delivering proteins from a single-cell to the MS instrument. Second, it is necessary to perform high-throughput and highly multiplexed detection to obtain dataset from sufficient numbers of cells. In 2018, Budnik and Slavov *et al.* developed a landmark single-cell MS method, single-cell proteomics by mass spectrometry (SCoPE-MS).[15] To address the challenges above, SCoPE-MS mechanically lyses single cells through sonication or freeze-thaw cycles, which can reduce protein loss during surfactant cleanup procedures (note: chemical detergents or urea for cell lysis are incompatible with MS). In addition, sample loss was further minimized by reducing the total sample volume and the number of sample transfers. Moreover, multiplexed detection was carried out using a tandem mass tag (TMT), which labels the samples with a different combination of isotopes that can be distinguished in tandem MS.[16,17] Simultaneous MS measurement with 10-plex TMT was done for a mixed sample with eight single-cell samples and a carrier cell sample, comprising between 10 and 200 cells. The carrier cell sample not only reduces sample loss caused by the adsorption to the tube surface but also provides sufficient peptide ions that can dramatically improve the number of identified peptides. The choice of the cell line for carrier cell samples usually corresponded to the cell type used in single-cell studies. As a result, over 1,000 proteins were identified from single mouse embryonic stem (ES) cells, including mostly high-abundance proteins of $>10^5$ copies per cell and a few low-abundance proteins of $<10^4$ copies per cell. In the second generation of SCoPE-MS (SCoPE2), the throughput and quantification accuracy were improved by optimizing experimental and data analysis protocols (Fig. 2A). This optimized method can identify over 3,042 proteins across 1,490 single monocytes and macrophages in 10 days of instrument time.[18,19]

Concerning the issues of sample loss and throughput for sensitive detection of single-cell proteome, it is essential to develop automated and miniaturized sample preparation methods. One example is nanoPOTS, a chip-based nanodroplet platform for all-in-one sample preparation.[20] With the nanoPOTS platform, all sample preparation steps from protein extraction, alkylation, and digestion to surfactant cleavage were performed within a submicroliter single droplet reactor, and approximately 3,000 proteins were identified from a pool of ten cells using ultrasensitive LC and MS. It is noteworthy that RapiGest, an acid labile surfactant that undergoes hydrolysis in acidic condition,[21] was used as a surfactant in nanoPOT instead of chemical detergents that interfere with peptide ionization. Single-cell analysis became possible with the second generation of nanoPOTS, termed nested nanoPOTS (N2) chip, which can quantify 1,500 proteins from 100 individual cells (Fig. 2B).[22] Such improvements were achievable due to the 2.3-fold increased peptide/protein recovery by further minimizing the processing volume ($\sim$30 nL), which reduced the contact area. In addition, the N2 chip was designed to accommodate 243 cells on a single chip, and the processing throughput was enhanced by ten-fold with a measurement time of 18 min/chip and 0.07 min/cell. As illustrated in Fig. 2B, nine single-cells in a droplet were labeled differently by TMT, leading to through-





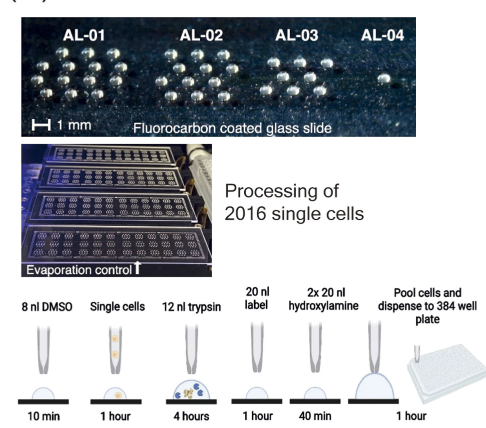


Fig. 2. (Color online) MS-based single-cell proteomics. **A**. Single-cell proteomics with SCoPE2.[18] First, heterogeneous cell samples are sorted into three groups, single-cell, reference cells, and carrier cells, with a cell number ratio of 100:5:1, respectively, in the multiwell plate. The choice of reference and carrier cells corresponds to the cell types employed in single-cell samples. Next, cells are lysed using a freeze-heat cycle on the plate and digested, with minimal proteomic sample preparation (mPOP) or nano-proteomic droplet sample preparation (nPOP) (Fig. 2C), followed by tandem mass tags (TMTs) labeling of the three samples. The peptide mixtures are then analyzed by LC-MS/MS, and incorporation of direct analysis in real time (DART)-ID and data-driven optimization of MS (DO-MS) further enhancing peptide identification and optimization of LC-MS/MS experiments, respectively. **B**. Second generation of nanoPOTS, called N2.[22] Nine nanowells for preparing single-cell samples are nested together, surrounded by a hydrophilic ring for one TMT set, resulting in 243 nanowells on the chip. Single-cells are sorted using an image-based single-cell isolation system (cellenONE F1.4, Cellenion), and cell lysis and sample preparation are all conducted within a reaction volume of less than 30 nL. Finally, peptides in nine wells are collected together and subsequently injected into a nanoLC-MS/MS. **C**. Workflow of nPOP. Piezo acoustic dispensing is used to isolate individual cells in a 300 pL volume, and all subsequent sample preparation steps are performed within a droplet on a highly hydrophobic fluorocarbon-coated slide.[23] Panel adapted with permission from: **A**, ref. 18, Springer Nature Limited.

put enhancement (Fig. 2B). Inspired by these single-cell MS studies, many innovative methods to prepare samples with minimal volume have been reported, for example, sub-nanoliter volume droplet samples on a hydrophobic polymer film (Fig. 2C),[23] preparing a lysate water droplet-in-oil,[24] usage of a commercial picoliter dispenser,[25] and microfluidic chip,[26] and performing seamless sample preparation and nano-LC separation.[27]

3. Single-cell proteomics via fluorescence imaging

Fluorescence labeling and microscopic imaging of proteins of interest provide information about abundance and intracellular localization, which are useful for single-cell proteomics. Compared with MS, fluorescence imaging can be carried out with relatively simple instruments, such as a bench-top fluorescence microscope, and its detection limit can ultimately reach a single-molecule level. Traditionally, single-cell proteomics were accomplished by imaging individual cells in which the protein of interest was endogenously labeled with a fluorescent protein (FP).[1),28),29] Generally speaking, however, it is extremely demanding to prepare proteome-scale FP libraries. In addition, FP fusion probably affect protein function or translocation.

Although single-cell proteomics with FP tags holds great advantages in live-cell imaging, because

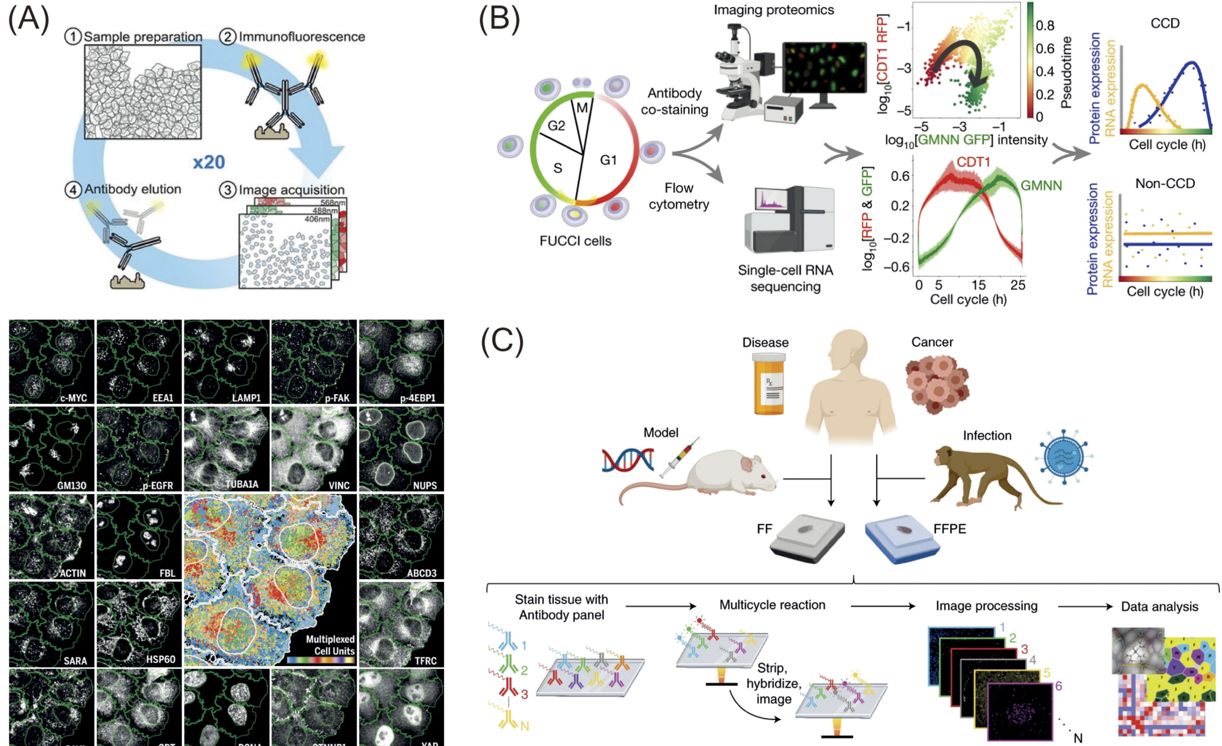


Fig. 3. (Color online) Fluorescence imaging-based single-cell proteomics. **A**. Through repeated antibody staining and elution, 40-plex protein mapping was carried out at high spatial detail in thousands of cells.[31] The obtained multiplexed protein maps provided a comprehensive quantitative description of compartmentalized intracellular protein composition. **B**. Spatiotemporal dissection of the cell cycle studied at the single-cell level with FUCCI-expressing U2OS cells.[36] By fitting a polar model to two FP intensities, a linear pseudotime representation of the cell cycle was obtained. Independent measurements of single-cell RNA and protein expression were compared after cell-cycle alignment of individual cells. **C**. CODEX pipeline for single-cell proteomics of tissue specimens.[38] Multiplexed protein mapping was carried out by repetitive hybridization and stripping of the fluorescently labeled oligo readouts. Panels adapted with permission from: **A**, ref. 31, AAAS; **B**, ref. 36, Springer Nature Limited; **C**, ref. 38, Springer Nature Limited.

of the practical reasons described, it is becoming more popular to exploit fluorescently labeled antibodies in the recent studies. Uhlen and Lundberg *et al.* constructed a subcellular map of the human proteome by mapping 12,003 human proteins with 13,993 fluorescently labeled antibodies and enabled the definition of the proteomes of 13 major organelles.[30] This work, however, did not detect multiple proteins in the same single-cell, resulting in limited information about functional linkages among subcellular distributions of the proteome. In the following year, 40 kinds of proteins per cell were detected by the iterative immunofluorescence imaging, in which different sets of protein kinds were stained and imaged in every cycle. In this study, 20 cycles of antibody staining, two-color fluorescence imaging, and chemical antibody elution were conducted with an automated liquid handling platform. As a result, 40 multiplexed protein maps can be generated that allow systematic comparisons of subcellular spatial protein distribution with cell crowding, cell cycle, and pharmacological perturbations at single-cell level (Fig. 3A).[31] In place of antibody elution, chemical bleaching and iterative immunolabeling,[32] sequential photocleavage of labeled dyes,[33] and post-imaging spectral unmixing[34] can be used to obtain multiplexed protein maps via fluorescence immunostaining. These spatial proteomic studies revealed that the subcellular localization of proteins and their functions are closely correlated.

Obtaining additional information about individual cell status using fluorescent indicators is another advantage of fluorescence imaging-based single-cell proteomics. For instance, fluorescent, ubiquitination-based cell cycle indicator (FUCCI) is a genetically encoded optical sensor for live-cell cycle monitoring. FUCCI is composed of two fluorescently tagged cell cycle markers: chromatin licensing and DNA repli-

cation factor 1 (CDT1) for G1 phase and geminin DNA replication inhibitor (GMNN) for S and G2 phases, tagged by red fluorescent protein (RFP) and green fluorescent protein (GFP), respectively. These markers are co-expressed during the G1–S transition, resulting in yellow color.[35] Thus, the color ratio between GFP and RFP in a nucleus was exploited as a pseudotime measure of the cell cycle. By fluorescence imaging of antibodies and FUCCI and comparing between single-cell proteome and transcriptome results (Fig. 3B), hundreds of proteins that exhibit cell-cycle dependency were identified by fluorescence immunostaining, and most cycling proteins were found to be regulated post-translationally.[36]

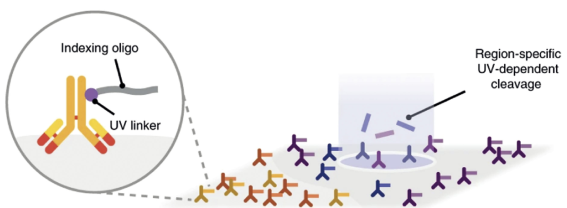
In spite of the advantages described above, fluorescence imaging can only distinguish 3–5 colors. Hence, imaging of fluorescent antibodies or FPs bears a limitation in its multiplexity and throughput. To transcend this limitation, oligonucleotide tags (oligo tags) composed of more than several bases of DNA have often been exploited. Oligo tags can be used as an identifier of molecular species according to their sequences consisting of adenine, thymine, guanine, and cytosine. In particular, the Nolan group proposed the use of oligo duplexes with 5′ overhangs with different lengths as tags to recognize many species of antibody. This overhang region is later extended with fluorophore labeled bases using polymerase, and by reading the timing of the florescence occurrence with imaging, the oligo species are distinguished. This method is named co-detection by indexing (CODEX).[37] The current version of CODEX uses fluorescent-labeled complementary strands to image the oligo tag and can visualize up to 60 markers both in cell and formalin fixed paraffin embedded (FFPE) tissue samples (Fig. 3C).[38] FFPE tissue is the most common form to preserve biopsied clinical specimens, while it has been considered to be tricky samples for fluorescence microscopy due to its intrinsic autofluorescence. With substantially brighter oligo reporters with 15–60 fluorescent dyes and usage of photocleavable (PC) linkers for iterative imaging and removing of a reporter oligo, a 108-plex assay in FFPE tissue samples was reported recently.[39] Furthermore, novel oligo designs that control hybridization and amplify a signal from one oligo tag may improve the sensitivity of this approach for single-cell spatial proteomics.[40]

4. Single-cell proteomics via sequencing

DNA sequencing analysis has begun to be applied to single cell proteome analysis. Such analysis has been made possible by treating cells with a mixture of antibodies against different protein species.[41] Here, antibodies for different targets are respectively conjugated with oligo tags composed of different DNA sequences, and their amounts bound to individual cells can be quantified by performing sequencing analysis of the oligo tags to obtain their numbers as sequencing read frequencies. The number of oligo tags can be expanded by the exponent of the oligo length on the four. Thus, oligo tags longer than 11 bases can cover a whole human proteome in theory. In addition, amplified oligo tags enable detection of low-copy proteins effectively. Furthermore, recent advances in next generation sequencing (NGS) technology facilitate reading with high throughput and low cost. Hence, speed and throughput of sequencing-based single-cell proteomics has been greatly promoted by exploiting NGS. Nowadays, there are many commercially available antibody panels with oligo tags depending on the purpose of the study, and access to NGS instruments is becoming easier.

Towards highly multiplexed analyses of single-cells and facile collection of oligo tags, labeling of the oligo tag via a PC linker was developed in 2012.[42] Using this technology, after 2 years from its development, the same group succeeded in profiling 90 kinds of protein in cancer cells and clinical samples.[43] In this work, oligo tags bound to antibodies via a PC linker were released by ultraviolet (UV) irradiation, collected using a needle aspirate, and sequenced. Furthermore, this strategy was recently exploited for spatial single-cell transcriptomics and proteomics with FFPE samples. This was realized by point or patterned UV irradiation with a digital mirror device (Fig. 4), and spatial profiling of pathological tissue specimens that consist of various types of cells became possible.[44] Unlike imaging-based methods, sequencing oligo tags is free from the problem of autofluorescence and thus is of benefit for samples with high background fluorescence, such a FFPE specimens.

The most remarkable advantage of the sequencing-based proteomics is that it realizes multi-omics analyses for the same single-cell. The seminal work in single-cell multi-omics is cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), a high-throughput multi-omics approach for capturing cellular surface proteins and mRNAs from thousands of single-cells.[45] Although early studies with CITE-seq were limited to surface proteins that were easier targets for antibodies, a novel method, single-cell protein and RNA co-profiling (SPARC), succeeded in



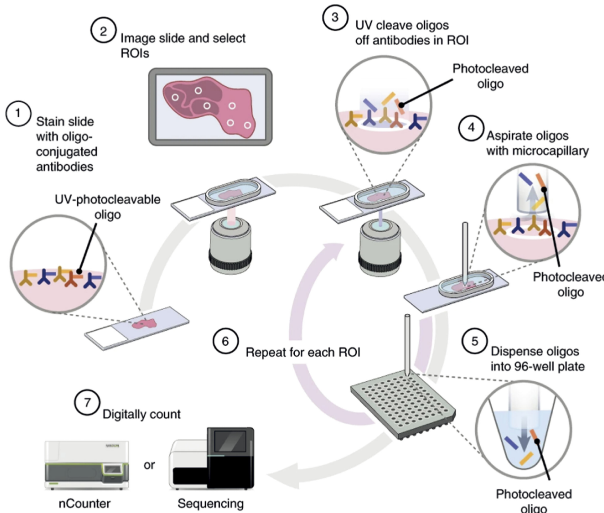


Fig. 4. (Color online) Workflow of digital spatial profiling.[44) (Top) Antibodies were covalently linked to a indexing oligo with a UV-cleavable linker (PC-oligo). Using tens of PC-oligos, a tissue section was stained with the panel of interest. (Bottom) By UV irradiation of the region of interest (ROI), indexing oligos within the ROI were liberated, followed by aspiration and analysis using a NanoString nCounter analysis system or NGS. The area and shape of the ROI could be freely chosen with the aid of a digital mirror device. Panel adapted with permission from: ref. 44, Springer Nature Limited.

measuring intracellular proteins as well as global mRNA in single-cells (Fig. 5A).[46)] The key advance was that single-cell proteins were detected through proximity extension assay (PEA)[47)]; the target protein is recognized by two or more antibodies with oligo-tags, then labeled oligo-tags are ligated, amplified, and sequenced (Fig. 5A). When exploring the correlation between protein and mRNA levels using SPARC, it was found that the mRNA level is a poor indicator of protein abundance at the time of measurement. For instance, there was a weak correlation between transcription factors and their downstream effects when measured at the RNA level, but a good correlation at the protein level.

In addition to transcriptomics, chromatin structures have received attention as an effective target to examine further upstream gene regulation mechanisms by transcription factors or other molecules.[48)] Recently, chromatin structures can be studied with a sequencing technique, assay for transposase accessible chromatin sequencing (ATAC-seq),[49),50)] specifically designed to measure chromatin accessibility across the genome. It analyzes DNA sequences that can interact with Tn5, a highly active transpose, which preferentially inserts into open chromatin sites, cleave, and add sequencing primers. As a result, the sequenced DNA informs the open chromatin regions, providing insights into the epigenetic state of chromatin. There have been several multi-omics approaches combining ATAC-seq and transcriptome/proteome analyses at single-cell level; DOGMA-seq is a trimodal single-cell assay of chromatin accessibility, transcriptome, and proteins, which can cover multiple layers in the central dogma of the gene regulation (Fig. 5B).[51)] It revealed cellular programming occurring in chromatin, transcriptional and post-translational regulation during monocyte development in bone marrow, activation of T cells, and downstream signaling of the T cell receptor.

With oligo-tagged antibodies, however, it is difficult to perform scalable and pooled library construction. To address this difficulty, developing phagemids as genetic barcodes, which are highly diverse ($\sim 10^{10}$ clones) and packed within a virus particle that expresses an antibody protein on its surface, has been reported.[52),53)] Using this technology, PHAGE-ATAC was established, as a multimodal single-cell approach combining nanobody (Nb)-displaying phages and droplet-based single-cell ATAC-seq for simultaneous measurement of proteins and chromatin accessibility profiling (Fig. 5C).[54)] As a demonstration, PHAGE-ATAC has been exploited to detect coronavirus disease 2019 (COVID-19)-infected cells. For this purpose, a synthetic phage Nb library was first constructed and the top seven phage-Nbs with the highest signal against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein (SARS-CoV-2 S) were selected. By utilizing a 12-plex panel (seven anti-SARS-CoV-2 S Nbs, two anti-EGFP Nbs, and three peripheral blood mononuclear cells-recognizing Nbs), PHAGE-ATAC examined nearly 5,000 single-cells and distinguished between cells expressing SARS-CoV-2 S and immune cells in human cell populations.

5. Combined methodologies towards single-cell proteomics

Combined methodologies of MS and other techniques for single-cell proteomics have received

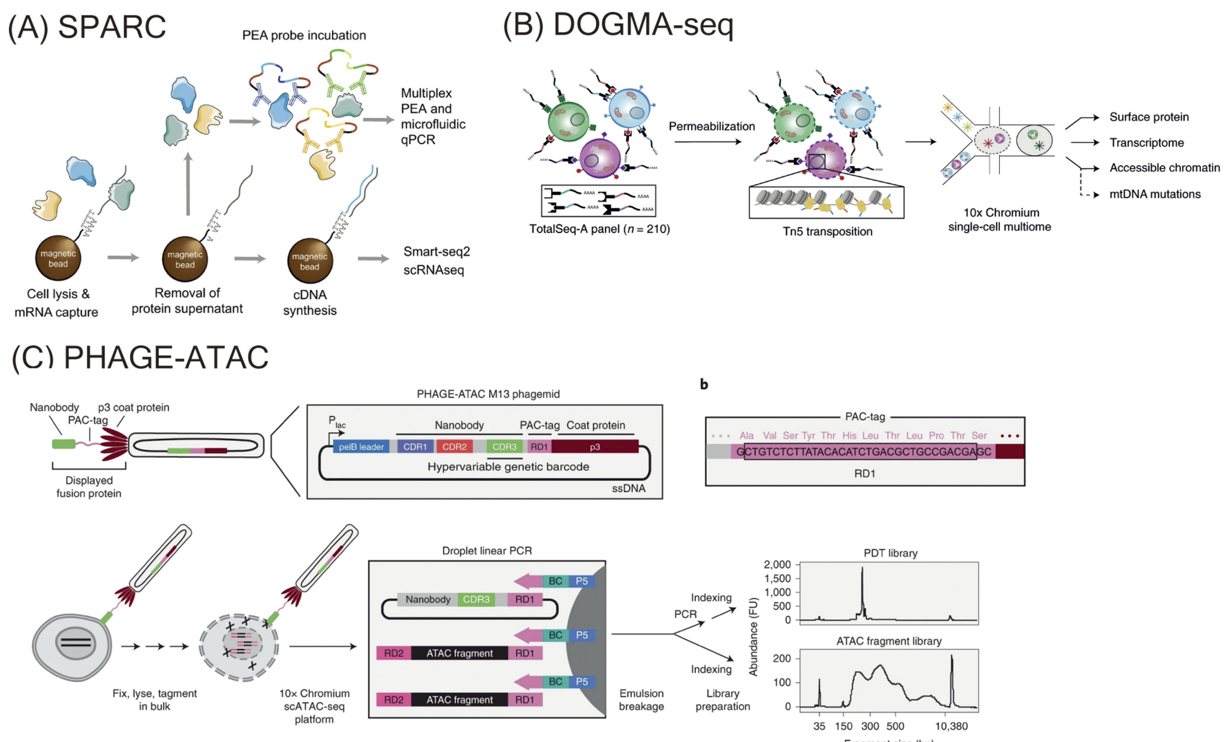


Fig. 5. (Color online) Joint single-cell proteomics with affinity tags. **A**. SPARC.[46] Single-cell isolation and cell lysis were performed in the presence of oligo-dT conjugated beads, followed by oligo-dT mRNA hybridization. Multiplex PEA and the modified Smart-seq2 approach were then used to process the proteinaceous supernatant and mRNA, respectively. **B**. DOGMA-seq.[50] Prior to fixation, permeabilization and transposition with Tn5, cells were labelled with oligo-conjugated antibodies. Within the droplet, bridge oligos spiked into the barcoding mix facilitated template elongation of the antibody tags during the first cycle of amplification, making them complementary to bead-derived barcoding oligos. Extended antibody tags were then barcoded along with the transposed chromatin fragments. **C**. PHAGE-ATAC.[54] After targeting the protein using a nanobody on the phage surface, fixation, lysis, and tagmentation processes were conducted in bulk, followed by droplet encapsulation of single-cells and 10× ATAC gel beads using 10× Genomics microfluidics. Following hybridization of the 10× barcoding primers to the RD1 sequence, linear amplification was performed with simultaneous droplet barcoding of chromatin fragments and phagemids, resulting in separate phage-derived tag (PDT) and ATAC-seq libraries. Panels adapted with permission from: **B**, ref. 50, Springer Nature Limited; **C**, ref. 54, Springer Nature Limited.

attention recently. Herein, three successful attempts are presented that have already been applied to or will be beneficial for single-cell proteomics.

First, Leonetti's group proposed the OpenCell platform, which provides a systematic proteome map of their intracellular localizations and interactions (Fig. 6A).[55] Analyzing the proteome using FP tags has been tried in early proteomics studies, but is not popular nowadays due to laborious library preparation requirements. OpenCell circumvented this problem by using CRISPR-mediated genome editing and constructed 1,310 cell lines with split FP tags. Because only one-eleventh of the partial unit of the FP is fused to the target protein, this tag does not alter the intrinsic properties of the target protein. Furthermore, this split FP tag can be used as a tag for immunoprecipitation for MS analysis, hence, protein-protein interactions with target proteins can be studied together. As a result, it was found that most protein-protein interactions are made of low stoichiometry and without strong similarities in their spatial distribution. On the other hand, high-stoichiometry interactors share very similar localization patterns, indicating that similarity in spatial distribution is a strong predictor of molecular interactions. In addition, through unsupervised segregation of the obtained spatial and interaction information, the proteome was largely categorized by three groups: soluble factors, membrane proteins, and RNA binding proteins. The last one, which was an unprecedented category, presumably plays an important role in gene expression control.

Another combined workflow is composed of AI-driven image analysis of cellular phenotypes, auto-

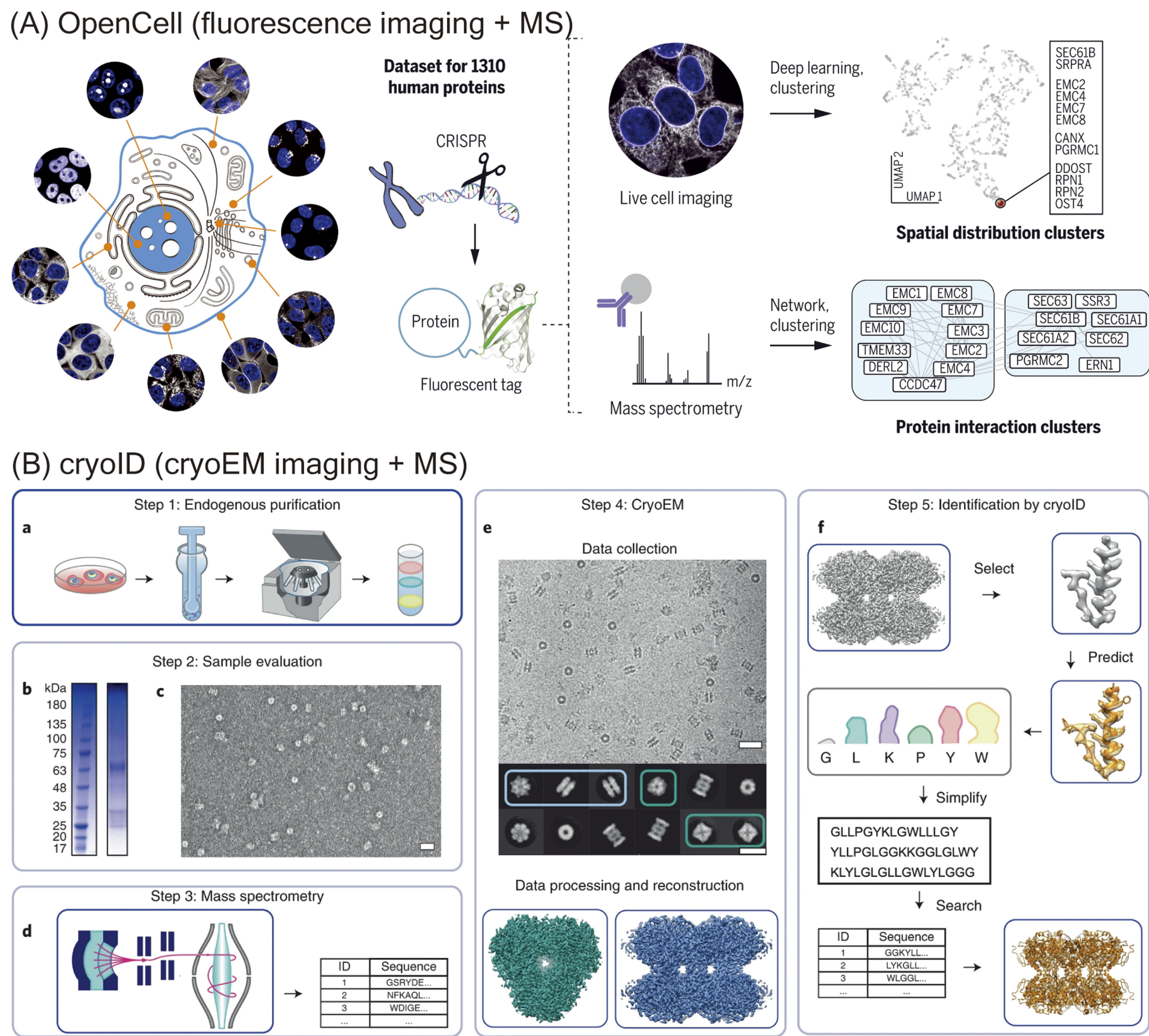


Fig. 6. (Color online) Combined approaches for single-cell proteomics. **A**. OpenCell. This approach uses a combination of endogenous tagging, live-cell imaging, and interaction proteomics to map the architecture of the human proteome.[55] A library of engineered cell lines was created using CRISPR to introduce split FP tags into 1,310 individual human proteins. The localization of each protein in live cells was imaged using fluorescence microscopy, and the interactions between a given target and other proteins within the cell were studied using MS. **B**. Endogenous structural proteomics workflow with cryoID.[58] Steps 1 and 2: Protein complexes were enriched by sucrose gradient fractionation, followed by SDS-PAGE and negative stain electron microscopy for sample evaluation prior to cryoEM. Step 3: MS identified a list of all proteins in each fraction. Steps 4 and 5: CryoEM analysis yielded near-atomic resolution cryoEM maps, and the major protein species in the cryoEM maps were identified using cryoID pipeline. Panel adapted with permission from: **B**, ref. 58, Springer Nature Limited.

mated laser microdissection, and highly sensitive MS, namely Deep Visual Proteomics.[56] To build a deep-learning-based algorithm for cytoplasm and nucleus segmentation, a large number of high-quality training data sets are required, which is challenging in practice. To solve this problem, artificial microscopic images that resemble real cells were constructed to train a deep neural network (DNN). In addition, feature-based phenotypic classification based on machine learning (ML) was combined with the expression level of biomarkers for precise cell classification. Once cells in the specimen were analyzed through their morphology as well as antibody and nucleus staining, cells or nuclei classified

as the same phenotypic group were excised by laser microdissection, and the contents were analyzed by MS. The spatial precision of laser microdissection used in this study was approximately 200 nm and throughput of 1,250 contours per hour. With this workflow, it can be possible to classify distinct cell states with spatial proteomic profiles defined by known and uncharacterized proteins, which will be useful for the molecular profiling of clinical samples in the future.

Finally, label-free visual proteomics that couples MS and cryo-electron microscopy (cryo-EM) is an intriguing approach and will be useful for single-cell proteomics in the future.[57] In the past, it was necessary to prepare homogeneous, pure samples when unidentified protein are analyzed using cryo-EM. Thus, identifying proteins from heterogeneous mixtures enriched directly from the endogenous source has been extremely challenging with cryo-EM because of a large pool of potential candidates, varied local resolution, and low overall resolution. Zhou *et al.* addressed this challenge by developing a targeted bottom-up structural proteomics approach (Fig. 6B).[58] In this study, a fraction of cell lysates was first analyzed by MS to obtain a list of proteins in the fraction, followed by cryo-EM imaging of the same fraction to construct 3D structural data on individual protein particles. Subsequently, the authors used their original data analysis pipeline, namely cryoID: predict the peptide sequence from the cryo-EM images, degenerate peptide sequences into 6-letter code, and search for the degenerate sequences from the list of proteins obtained using MS. It allows protein identification with high fidelity. A reverse order to this workflow was also reported. The 'Build and Retrieve' method, which conducts cryo-EM imaging first then performs iterative *in silico* purification and 3D *ab initio* classification until sufficient 3D structural information of the target protein is achieved.[59] With the constructed protein maps, the identities of proteins can be determined, and MS is performed finally to confirm the presence of the target protein. This method was found to be particularly useful for analyzing membrane proteins and protein complexes.

All three methodologies introduced above exploit MS and microscopic imaging such as fluorescence imaging or cryo-EM. It is noteworthy that the imaging methods supplement spatial or structural information on the proteome, which cannot be obtained easily with current MS technologies. Vice versa, the protein interactome was investigated by MS in OpenCell, which would be difficult to study with imaging-based approaches. Although combining multiple methodologies requires efforts to conduct thorough collaboration across varied specialized fields and data integrations obtained by different methods, once developed, it promises richer and deeper profiling of single-cell proteomes than any single approach.

6. Emerging technologies towards single-cell proteomics

6.1. AI and proteomics. AI technologies have progressed substantially in facilitating the analysis of complex and massive datasets. In the proteomics field, many studies are seeking ways to empower analysis pipelines with AI to reduce the number and time of measurements, leading to ultra-high-throughput for whole experiments. A successful example is DIA-NN, a software based on DNNs for data processing in data-independent acquisition (DIA) of MS (Fig. 7A).[60] Unlike data-dependent acquisition (DDA) that selects the most abundant precursor ions for further analysis, DIA analyzes all the precursors within the mass range of interest, resulting in high reproducibility and in-depth proteomic analysis.[61] Nonetheless, the computational processing of DIA datasets is complicated because each precursor ion gives a set of chromatograms corresponding to many fragment ions that interfere with other co-fragmenting precursors. Moreover, the analysis becomes more challenging when short chromatographic gradients are used, limiting the application of DIA-MS in high-throughput workflows. DIA-NN can solve these analytical bottlenecks by recognizing the characteristics of each elution peak in the DIA dataset and selecting the best candidate peak per precursor using iterative training with a linear classifier. Here, DIA-NN uses fully connected DNNs, trained in distinguishing the target from decoy precursors. As a consequence, this algorithm has enabled ultra-high-throughput clinical proteomics of COVID-19 infection (*cf.* DIA-MS analysis of 180 samples per day)[62],[63] and been incorporated in various cutting-edged MS techniques, such as plexDIA that quantified ∼1,000 proteins per single-cell with 5-minutes of active chromatography.[64]

Furthermore, an ML-based pipeline, Infinity Flow, allows overlapping flow cytometry panels for the simultaneous analysis of the co-expression patterns of hundreds of proteins across millions of individual cells (Fig. 7B).[65] The key feature of Infinity Flow is the labeling the samples with a

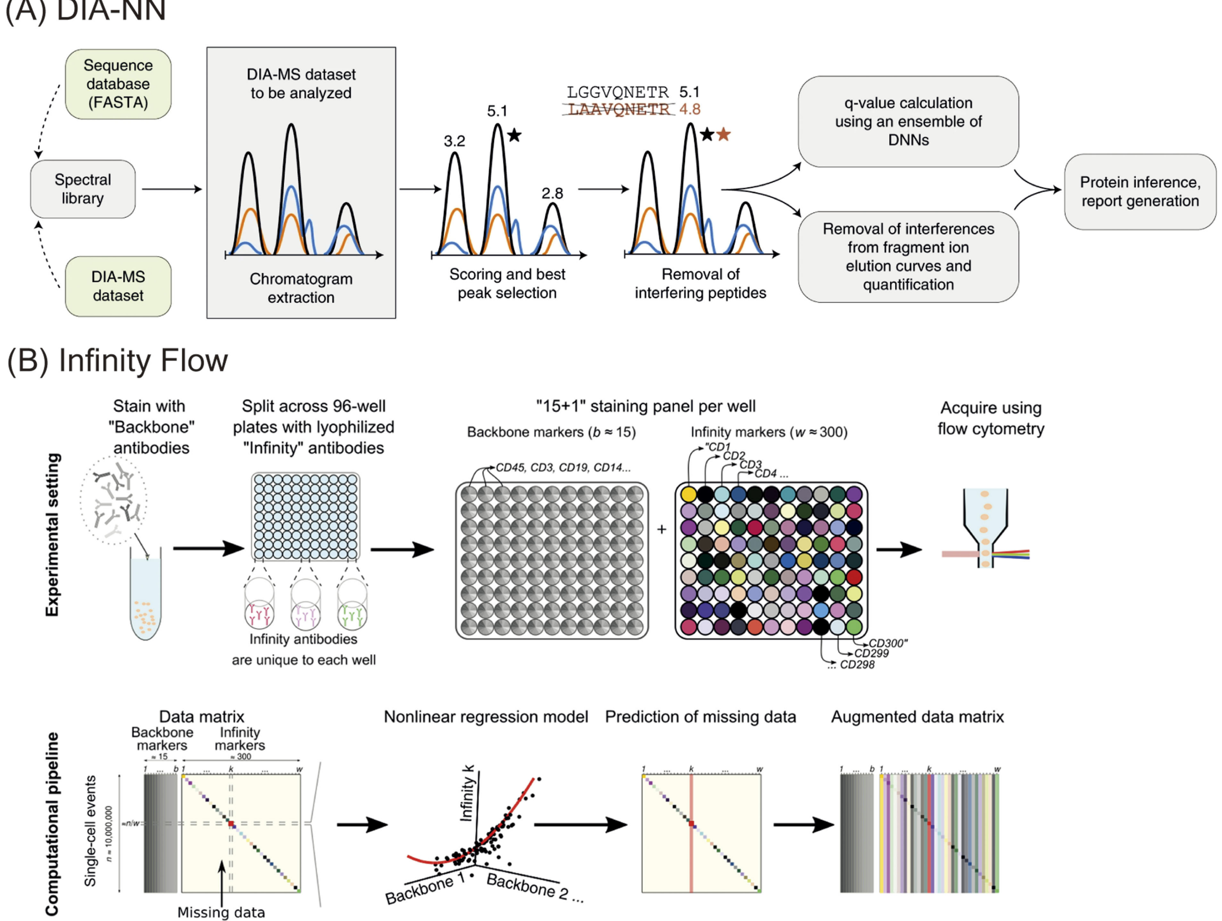


Fig. 7. (Color online) AI-aided high-throughput protein analyses. **A**. DIA-NN.[60] In light of spectral library that can be prepared either experimentally or *in silico*, mass chromatograms were extracted for an individual precursor ion and its corresponding fragment ions. Next, the 'best' candidate peak was selected based on the scores for probable elution peaks, and other interfering peptides were then detected and removed. The precursor-peak matches enabled the q-value calculation using an ensemble of DNNs and the removal of interferences from the fragment elution curves, improving the reliability and robustness of this method. **B**. Infinity Flow.[65] In the experimental setting, protein mixtures were stained with a backbone panel, followed by infinity panel staining, per-well staining panels, and data acquisition. In the computational pipeline, the obtained data matrix with dense backbone and sparsely non-missing Infinity marker measurements were subjected to the fitting of per-well nonlinear regression models and missing data prediction. Panel adapted with permission from: **A**, ref. 60, Springer Nature Limited.

cocktail of fluorescently labeled antibodies with at least one empty fluorescence channel (backbone) and a distinct and highly-specific antibody clone labeled with a fluorescent dye not used for the backbone (infinity panel). After flow cytometry experiments, a dataset composed of the intensities of forward scatter (FSC), side scatter (SSC), backbone, and the infinity panel is obtained. This data matrix is then analyzed using nonlinear multivariate regression to recast the disjointed data structure into a single cohesive expression matrix of the entire marker set across cells. For this purpose, an ML model is trained for every well, which predicts the expression of the infinity panel marker on a continuous scale from the measured intensities of FSC, SSC, and backbone marker. Once trained, these ML models are applied across the whole dataset to impute the intensity of each of the w Infinity antibodies across the n events, resulting in an $n \times w$ dense Infinity matrix of estimated intensities (Fig. 7B).[65] Using Infinity Flow, unknown cellular and functional heterogeneity were identified in the lungs of melanoma metastasis-bearing mice[65] and thymic epithelial cells.[66]

Not only to increase the throughput, but AI is also useful to extract biological implications from a limited number of results. This is the case for precious

samples such as patient-originated specimens and for certain types of biological assays with intrinsically low throughput such as 2D gel electrophoresis (2D-GE). Sawada and Hayashi *et al.* developed an AI-aided analytical algorithm for 2D-GE incorporating transfer learning for DNN.[67),68)] In brief, small-scale target domain data are directly inputted into a model constructed with source domain data, which are collected from a different domain from the target. Herein, target vectors are imputed with the outputted target domain data and further used to refine the model. Recognition performance of small-scale data is improved by reusing whole layers, including the output layers of the neural network. Using this algorithm, healthy individuals can be distinguished from those with disease using their serum proteome profiles.

There are numerous other recent studies that have employed AI for unsupervised data analysis, such as feature-based phenotypic classification of Deep Visual Proteomics[56)] and self-supervised profiling of protein subcellular localization of Open Cell,[55),69)] suggesting that AI has become an essential tool in modern proteomics.[70)]

6.2. Single-molecule peptide sequencing. Taking advantage of molecular sensitivity, single-molecule peptide sequencing holds great promise in single-cell proteomics as an alternative to MS.[71)] By cleaving a fluorescently labeled residue of a digested peptide sequence chemically or enzymatically and monitoring each reaction with a total internal reflection fluorescence (TIRF) microscope,[72)] it is feasible to obtain sequence data of individual peptides and may lead to protein identification. This idea was computationally justified by the groups of Joo and Marcotte; by ordered detection of cysteine and lysine labeled with two different fluorescence colors and selective enzymatic digestion resulting in a known terminal residue, in theory, one can identify almost the whole human proteome (Figs. 8A and 8B).[73),74)] Three years later, this concept was experimentally proved by both groups via analyzing single-molecule fluorescence trajectories during scanning of peptides with a fluorescently labeled protein translocase[75)] or removing N-terminal residues sequentially by Edman degradation.[76)]

Nanopore is an emerging single-molecule detection platform to read the sequence of biomolecules such as nucleic acids and peptides. Compared with MS and fluorescence microscopy with high-power lasers, in general, nanopore-based analyzers are easily accessible platforms because they are low cost, with no need for highly skilled operators, and sometimes portable.[77)] Recently, Oukhaled *et al.* identified 13 out of the 20 natural amino acids and 2 additional amino acids with an aerolysin nanopore by detecting ionic currents (Figs. 8C and 8D).[78)] The difference in ionic currents originates from the charged side groups and molecular shape of amino acid residues. With single-molecule peptide sequencing with nanopore technologies, it may become possible to identify several model proteins[77),79)] and the sites of PTMs.[80),81)] Furthermore, there have been substantial efforts made to develop novel pore components that enable multiple rereads,[82)] unfolding and threading of linearized proteins,[83)] and analyzing intact protein species.[84)] With advances in AI-aided analysis of sequencing data,[85),86)] nanopore platforms will become one of the central technologies for single-cell proteomics.

In addition, there have been many novel approaches to read peptide sequences, such as using aptamers to recognize amino acids,[87)] fluorescently labeled N-terminal recognizers,[88)] single-molecule Förster resonance energy transfer system that generates a structural fingerprint of a protein,[89)] and single-molecule Raman detection that discriminates amino acid residues.[90)] The rapid progress in the field of single-molecule peptide sequencing implies that there is room for new technologies to outperform conventional Edman degradation- and nano-pore-based methods.

6.3. Potential of single-molecule bioassays. Regarding single-molecule peptide sequencing, however, Slavov *et al.* recently raised a concern that it will face throughput and scalability problems in the future as MS did.[91),92)] It is known that the magnitude of the total number of protein molecules is 4–5 orders higher than that of mRNA molecules (*i.e.*, 1×10^{10} and 3×10^{5} molecules in a HeLa cell, respectively). If the current NGS and nanopore platforms for transcriptomics are used for proteomics, in other words, the running time and cost would be substantially extended. In imaging and nanopore-based sequencing platforms, the current throughputs per run are 1×10^{7} and 5×10^{7} molecules at best, respectively, and both require 1–2 days for data acquisition. Nonetheless, it does not mean one can simply increase running time because throughput does not increase linearly with running time (*cf.* a sixfold increase in time often increases the number of peptides that can be measured by only twofold).[91)] In addition, there is a clear limitation to the maximum number of molecules that can be

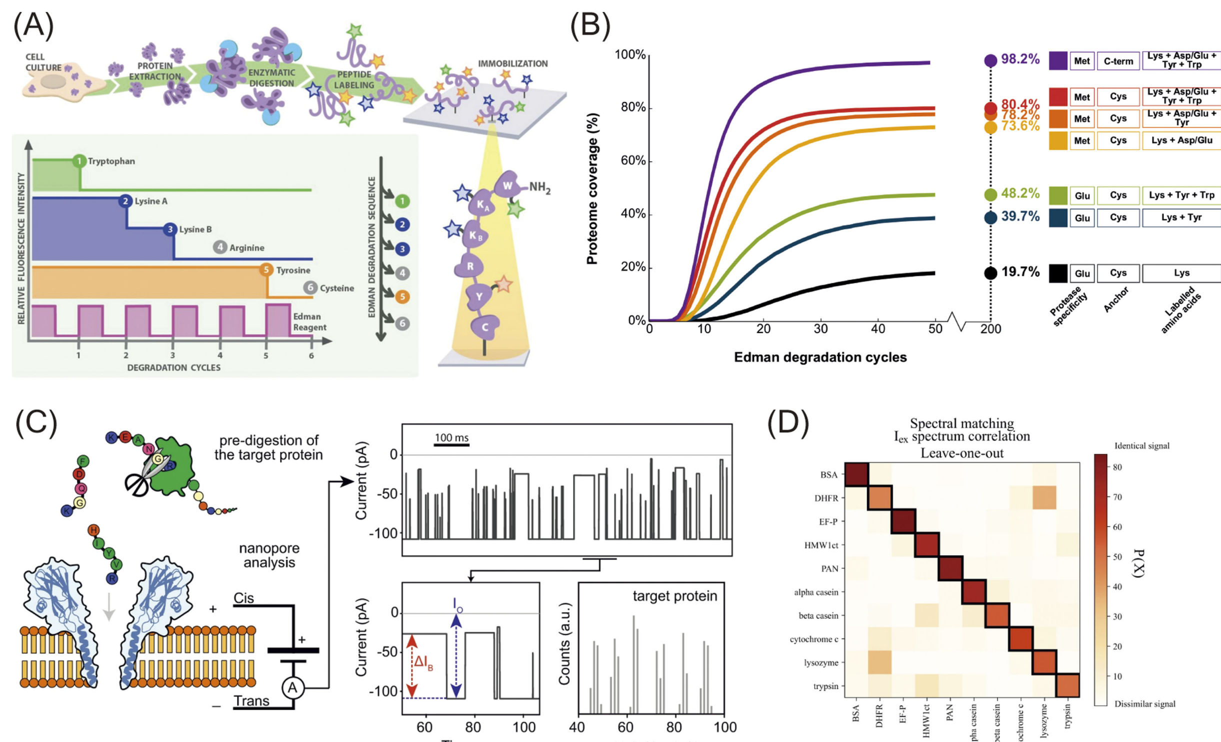


Fig. 8. (Color online) Single-molecule peptide sequencing. **A**. Illustrative scheme of the workflow of sample preparation and single-molecule peptide sequencing during Edman degradation. Herein, particular amino acids, such as tyrosine, tryptophan, and lysine, were fluorescently labeled, and proteins were treated with endo-peptidase for sequence-specific digestion.[74] Because Edman degradation removes the most N-terminal amino acids one by one, the step-drop of fluorescence intensity indicates the presence of the amino acid residue labeled with dye. **B**. Simulations of ideal experimental conditions of single-molecule fluorescence peptide sequencing with Edman degradation. **C**. Nanopore-based protein fingerprinting proposed by G. Maglia *et al.*[77] (Left) The target protein was pre-digested using a specific protease (*e.g.*, trypsin), and the cleaved peptides were measured as they translocated the nanopore, G13F-Fragaceatoxin C (G13F-FraC). (Right) The open pore current (I_O) decreased to the blocked pore current (I_B) when a peptide molecule entered the nanopore. The resulting excluded current ($\Delta I_B = I_O - I_B$) inferred the volume of the peptide. As a result, a histogram of the percentage of excluded current ($I_{ex}\% = \Delta I_B/I_O\%$) was used as a fingerprint spectrum to identify the protein. **D**. Spectral matching for 10 proteins with molecular weights ranging between 12.4 and 66.5 kDa using Euclidean cosine cross-correlation.[77]

detected at once using imaging-based detection because of the diffraction limit. Assuming an ideal placement of target molecules, the required sensor areas for the number of reads are 1 mm^2 for 10^6 reads, 1 cm^2 for 10^8 reads, and 10 cm^2 for 10^{10} reads, which becomes too large to perform single-molecule imaging, while maintaining practical throughput.[91] Furthermore, the wide dynamic range of proteome of about 10^6 orders has been a long-lived bottleneck for analyses. If the detection sensitivity and loading efficiency are not sufficient, which is the case for conventional MS and nanopore sequencing, respectively, low-copy proteins (*i.e.*, less than 10^3 molecules) cannot be investigated.

To address all these technical issues, we have sought the possibility of a combined methodology between single-molecule fluorescence imaging and conventional bioassays, such as gel electrophoresis. Previously, we investigated the labeling efficiency of reactive fluorescent dyes to protein molecules with varied molecular weights using a TIRF microscope.[93),94] In this study, dye labeled proteins along the whole lane of the polyacrylamide gel were extracted after electrophoresis, and fluorescence intensities of the individual protein molecules were measured (Fig. 9). As a result, the total proportion of proteins labeled with at least one dye (labeling occupancy) was 71%, whereby the values varied between 50% and 90% for proteins smaller than 32 kDa and the rest, respectively (Fig. 9C). In addition, for proteins with more than 30 lysine residues, over 80% of species were labeled by

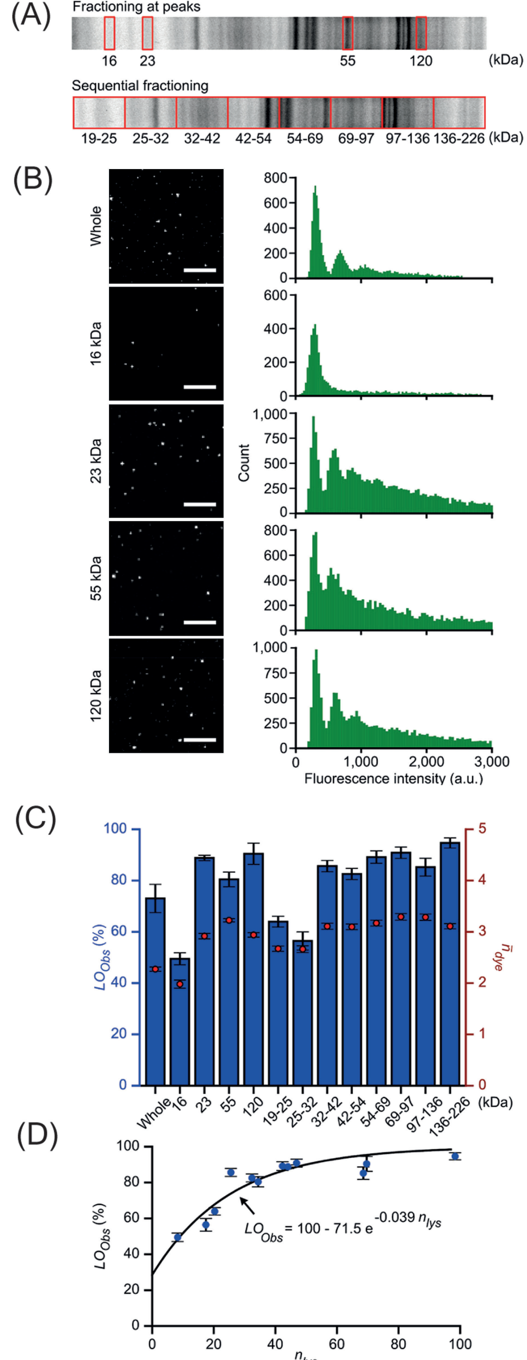


Fig. 9. Protein analysis with SDS-PAGE and single-molecule fluorescence imaging.[94] **A**. SDS-PAGE gel images of the HeLa cell proteome labeled with Cy5 dyes. **B**. TIRF microscopic imaging of the proteome sample within a certain molecular weight fraction extracted from the SDS-PAGE gel (left) and histograms of spot intensities (right). The scale bar is 10 µm. **C**. Labeling occupancy (LO_{Obs}) (blue) and the averaged number of labeled dye molecules ($\bar{n}_{dye}$, red) calculated from the single-molecule fluorescence imaging results (Fig. 9B). **D**. LO as a function of the estimated number of lysine residues (n_{lys}).

approximately three dyes on average (Fig. 9D). Taken together, our single-molecule analysis provided in-depth information about N-hydroxysuccinimide (NHS) ester mediated protein labeling; labeling occupancy was significantly affected by the size and number of lysine residues in the protein as well as the labeling reaction conditions such as pH and surfactant. This study suggested a possibility of single molecule proteome-level analysis by counting and measuring fluorescence signals from the dye-labeled protein molecules.

Provided that the polyacrylamide gel after electrophoresis can be analyzed with single-molecule fluorescence imaging, many of the current problems can be solved. Using single-molecule microscopy, individual protein or peptide molecules can be detected. In addition, the entire context of the sample can be analyzed, which is not feasible with the diffusion-dependent sample injection such as in a nanopore platform. Furthermore, if rapid 3D single-molecule imaging of a gel is realized, the theoretical limitation of imaging-based detection discussed earlier will be overcome, and detecting vast numbers of molecules at the proteome scale can be achieved. Based on the outlooks above, we are currently developing single-molecule bioassays aiming at single-cell proteomics using advanced microscopic techniques.

7. Summary and outlook

To conclude, let us summarize the advantages, disadvantages, and future opportunities of the major approaches in single-cell proteomics. First, MS has been the first choice for proteomics, and state-of-the-art MS techniques can identify a few thousands of proteins in a typical mammalian cell with high throughput (*cf.* 150 cells/day with DIA-NN).[60),95)] To achieve such sensitivity and throughput, minimizing the sample volume within sub-microliter scales and with an automatic robotic dispenser become prerequisite in recent studies. Nonetheless, biologically important information such as intracellular localization of protein, conformation, and other related omics cannot be investigated at the single-cell level with the current MS techniques. It is worth noting that there are several MS techniques for the purposes listed above,[96)–99)] but their application to single-cell proteomics has not been reported as yet. This might be due to the sensitivity problem, with 5,000–20,000 copies required to be quantified by MS because of the inefficient ionization of protein molecules. Although a few studies on single-cell

phosphoproteomics have been reported,[100),101)] it is still technically challenging for MS to investigate biologically important low-copy proteins (*i.e.*, transcription factors) or modified proteomes, of which the intracellular concentrations are generally low and fluctuate according to the cell state at single-cell level. In addition, the MS techniques utilized in previous decades were relatively weak in terms of the quantitative performance; peptides with different sequences have different efficiencies of ionization, for which intensities in a mass spectrum cannot accurately reflect their abundances.[102)] If these limitations are solved by technical advances, mass imaging[103)–105)] and top-down MS[8),106)] would be powerful tools to conduct protein identification and study additional biologically important information such as subcellular localization and various proteoforms, respectively.

Second, fluorescence imaging of proteins at the proteome level can determine their intracellular localizations, which is mostly found to be closely related to the protein function and protein-protein interactions. Using FP tags and fluorescently labeled antibodies, it is currently possible to map over 1,000 and 10,000 proteins in a single mammalian cell, respectively.[30),55)] In addition, the spatial resolution can be beyond the diffraction limit through adopting expansion microscopy.[107)] Although typical fluorescence imaging can distinguish 3–5 colors at once, the multiplexity of fluorescence imaging can be greatly expanded by using oligo tags. To detect oligo-tagged antibodies, most of the current methods rely on the same principle of fluorescence *in situ* hybridization (FISH). It is important to note that targets of multiplexed imaging can be extended to transcriptome in single cells. A typical method for such spatial transcriptome analysis is sequential FISH (seqFISH).[108),109)] This method pioneered microscopy-based omics analysis based on iterative probing and imaging of the targets. Here, many species of RNA in a cell can be identified and quantified through a cycle of hybridization of fluorescently labeled DNA to different RNA targets, imaging and de-hybridization or fluorescence quenching. In principle, proteome analysis using oligo-tagged antibodies and immunofluorescence imaging such as CODEX can be done in parallel with seqFISH-based transcriptome analysis, by applying different fluorescent probing methods in each iterative imaging cycle. Hence, microscopy-based approaches possess a clear potential to achieve single-cell multi-omics analysis with spatial information.[110),111)] On the other hand, the preparation of an FP-tagged library at the proteome level or iterative staining and elution of antibodies for more than thousands time are sufficiently laborious and time-consuming to limit the application of this method to other cell lines or various biological models. In addition, the efficiency of FP fusion may differ depending on the protein species, which makes quantitative proteome analyses difficult. Regarding antibody staining, it exhibits the same problems as conventional immunoassays, for instance, variation in the affinity of the antibodies depending on the manufacturer or batch and false positive signals caused by non-specific binding.

Third, single-cell proteomics with sequencing can take advantage of the commercial oligo-labeled antibody panels and up-to-date NGS platforms for other single-cell studies, such as 10× Genomics microfluidics. Accordingly, among the three major approaches above, sequencing-based approaches have been the most successful for investigating multiple layers of gene regulation in a single cell as DOGMA-seq did.[50)] Additionally, low-copy proteins can be detected because oligo tags can be amplified. Using a PC-antibody and spatially controlled UV irradiation, the intracellular localization of a protein can be also investigated.[44)] However, this approach requires the use of affinity reagents, mostly antibodies, to detect proteins. As explained above, this method has the same problems as conventional immunoassays. Furthermore, preparing oligo-labeled antibodies at the proteome scale is laborious and requires synthesis skills to label oligonucleotides onto antibodies while maintaining the affinity of the antibody.

As summarized in Table 1, it is advisable to choose a suitable method based on the purpose of the single-cell analysis. In the near future, combined approaches with MS and other visualization methods are expected to be widely used, and many emerging technologies such as AI and single-molecule detection may break the technical limits of currently available methods.

With the aid of anticipated methodological advances, it is then crucial to find adequate targets for single-cell proteomics. Analysis of cycling proteins that are regulated post-translationally is a good example.[36)] In this sense, PTM, conformational changes, complex formation, and protein-protein interactions will be important targets in single-cell proteomics. These are closely related to the activation and deactivation of particular signaling pathways, which cannot be studied at genomic and transcriptomic levels. If modified single-cell proteo-

Table 1. Comparison of the major methodologies for single-cell proteomics

Methods	Advantages	Disadvantages	Opportunities
(Bottom-up) MS	• In general, MS directly detects proteins in a label-free manner. • Workflow can be easily applied to high-throughput analyses. • Various compatible separation technologies have been developed (*e.g.*, LC, CE, *etc.*).	• Requires high-cost instrumentation • Relatively low sensitivity (*cf.* 5,000–20,000 copies required) • Depending on the properties of protein species (*e.g.*, solubility, ionization, *etc.*), it is not facile to perform highly quantitative analyses for some protein groups.	• There have been several attempts to use MS to identify protein species detected by different detection methods (mostly microscopy). • With the improved sensitivity, mass imaging and top-down MS may be powerful tools for single-cell proteomics in the future.
Fluorescence imaging	• Can be conducted with bench-top microscopy. • Exhibits high sensitivity up to single-copy. • Spatial information (intracellular localization) can be obtained. • (With FP-tags) Can perform live-cell analysis.	• Efficiency of FP fusion may differ depending on protein species. • As for antibody staining, preparing antibodies at the proteome scale is high cost. • As for antibody, variance in affinity and non-specific binding may affect the results.	• With modern spatial transcriptomic methods (*e.g.*, seqFISH), joint single-cell spatial omics will be realized. • Advanced microscopy may enable high-throughput and single-molecule sensitive protein analyses.
Sequencing	• Exhibits high multiplexity (the number of barcodes = $4^{\textit{length of the oligo}}$). • Can amplify the oligo tag to detect low-copy proteins. • Can exploit commercial reagents and platforms for single-cell omics.	• Exhibits common problems of antibody staining. • Preparing proteome-scale oligo-labeled antibodies is demanding.	• With a PC linker, spatial information can be obtained. • Most compatible with joint omics studies (*e.g.*, transcriptomics, chromatin accessibility, *etc.*)

mics are investigated, 'protein activation velocity' can be calculated from the ratio between phosphorylated and dephosphorylated proteins, which may be useful to predict the future of cells. This prediction might be nearer future than that expected from RNA velocity (*cf.* RNA velocity = [unspliced mRNA] − $k_{\text{degradation}}$[spliced mRNA])[112),113)] or might contain a completely different picture that cannot be demonstrated at the transcriptome level.

Furthermore, we expect that single-cell proteomics will widely benefit the medical field; AI-based image analysis of 2D gels has been used for sepsis diagnosis,[67)] digital spatial profiling has enabled thorough inspection of tumor microenvironments (Fig. 4),[44)] and deep-phenotyping of rare cells, such as circulating tumor cells, have been performed.[114)] Single-cell proteome analysis can be described as ultimately multiplexed and sensitive protein detection methods providing a system-wide overview of the current physiological status of individuals. Thus, single-cell proteomics will be an effective tool in early diagnosis, precision and personalized medicine,[115),116)] and become a new paradigm of *in vitro* diagnostics in the future.

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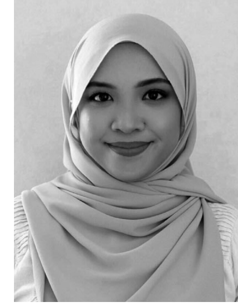

Profile

Yuichi Taniguchi was born in 1979 in Gifu, Japan. He earned his Ph.D. in biophysics under the supervision of Prof. Toshio Yanagida at Osaka University in 2006. He then worked as a postdoctoral fellow with Prof. Sunney Xie at Harvard University until 2011. Then, he founded his laboratory at RIKEN as a unit leader. In 2020, he started a career as a professor at the Institute for Advanced Study and Graduate School of Biostudies in Kyoto University. He works on biophysics research aimed at developing new approaches for life sciences. He has developed a method for nucleosome-resolved 3D genome structure analysis, a method for quantifying single-cell proteomes and transcriptomes with single molecule sensitivity, and light sheet microscopy, which has now been commercialized by Carl ZEISS.