

Multiplexed Single-Cell in Situ Protein Profiling

Yi Chen and Jia Guo*

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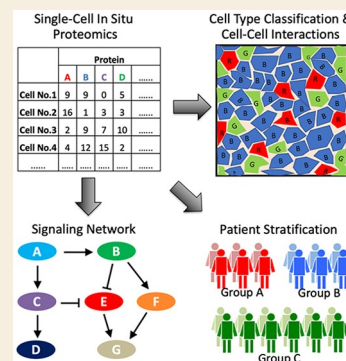
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ABSTRACT: The ability to profile a large number of different proteins in individual cells in their native cellular locations is critical to accelerate our understanding of normal cell physiology and disease pathogenesis. Bulk cell protein quantification masks the cell heterogeneity in complex biological systems, while conventional immunofluorescence or immunohistochemistry are limited by their low multiplexing capacity. Recent technological advances in multiplexed protein imaging approaches allow many distinct proteins to be analyzed in single cells in situ. These methods will bring new insights into various biological and biomedical fields, such as cell type and subtype classification, signaling network regulation, tissue architecture, and disease diagnosis and prognosis, along with treatment monitoring. In this Review, we will describe the recent advances of multiplexed single-cell in situ protein profiling technologies, discuss their unique advantages and limitations, highlight their applications in biology and medicine, present the current challenges, and propose potential solutions.

KEYWORDS: immunofluorescence, immunohistochemistry, proteomics, heterogeneity, microenvironment, antibodies



1. INTRODUCTION

The composition of molecularly and functionally different cells is a common feature of most of the biological systems.¹ Such cell heterogeneity exists not only in multicellular tissues but also in genetically identical bacterial and yeast cells.² Heterogenous cells are also observed in various biological processes, such as cancer metastasis,³ tumor response to treatment,^{4–6} stem cell differentiation,⁷ immune response,⁸ etc. Cell heterogeneity can be attributed to many factors, including distinct genetics or epigenetics, varied microenvironments, and stochastic gene expression, among others. The variations among the single cells in a biological system may play important roles in the function, health and survival of the entire system. Nevertheless, many experiments are carried out using groups of cells, which can obscure the important cell differences in the system. Thus, the single-cell assays are critically needed to advance our understanding of biology and medicine.

Each individual cell is composed of a huge collection of different biomolecules, which are involved in various signaling pathways. To understand how these complex signaling networks function in normal cells and malfunction in diseases, comprehensive molecular profiling is required.⁹ Among all the biomolecules, proteins are crucial to many cell functions and processes, including biochemical reaction catalysis, gene expression regulation, biomolecule transport, and cellular structure support, etc. Thus, the development of single-cell comprehensive protein profiling technologies is critical to accelerate our understanding of health and disease.

The well-defined locations of biomolecules in a cell and cells in a tissue are crucial for effective biomolecule-to-biomolecule or cell-to-cell interactions, which can determine the organization, function and regulation of the biological systems.¹⁰ For example, neurons develop and maintain their highly polarized structures by tightly regulating the synthesis and transportation of the different RNAs and proteins at their distinctive compartments, such as cell bodies, dendrites, axons, and synapses. And to have effective signal transmission in neural circuits, the locations of presynaptic and postsynaptic neurons in the brain tissue are also precisely regulated. In addition, it has been demonstrated that chromosomes are hierarchically organized into various compartments composed of different topologically associating domains to form 3D genome architecture;¹¹ disease-related genes can mislocate in cancer cells;¹² and the locations of stem cells in specialized niches can determine their fates.¹³ Thus, to better understand the architecture, regulation, and interactions in these complex biological systems, there remains substantial need to develop single-cell in situ comprehensive protein profiling technologies.

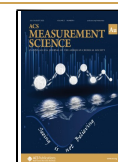
Because of the low abundances of the proteins in single cells and the inability to amplify those proteins, extremely sensitive methods are required for single-cell protein profiling.¹⁴

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Fluorescence microscopy has high detection sensitivity and, thus are widely applied for analyzing proteins in individual cells. Nonetheless, the spectral overlap of the common fluorophores limits the multiplexing capacity of these imaging based approaches.¹⁵ Conventional protein microarray¹⁶ and mass spectrometry¹⁷ allows comprehensive protein analysis. However, as these methods require proteins in a sample to be isolated and purified from other cellular components, the location information on the protein targets is lost. To address these limitations and enable single-cell in situ comprehensive protein profiling, novel methods with high sensitivity and multiplexing capacity are critically needed.

Here, we review the recent advances in the development of single-cell in situ proteomic technologies, including mass spectrometry imaging, reiterative immunofluorescence with fluorescent antibodies, and reiterative immunofluorescence with signal amplification. These imaging-based approaches allow multiplexed protein profiling in individual cells at the subcellular resolution. Readers are referred to other articles^{9,10,18–21} concerning alternative single cell proteomic or spatial proteomic technologies. We will introduce their advantages and limitations, and highlight their applications to explore cell signaling networks, classify the distinct cell types, and investigate cell–cell interactions in their microenvironment. The broad impact of these techniques on understanding, diagnosis, prognosis, and treatment of various diseases will also be discussed. In the end, we will describe the current challenges of these technologies and propose potential solutions.

2. MASS SPECTROMETRY IMAGING

To enable multiplexed single-cell in situ protein profiling, ion beam imaging²² and imaging mass cytometry²³ have been developed. In these approaches (Figure 1), the different

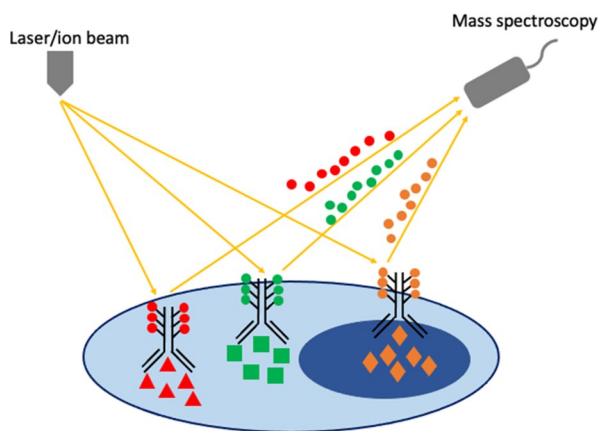


Figure 1. Mass spectrometry imaging. After incubation with metal isotope labeled antibodies, the specimen is transferred pixel-by-pixel into a mass spectrometer by an ion or laser beam. Then, the protein abundances are calculated by computer software using the mass data of the identified metal isotopes.

protein targets are recognized by the corresponding antibodies conjugated with varied metal isotopes. Then, ion or laser beams are applied to convert the specimen pixel-to-pixel into streams of particles. The metal isotopes in the particles are detected by a mass spectrometer to decipher their compositions and abundances. Subsequently, the collected mass data of individual pixels is translated into protein

expression levels by computer software. As all the protein targets are stained simultaneously with antibodies labeled with distinct metal isotopes, these methods minimize the epitope decay during the assay and also facilitate image registration. Additionally, by selecting the metal isotopes that do not exist in the specimen, these approaches avoid the false positive signals generated by endogenous biomolecules. However, since the specimen is analyzed pixel-to-pixel, it takes these methods ~ 8 h to image 1 mm^2 sample.^{22,23} As a result, the current ion beam imaging and imaging mass cytometry have low sample throughput and long assay time. Another challenge is the instrument availability, as the expensive high-resolution imaging mass spectrometers are rare in clinical setting and academic institutions, which can hinder their broad applications.

3. REITERATIVE IMMUNOFLUORESCENCE WITH FLUORESCENT ANTIBODIES

To allow high-throughput in situ proteomic profiling with commonly available instruments, reiterative immunofluorescence approaches with fluorescent antibodies have been explored. Each analysis cycle of these methods is composed of three major steps. First, the protein targets in the specimen are recognized with antibodies labeled with different fluorophores. Then, fluorescence images are captured under a microscope to quantify the targets in their native spatial contexts. Finally, the staining signals are erased to initiate the next analysis cycle. Through cycles of target staining, fluorescence imaging, and signal removal, a large number of different proteins can be profiled at the optical resolution in situ. For instance, with M reiterative cycles and N proteins quantified in every cycle, a total of $M \times N$ proteins can be analyzed in the sample. Instead of examining the specimen pixel-to-pixel in mass spectrometry imaging, reiterative immunofluorescence can image millions of pixels simultaneously within milliseconds to seconds. As a result, reiterative immunofluorescence allows a large specimen or 3D tissue to be profiled in a relatively short time. Additionally, as a standard fluorescence microscope is the only instrument required, reiterative immunofluorescence can be easily adopted by various academic and clinical laboratories.

Two critical requirements exist for the success of the reiterative immunofluorescence approaches. First, the staining signals have to be efficiently removed at the end of each analysis cycle, so that the minimum signal leftovers will not interfere with the accurate protein quantification in the following cycles. Second, the signal removal process should not damage the integrity of the epitopes or the morphology of specimen. In this way, other protein targets can be precisely profiled in the later cycles. To fulfill these requirements, different approaches have been explored. In this section, we will discuss the recent technological advances of these reiterative immunofluorescence technologies.

3.1. Chemical/Photo-Bleaching and Antibody Stripping

In 2006, Schubert et al. have applied photobleaching²⁴ to eliminate the staining signals generated in each cycle of reiterative immunofluorescence (Figure 2A). With this method, multiplexed in situ protein profiling has been successfully achieved in cells and tissues. However, since the varied fluorophores have to be bleached individually and the different imaging areas have to be bleached sequentially, this method suffers from limited sample throughput. To allow all

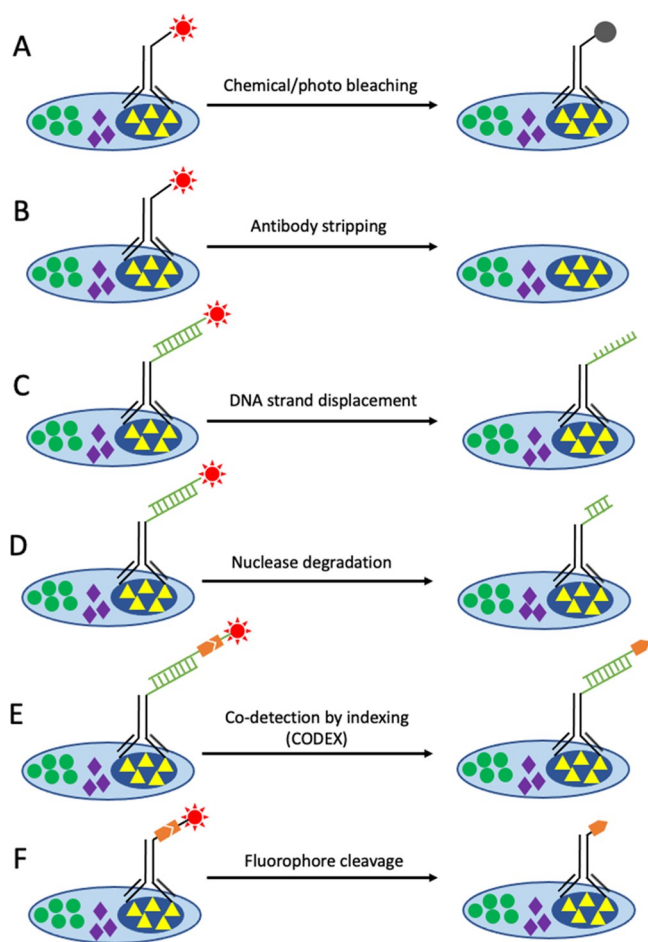


Figure 2. Approaches used in reiterative immunofluorescence to erase fluorescence signals. (A) Staining signals are removed by chemical- or photo-bleaching. (B) Fluorescent antibodies are stripped from their protein targets. (C) DNA strand displacement reactions remove the fluorescent oligonucleotides hybridized to oligonucleotide labeled antibodies. (D) Nucleases are applied to degrade the fluorescent oligonucleotides conjugated to antibodies. (E) In Co-detection by indexing (CODEX), the proteins are stained by incorporation of the fluorescent nucleotides into the primers hybridized to oligonucleotide labeled antibodies. Subsequently, the fluorescence signals are erased by chemical cleavage. (F) Fluorophores conjugated to antibodies through a cleavable linker are removed by chemical reactions.

the fluorescence signals in the whole specimen to be removed simultaneously, chemical bleaching (Figure 2A) and antibody stripping (Figure 2B) methods have been developed. In chemical bleaching,^{25,26} H_2O_2 in basic or acidic is used to deactivate the fluorophores. And in antibody stripping,^{27,28} high or low pH solutions containing sodium dodecyl sulfate is applied to elute the antibodies by breaking the antibody–antigen interactions. Although it has been demonstrated that the staining signals can be efficiently erased by these approaches within 1 h, the repeated harsh chemical treatments can lead to epitope damage and specimen degradation.^{28,29} Consequently, these methods have the limited number of analysis cycles and the accuracy of protein quantification in the later cycles can be compromised.

3.2. Mild Signal Removal Methods

To effectively erase the staining signals without damaging the integrity of epitopes, oligonucleotide conjugated antibodies have been explored to recognize the protein targets. These

oligonucleotides can recruit complementary fluorescent oligonucleotides or function as templates to incorporate fluorescent nucleotides to stain the proteins. After image capture, the fluorescent oligonucleotides can be removed by DNA strand displacement reactions³⁰ (Figure 2C) or degraded by nuclease³¹ (Figure 2D). Or a mild chemical reaction³² can be applied to cleave the fluorophores attached to the bases of the incorporated nucleotides (Figure 2E). Although these mild signal removal methods maintain the epitope integrity, some nonideal factors still exist. For example, the large sizes and the negative charges of the oligonucleotides could potentially interfere with the binding specificity and affinity of the conjugated antibodies. Moreover, the varied oligonucleotides on different antibodies could potentially mishybridize to each other or to endogenous nucleic acids, to generate false positive signals. And to minimize such cross-hybridization, the number of the varied oligonucleotides that can be applied and corresponding multiplexing capacity of the assay are also limited.

To avoid the issues caused by oligonucleotides conjugated antibodies, cleavable fluorescent antibodies (Figure 2F) have been developed.³³ Instead of using bulky and negatively charged oligonucleotide to tether the fluorophores to antibodies, this approach applies a neutral and small molecule linker to attach the fluorophores and antibodies. Following target recognition and fluorescence imaging, the fluorophores are efficiently cleaved using tris(2-carboxyethyl)phosphine (TCEP) to reduce the azide in the cleavage function group $-OCH(N_3)-$. With this mild and biorthogonal cleavage reaction, it has been demonstrated that the epitope integrity is maintained after this chemical reaction. And as the small molecule linker is neutral and has much smaller size than oligonucleotides, the interference from the conjugation on the binding specificity and affinity of the antibodies could be reduced. Finally, by avoiding the nonspecific binding between the conjugated antibodies and also between the antibodies and endogenous biomolecules, cleavable fluorescent antibodies enable accurate in situ proteomic analysis.

4. REITERATIVE IMMUNOFLUORESCENCE WITH SIGNAL AMPLIFICATION

In the reiterative immunofluorescence approaches discussed above, only a couple of metal isotopes or fluorophores are conjugated to each antibody molecule. Without any signal amplifications, these methods suffer from low detection sensitivity. As a result, their applications to profile proteins with low abundances are hindered. Additionally, their analysis accuracy is compromised when examining the specimen with high autofluorescence, such as formalin-fixed paraffin-embedded (FFPE) tissues,³⁴ which are the most commonly archived clinical tissues.³⁵ Moreover, the weak sensitivity of these approaches also leads to the long imaging exposure time and thus the low sample throughput. To tackle these issues, several reiterative immunofluorescence with signal amplification approaches have been developed recently. In this section, we will present the technological advances of these methods.

4.1. Cleavable Fluorescent Streptavidin

To enable highly sensitive and multiplexed in situ protein profiling, a layer-by-layer signal amplification method with cleavable biotin conjugated antibodies and cleavable fluorescent streptavidin has been explored.³⁶ In this approach (Figure 3), antibodies labeled with cleavable biotin are first applied to

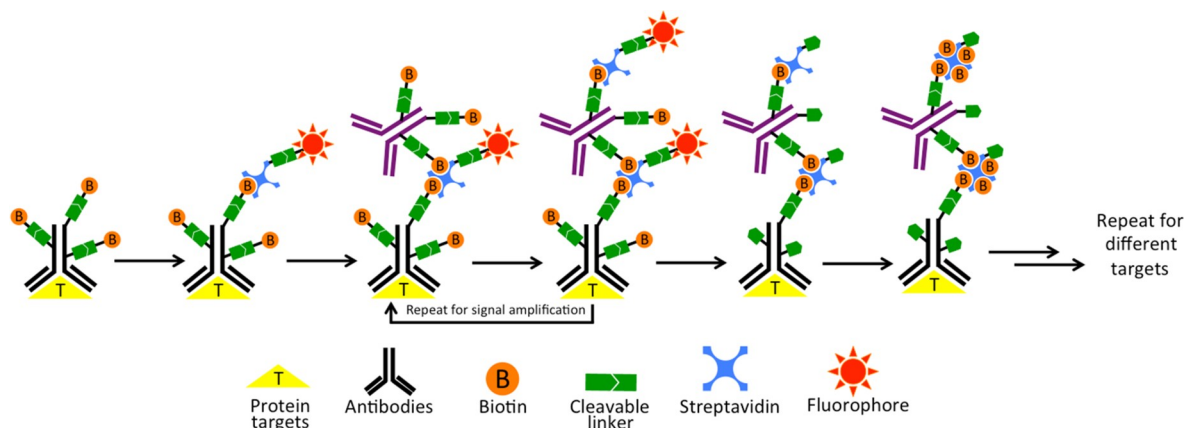


Figure 3. Highly sensitive and multiplexed in situ protein profiling with cleavable fluorescent streptavidin. In every cycle, the proteins of interest are first recognized by antibodies conjugated with cleavable biotin, and subsequently stained with cleavable fluorescent streptavidin. Then, the staining signals are amplified by the repeated incubation with cleavable biotin conjugated orthogonal antibodies and cleavable fluorescent streptavidin. Once the desired signal intensities are obtained, all the fluorophores and the unbound biotins are cleaved by a mild chemical reaction. After blocking the leftover streptavidin with biotin, the next cycle is initiated. Reproduced with permission under a Creative Commons CC-BY license from ref 36.

recognize the proteins of interest. Subsequently, cleavable fluorescent streptavidin and cleavable biotin conjugated orthogonal antibodies are applied repeatedly, to amplify the staining signals layer-by-layer until the preferred signal intensities are obtained. After imaging, the fluorophores and the unbound biotins are cleaved by a mild chemical reaction. Following streptavidin blocking with free biotin, the next analysis cycle is initiated. Through reiterative cycles of target recognition, signal amplification, fluorescence imaging, fluorophore and biotin removal, and streptavidin blocking, a large number of proteins can be sensitively detected in single cells in situ. It has been demonstrated that this approach enhances the protein detection sensitivity by at least 1 order of magnitude. Additionally, almost all the reiterative immunofluorescence approaches require the prior knowledge of the relative expression levels of the protein targets, so that proteins can be quantified from low to high abundances. This prior knowledge requirement is eliminated by the cleavable fluorescent streptavidin method, as the staining signals for different proteins can be amplified until the desired intensities are generated. In this way, the staining intensities in the latter cycles are always higher than those in the previous cycles. Nonetheless, the layer-by-layer signal amplification process in this approach can be time-consuming. And the protein detection sensitivity of this technology needs to be further improved.

4.2. Immuno-SABER and CosMx

Recently, two alternative sensitive in situ proteomics methods named immunostaining with amplification by exchange reaction (Immuno-SABER)³⁷ and CosMx³⁸ were explored. In both methods (Figure 4), protein targets are recognized by antibodies conjugated with oligonucleotides, which subsequently recruit the DNA concatemers generated by primer exchange reaction or photocleavable oligonucleotides. By sequentially applying multiple DNA concatemers or photocleavable oligonucleotides, the number of binding sites for fluorescent oligonucleotides is significantly increased. Following signal amplification, target staining and image capture, the fluorescence signals are erased by oligonucleotides dehybridization with hot formamide solutions, or by photocleavage. With repeated cycles of target staining and signal removal,

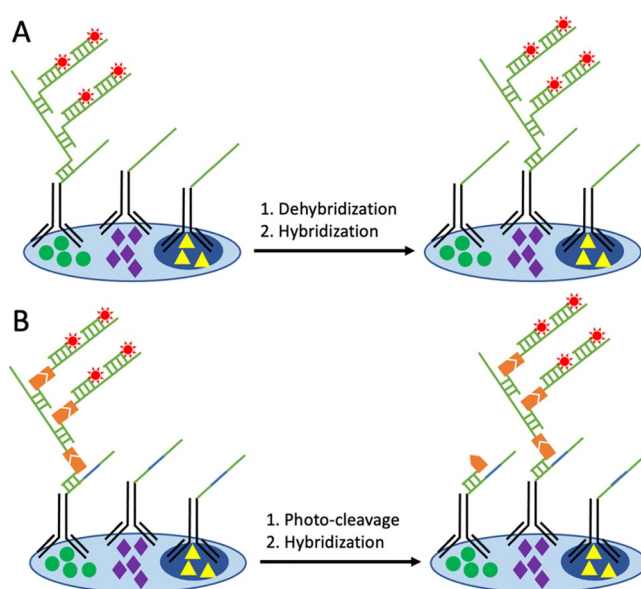


Figure 4. (A) Immunostaining with signal amplification by exchange reaction (Immuno-SABER). The protein targets are recognized by oligonucleotides conjugated antibodies. Subsequently, DNA concatemers and a large number of fluorescent oligonucleotides are recruited to stain the targets by hybridization. With reiterative cycles of dehybridization and hybridization of the fluorescent oligonucleotide probes, the different protein targets can be quantified in situ with high sensitivity. (B) Immunostaining with signal amplification in CosMx. After the protein targets are recognized by oligonucleotides conjugated antibodies, photocleavable and fluorescent oligonucleotides are sequentially recruited to stain the targets by forming a large branched DNA structure. With repeated cycles of photocleavage and hybridization of the branched DNA, the varied protein targets can be sensitively quantified by a color barcode in single cells.

highly sensitive and multiplexed in situ protein profiling can be achieved. However, similar to the other methods using oligonucleotides conjugated antibodies, these approaches could potentially suffer from the potential cross-hybridization between the oligonucleotide probes and endogenous nucleic acids. Additionally, to prepare oligonucleotides conjugated antibodies while maintaining their binding specificity and affinity can be technically demanding.

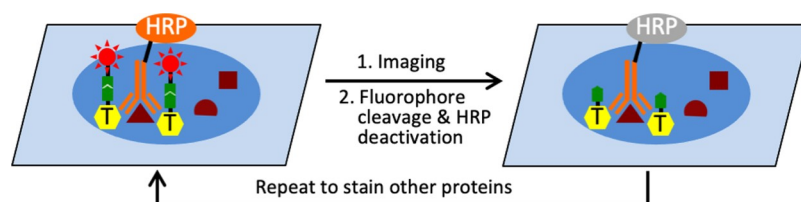


Figure 5. Highly sensitive and multiplexed in situ protein analysis with cleavable fluorescent tyramide. In each cycle, the protein targets are stained with horseradish peroxidase (HRP) conjugated antibodies and cleavable fluorescent tyramide. Following image capture, the fluorophores are cleaved and the HRP is deactivated simultaneously by a mild chemical reaction. Through repeated cycles of target staining, imaging, fluorophore removal, and HRP quenching, a large number of varied proteins can be profiled in situ with high sensitivity.

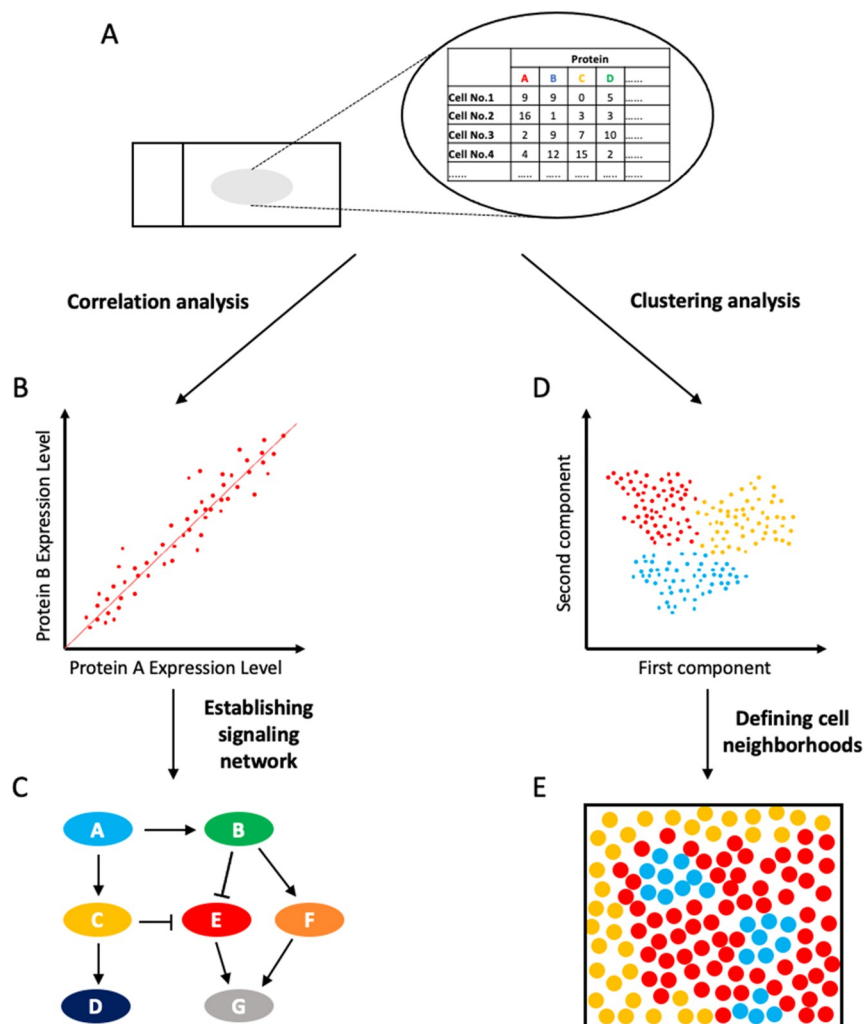


Figure 6. (A) By multiplexed in situ protein profiling, the abundances of various proteins in individual cells of a biological system are obtained. (B) With the single-cell protein expression profiles, the pairwise protein expression correlation analysis can be carried out. (C) Generated protein expression correlation coefficients can be applied to establish the protein signaling networks. (D) On the basis of their unique protein expression patterns, individual cells can be partitioned into distinct subgroups. (E) By mapping the cells back to their original locations in the tissue, different cell neighborhoods composed of cells from specific subgroups can be identified.

4.3. Cleavable Fluorescent Tyramide

To enable highly sensitive and multiplexed protein in situ analysis, an approach using cleavable fluorescent tyramide has also been developed.^{39–41} In this method (Figure 5), antibodies conjugated with horseradish peroxidase (HRP) are applied to stain the protein targets. HRP can enzymatically catalyze the coupling reaction between cleavable fluorescent tyramide and the tyrosine moieties on the antibodies or the endogenous proteins close to the targets. Through this

enzymatic reaction, one HRP molecule will deposit hundreds of fluorophores in close proximity to the protein targets. After imaging, mild reducing reagents, such as TCEP, are applied to cleave the azide based linker to remove the fluorophores from tyramide and simultaneously deactivate HRP. With reiterative cycles of target staining, fluorescence imaging, fluorophore removal, and HRP deactivation, a large number of varied proteins can be sensitively detected in single cells in situ. With a ~10 min enzymatic reaction, this approach increases the

detection sensitivity by 2 orders of magnitude. And as a large collection of HRP conjugated antibodies are commercially available, the time-consuming, technically demanding and expensive antibody conjugation step is avoided. Thus, this approach can be easily adopted by different research laboratories and in clinical settings. However, as HRP has to be deactivated for each protein, the current cleavable fluorescent tyramide approach can only profile one protein in each analysis cycle.

5. BIOLOGICAL AND BIOMEDICAL APPLICATIONS

Single-cell in situ proteomic technologies are powerful tools to interrogate intracellular signaling networks. To explore protein inhibitory and activating interactions using populations of cells, protein expression variations have to be generated by interfering RNA, small molecules, knockdown or knockout models, etc. With stochastic protein expression naturally occurring in individual cells,⁴² single-cell analysis eliminates the requirements of those external stimuli. By quantifying a large number of varied proteins in single cells (Figure 6A), expression correlation analysis between each pair of the profiled proteins (Figure 6B) can be performed to explore the protein signaling networks (Figure 6C). Using this approach, the signaling pathways in cancer cells have been studied by reiterative immunofluorescence.³³

Another exciting application of single-cell in situ proteomic technologies is to investigate cell heterogeneity in complex biological systems. By comprehensive protein profiling in individual cells of a heterogeneous cell population (Figure 6A), those single cells can be classified into different subtypes based on their protein expression patterns (Figure 6D). By mapping these identified cell subtypes back to their native locations in the tissue, distinct cell neighborhoods consisting of varied cell subtypes can be determined (Figure 6E). Applying this method, mass spectrometry imaging^{22,23} and reiterative immunofluorescence^{25–27,39} have been used to understand the cell subtype compositions, cell–cell interactions, and 3D architectures of tumor and brain tissues.

In addition to studying brain functions and molecular mechanisms of cancer, in situ proteomic technologies can be applied in many other biological and biomedical fields. For example, individual immune cells before and after the immune response can be profiled using these methods. By comparing the immune cells catalogue under the different conditions, we can investigate how the immune systems respond and evolve after antigen activation. Also, in situ proteomic technologies can be applied to monitor the embryo development at various stages, to investigate the molecular mechanisms of stem cell differentiation and organ formation. Moreover, by comparing the normal and diseased tissues, the altered protein expression profiles, cell subtypes or cell neighborhoods can be identified as new biomarkers. Applying these single-cell proteomic technologies, we can also advance our understanding of the regulatory network within individual cells and the communication among cells in their microenvironment. As a result, the disease mechanisms can be better understood and novel drug targets can be discovered. Furthermore, these technologies also enable the monitoring of the drug effects and immune responses during treatments. With the accurate therapy monitoring, the appropriate treatment can be selected and adjusted timely.

6. CHALLENGES AND FUTURE DIRECTIONS

While remarkable advances have been made in the recently developed in situ proteomic technologies, the current versions of these approaches still suffer from low multiplexing capacity. These methods can only profile dozens of varied proteins, which represents a small fraction of the entire proteome. To better understand the complex signaling networks and more precisely classify the cell subtypes, the number of proteins that can be quantified in each sample need to be increased. This challenge could be partially tackled by combining the in situ proteomic technologies with other systems biology approaches, such as genomics,⁴³ transcriptomics,⁴⁴ proteomics,^{16,17} and metabolomics,⁴⁵ among others. For example, a biological sample can be first profiled using these systems biology assays to select the most informative proteins for cell subtype differentiation and signaling pathways identification. Then, such selected proteins can be quantified in an adjacent tissue section by in situ proteomics technologies. Alternatively, the specimen can be first examined by multiplexed in situ protein profiling. Once the specific cell subtypes or regions of interest are selected, they can be isolated by microfluidics or microdissection⁴⁶ and further analyzed by the “omics” assays.

Other challenges for single-cell in situ proteomics involve image data analysis. To accurately quantify the protein expression levels in single cells of a specimen, the boundaries of each cell must be defined. Most of the current assays use the stained nuclei and membrane proteins to identify the presence of cells and their boundaries, respectively.⁴⁷ Nevertheless, the nuclei in certain cells are missing in the typical tissues with the thickness of less than 10 μm . Additionally, the selected membrane proteins may have different cellular locations in varied cell subtypes or in diseased tissues. To mitigate the cell segmentation errors caused these factors, all the stained proteins should be included in the algorithms to identify the cellular boundaries. Moreover, almost all the in situ proteomic technologies apply only the protein abundances information to classify the varied cell subtypes. To understand cell heterogeneity in more details, new algorithms should be developed by integrating protein expression levels, their cellular locations together with protein colocation information.

7. CONCLUSIONS

With the recent technological development, single-cell in situ proteomic technologies are emerging as powerful tools to bring new insights into many important biological and biomedical fields. Each approach discussed in this Review has its unique advantages. For example, mass spectrometry imaging allows all the antibodies to be applied simultaneously, to minimize the epitope decay during the analysis. Reiterative immunofluorescence with fluorescent antibodies enables the profiling of a large tissue in a short time using a standard fluorescence microscope. And with signal amplification integrated into reiterative immunofluorescence, low expression proteins in highly autofluorescent samples can be precisely quantified. In addition, the advantages of these complementary approaches could be combined by applying them sequentially on the same specimen. For instance, the integration of reiterative immunofluorescence with fluorescent antibodies and with signal amplification will allow proteins with a wide range of abundances to be accurately quantified in the same sample. And this specimen can be subsequently analyzed by mass

spectrometry imaging to further increase the number of proteins profiled in each cell.

Comprehensive in situ protein profiling technologies hold great promise to unlock many mysteries in biology and medicine. By revealing the gene expression regulation, spatial organization and interactions of the various cell types in complex organisms, such as developing embryos, brain tissues, and solid tumors, we can significantly advance our understanding of normal physiology and disease mechanisms. By pinpointing the altered protein abundances and locations in diseased cells, new biomarkers can be discovered for more precise diagnosis, prognosis and patient stratification, and novel drug targets could be identified for more effective cellular targeted therapy. We envision that single-cell in situ proteomic technologies will broadly complement other systems biology approaches and will have wide applications in biomedical research and precision medicine.

AUTHOR INFORMATION

Corresponding Author

Jia Guo – Biodesign Institute & School of Molecular Sciences, Arizona State University, Tempe, Arizona 85287, United States; orcid.org/0000-0001-8036-5349; Email: jiaguo@asu.edu

Author

Yi Chen – Biodesign Institute & School of Molecular Sciences, Arizona State University, Tempe, Arizona 85287, United States

Complete contact information is available at:

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Author Contributions

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Notes

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