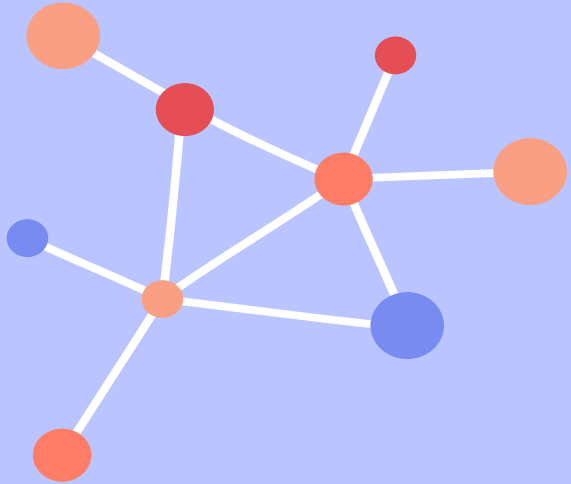
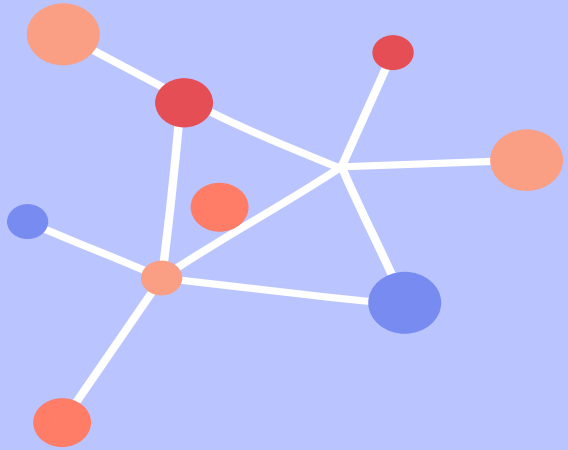
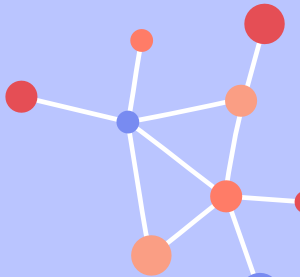
Single cell proteomic analysis

Supervised by Dr. Ibrahim Al-Awadi







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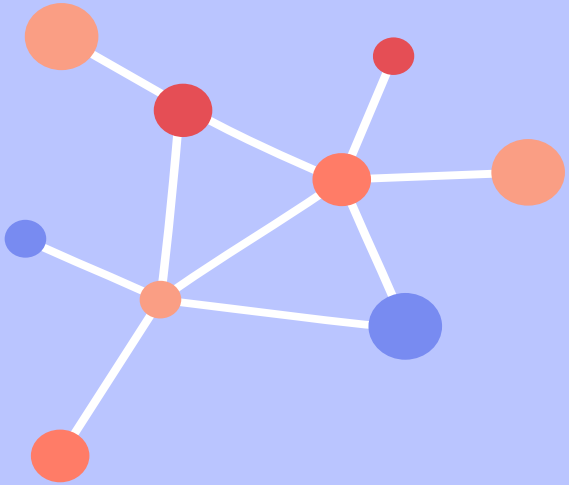
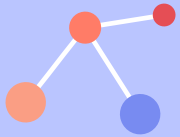


Abstract

It is essential to have the ability to comprehensively profile proteins in every one of the individual cells of complex biological systems if we are to gain a deeper understanding of physiology and disease.
As a result of the limited multiplexing capacity of single-cell imaging methods, bulk cell experiments mask the diversity in the population of cells.
Microchip-, mass spectrometry-, and reiterative staining-based technologies have recently made it possible to profile proteins in single cells in a comprehensive manner.
These approaches will provide new insights into biological and biomedical fields, such as signaling network regulation, cell heterogeneity, tissue architecture, and disease diagnosis.

introduction

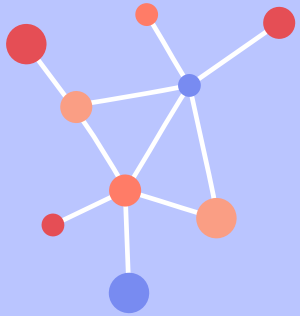
Cell heterogeneity is a common feature in most of the biological systems. The presence of molecularly and functionally different cells has been observed not only in complex multicellular organisms, but also in genetically identical yeast and bacteria cells. Such cell heterogeneity plays important roles in many biological processes, including cancer metastasis, tumor response to drugs, immune response, and stem cell differentiation, among others. Many factors can give rise to cell heterogeneity, such as varied genetics or epigenetics, different microenvironments, and stochastic gene expression, and so on. Being composed of many distinct cell types can be essential for the health, function, and survival of a biological system. Nonetheless, many biological assays are carried out using populations of cells, which can mask cell heterogeneity in the system. Thus, the development of single-cell analysis technologies is critical to accelerate our understanding of health and disease.

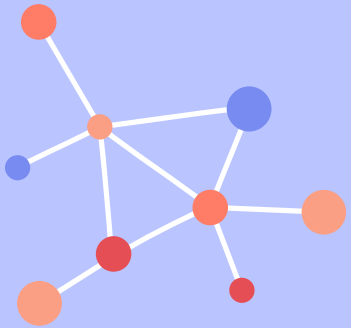


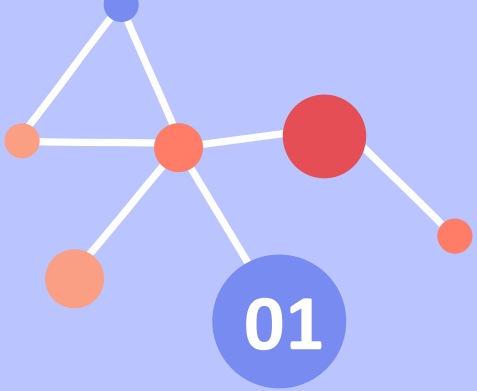
Every individual cell has a huge collection of distinctive biomolecules, which are regulated by its intrinsic signaling network. Because of the complexity of such signaling network, highly multiplexed molecular profiling is required to understand the functions of the various biomolecules in a pathway and their malfunction in diseases. Among these biomolecules, proteins are essential to a wide range of cellular processes and functions, such as regulating gene expression, catalyzing biochemical reactions, providing structural support, and transporting molecules, and so on. Therefore, the development of single-cell proteomic technologies is in a critical need to advance our understanding of normal cell physiology and disease pathogenesis.

Because of the limited number of proteins in individual cells, highly sensitive approaches are required for single-cell protein analysis. Fluorescence imaging-based methods have high detection sensitivity and thus are routinely used to quantify proteins in individual cells. However, due to the spectral overlap of the common organic dyes and fluorescent proteins, a fluorescence microscope has a limited number of imaging channels. As a result, only a handful of proteins in one specimen can be studied simultaneously using these imaging-based methods. Conventional proteomic assays, such as mass spectrometry and protein microarray, can quantify proteins in a sample comprehensively. However, because of their limited sensitivity, these proteomic technologies require the proteins from a population of cells to be combined and analyzed together. Consequently, the variations of the individual cells in the sample are masked by these approaches. To overcome the limitations of the traditional methods and enable single-cell proteomic analysis, novel approaches with both high sensitivity and multiplexing capacity need to be developed.

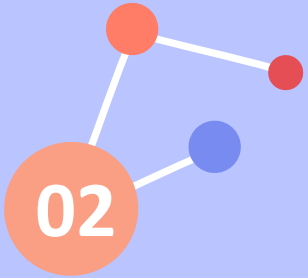
Proteomic Analysis In Isolated Single Cells



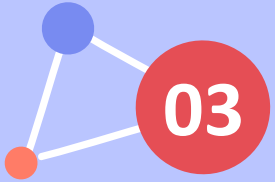




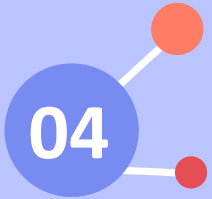
Multiplexed in situ targeting



Single cell barcode chips



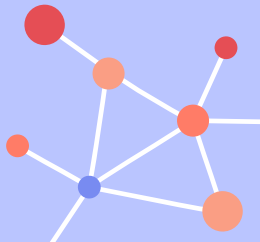
Single cell western blot



Mass cytometry

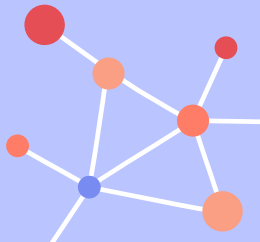
01) Multiplexed in situ targeting

In situ hybridization is a technique that is used to detect nucleotide sequences in cells, tissue sections, and even whole tissue. This method is based on the complementary binding of a nucleotide probe to a specific target sequence of DNA or RNA. These probes can be labeled with either radio-, fluorescent-, or antigen-labeled bases. Depending on the probe used, autoradiography, fluorescence microscopy, or immunohistochemistry, respectively, are used for visualization. *In situ* hybridization is extensively used in research, as well as clinical applications, especially for diagnostic purposes.



BASIC PRINCIPLES OF IN SITU HYBRIDIZATION

The objective of in situ hybridization is to determine the presence or absence of DNA or RNA sequences of interest, as well as to localize these sequences to specific cells or chromosomal sites. Sequences are identified within cells by taking advantage of a property of nucleic acids (i.e., their ability to specifically anneal to each other to form hybrids). This process can be used for two complementary strands of DNA, and for RNA-to-DNA and RNA-to-RNA combinations. Additionally, hybrids between natural and artificial nucleic acids are possible. After a labeled probe is annealed to matching sequences in fixed cells or tissue, the hybridized probe is visualized. When one of the two strands is labeled, the annealed hybrids can be detected by various methods, including isotopic and non isotopic (fluorescent and nonfluorescent) approaches.



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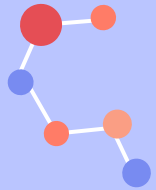
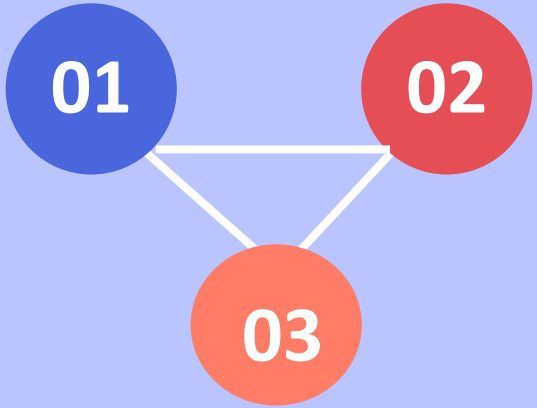
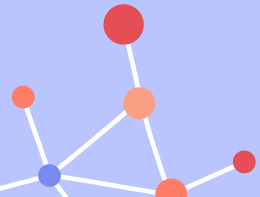


Preparation of Tissue



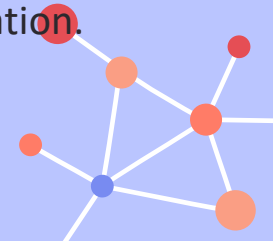
Probes

Probe Labeling and
Signal Detection



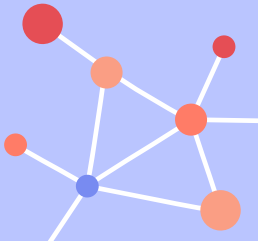
01) Preparation of Tissue

Pretreatment steps can be performed before hybridization to increase hybridization efficiency and reduce nonspecific background staining. Treatment with proteases (proteinase K is the most common) is an important step to facilitate access of the target nucleic acid. Acetylation of sections with 0.25% acetic anhydride/0.1 M triethanolamine reduces the binding of charged probes to tissues. Optimization of tissue processing, including fixation and storage, is important for detecting intracellular nucleic acids. Fixation for in situ hybridization needs to preserve DNA/RNA and tissue morphology. In situ hybridization can be performed on cell samples, such as smears and cytospins, and on tissue sections (e.g., frozen and paraffin). Frozen tissue is better than paraffin-embedded tissue for preserving nucleic acids. Optimally preserved cells should be used for in situ hybridization to avoid damage to nucleotide sequences. Frozen tissue and formalin-fixed tissue that has been stored for several years may be used for in situ hybridization.



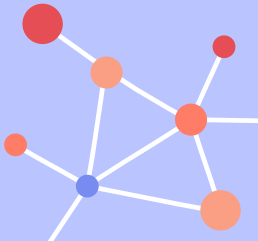
02) Probes

Many different types of probes are used for in situ hybridization; these include cDNA, cRNA, and synthetic oligonucleotide probes. When choosing a probe for in situ hybridization, the researcher must consider sensitivity and specificity, production facilities, how easily the probe penetrates the tissue, stability of hybrids, the application, and how reproducible the method is. The optimal size of the probe is 50–300 bases. Probes that can be used include double-stranded DNA probes, single-stranded antisense RNA probes (riboprobes), single-stranded DNA probes generated by polymerase chain reaction (PCR), synthetic oligodeoxynucleotide probes, and oligoriboprobes. The details of these probes, including their advantages and disadvantages, are beyond the scope of this article.



03) Probe Labeling and Signal Detection

There are two main approaches for labeling a probe: radioisotope labeling and nonisotope labeling. Radioisotope labeling is considered as the most sensitive method of labeling by many researchers, but others believe that nonisotopic methods are just as sensitive.
The results of radioisotope labeling are easily quantified or semiquantified using densitometry counting on film or by silver grain counting. Hybridization sites are observed by autoradiography with X-ray film or liquid emulsion. Problems associated with radioisotope labeling include a long exposure time, poor spatial resolution, risk of exposure to radioactivity, and disposal of radioactive waste. For nonisotopic labeling, compounds including biotin, fluorescein, digoxigenin, alkaline phosphatase, or bromodeoxyuridine are used and are visualized by histochemistry or immunohistochemistry. Problems associated with nonisotopic labeling are that it is generally considered not as sensitive as radioactive labeling, and the hybridization results are difficult to quantify.

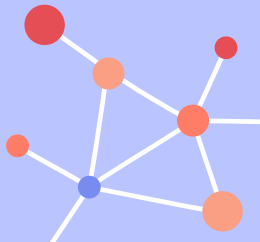


Advantages and Disadvantages of In Situ Hybridization

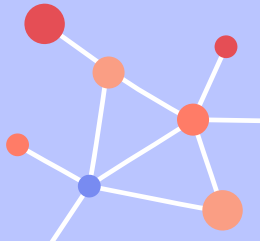
A major advantage of in situ hybridization is that it enables maximum use of tissue that is difficult to obtain (e.g., embryos and clinical biopsies). Hundreds of different hybridizations can be performed on the same tissue. Libraries of tissues can be formed and stored in the freezer for future use. A disadvantage of applying in situ hybridization techniques is the difficulty in identifying targets that have low DNA and RNA copies. However, approaches are continually being developed to improve the sensitivity of in situ hybridization by amplifying either target nucleic acid sequences before in situ hybridization or by detecting the signal after completion of hybridization. These approaches are discussed in the following section.

Fluorescence In Situ Hybridization

FISH is an effective technique that enables direct visualization of genetic alterations in the cell. This technique has many applications and is generally used to examine either imbalances, as a gain or loss of segments of chromosomal materials, or to show specific breakpoints with or without imbalance. FISH was initially used for classification of chromosomes, but this technique has since been adopted in a range of applications in the medical and biological fields. The driving force of FISH technology has been increased by geneticists and pathologists interested in human disease because these specialists need to characterize genotype-phenotype correlations. Common uses of FISH in cytogenetic analysis are chromosomal gene mapping, characterizing genetic abnormalities, identifying genetic abnormalities related to genetic disease or neoplasmic disorders, and detecting viral genomes in interphase nuclei or metaphase chromosomes.

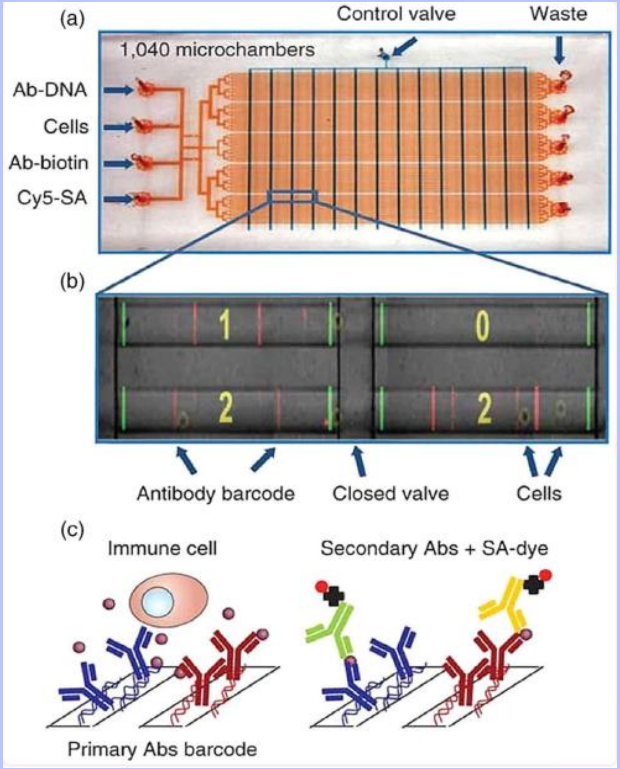


Probes for FISH are labeled by fluorescein, biotin, or digoxigenin. Detection of a second signal coupled with fluorochromes is then performed. Probes for FISH can mainly be classified into two categories: locus-specific or chromosome paint probes. Locus-specific probes are used for detecting a particular gene or chromosomal area and are usually applied for evaluating deletion or amplification of DNA sequences. Whole chromosome paint probes are derived from the complete chromosome. These are good for detecting the origin of structurally abnormal chromosomes and for identifying rearrangements involving different (i.e., nonhomologous) chromosomes. The signals from hybridization (fluorescent spots) are visualized by fluorescent microscopy. FISH tests are highly sensitive.



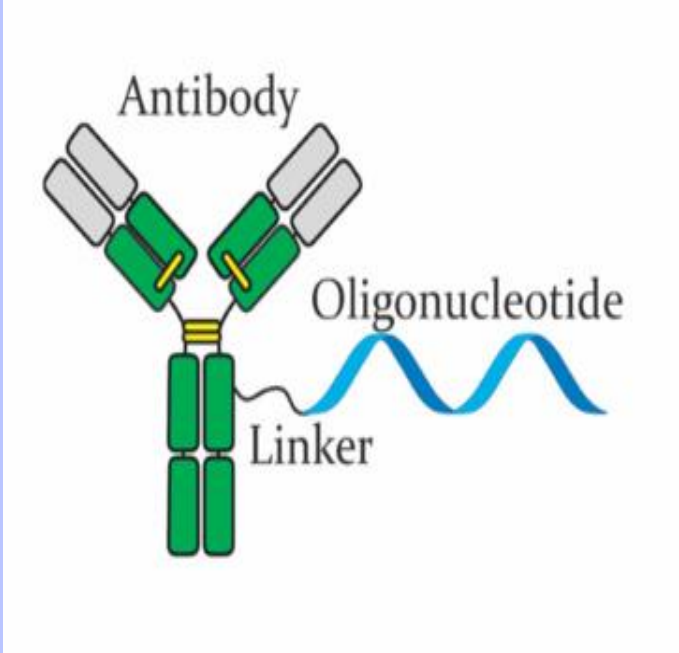
02) Single cell barcode chips

Single cell barcode chips: is an approach developed by the Heath group to enable comprehensive protein analysis in single cells. In this method, individual cells are compartmentalized into microchambers as shown in figure a. In each microchamber, an antibody barcode array, patterned as parallel stripes as shown in figure b. The antibody array, patterned as a barcode for capturing and spatially selective detection of a dozen secreted proteins from single cells as shown in figure c.



Multiplexed single-cell protein analysis with single-cell barcode chips (SCBC). (a) Image of SBSC. The control and flow channels are shown in blue and red, respectively. (b) Image of the microchambers together with the fluorescence signals detected in each chamber. (c) DNA-encoded antibody library technology, which enables the capture and detection of proteins secreted from individual cells

The captured protein targets are stained with the corresponding biotinylated antibodies and fluorescent streptavidin. A biotinylated antibody is one of these blood proteins with biotin attached to it, fluorescent streptavidin used to detect biotinylated secondary antibodies and other macromolecules. By comparing the generated fluorescent signals with the calibration curve, the abundances of the captured proteins can be determined. calibration curve is method used for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration. Also, mass spectrometry can also be applied to identify and quantify the captured proteins directly. One of the important requirements for the success of SCBC is the preparation of the **miniaturized antibody barcodes**. To achieve that, a technology called **DNA-encoded antibody library (DEAL)** has been developed. In this approach, oligonucleotides with different sequences are prevented from moving (immobilized)on a polylysine-coated surface that enhances electrostatic interaction between negatively-charged ions of the cell membrane and positively-charged surface ions on the surface as highly dense and uniform barcodes. The immobilized oligonucleotides are then hybridized by the DNA–antibody conjugates, to convert the oligonucleotide barcodes into the **antibody barcodes** as shown in figure. With the DEAL technology, 20 antibody barcodes can be patterned in each microchamber. As a result, this spatial barcoding platform allows the analysis of up to 20 different proteins secreted from single cells.



Antibody–oligonucleotide conjugates

To further increase the number of proteins that can be quantified in each chamber, spatial and spectral barcodes are combined on one chip. In this platform, 15 spatially separated antibody stripes are patterned in each microchamber, and each stripe contains three varied antibodies.

To distinguish the three different antibodies in the same stripe, they are stained with blue, green, and red detection antibodies. In this way, up to 45 secreted proteins from individual cells can be quantified in each chamber.

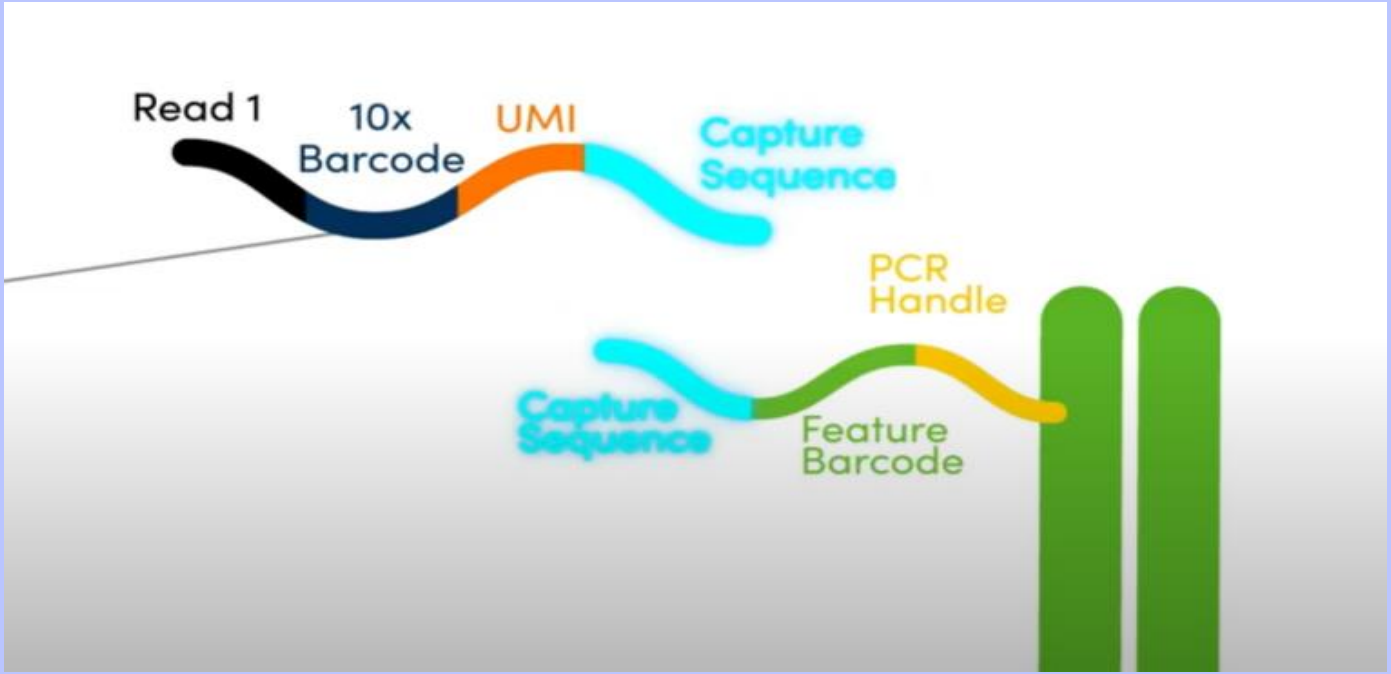
Advantages and Disadvantages of Single cell barcode chips

1) It enables the multiplexed analysis of secreted proteins from live cells as Multiplexed protein analysis is about using an antibody-DNA barcoding approach could accelerate early detection and monitoring of cancer biomarkers in patient samples, whereas other single-cell proteomic technologies can only detect membrane and intracellular proteins.

2) With the cells lysed in its microchamber, SBSC is also capable of simultaneously quantifying the secreted, membrane, and cytoplasmic proteins together with metabolites from the same cell.

1) The multiplex capacity and detection sensitivity of SCBC must be improved.

By increasing the number of antibody stripes in each microchamber, the multiplexing capacity of the assay can be enhanced. this will lead to the enlarged volume of the chamber and the reduced concentrations of the secreted proteins. As a result, the detection sensitivity of the assay is sacrificed. The multiplexing capacity of SCBC can also be improved by immobilizing more different antibodies in each stripe. Nonetheless, the amount of each antibody in these stripes will be reduced, resulting in the decreased sensitivity.

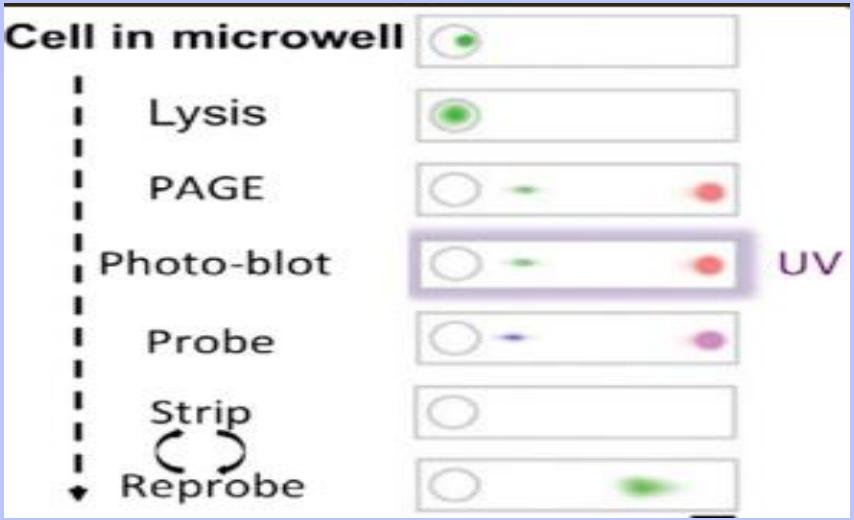


Analysis of cell surface protein expression using feature barcode antibodies.

03) Single cell western blot

In most of the single-cell proteomic assays, the protein targets are detected by antibodies. However, the nonspecific binding and cross-reactivity of antibodies can generate false positive signals and restrict the analysis accuracy.

To overcome these limitations, the Herr groups developed single-cell Western blots (scWesterns) (Hughes et al., 2014) (Figure 5).

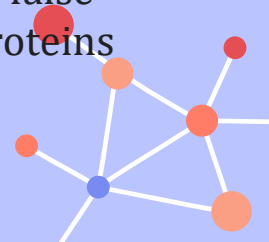


Workflow of single-cell Western blots. The process begins as individual cells are settled and lysed in the microwell, followed with single-cell PAGE, immobilization of proteins onto the gel by UV, and in-gel probing with fluorescent antibodies. Through reiterative cycles of antibody removal and protein relabeling, comprehensive protein analysis can be achieved in single cells

How does the Single cell western blot work?

1) The scWesterns array allows more than 6,000 cells to be simultaneously on a microscopic slide.
2) Each cell is settled to a microwell and lysed with a denaturing buffer.
3) Subsequently, the proteins are separated by polyacrylamide gel electrophoresis, based on their different molecular mass.
4) Afterwards, UV light is used to immobilize the separated proteins on the benzophenone-containing gel. Finally, target proteins are labeled with fluorescent antibodies. As proteins are covalently linked to the gel, antibodies can be stripped using a strong denaturing buffer.

By repeating cycles of the protein labeling and antibody stripping, a large number of different proteins can be quantified. In comparison to other approaches, scWesterns eliminate the false positive signals generated by antibody cross-reactivity and nonspecific binding, as the proteins are separated by their mass before antibody labeling.

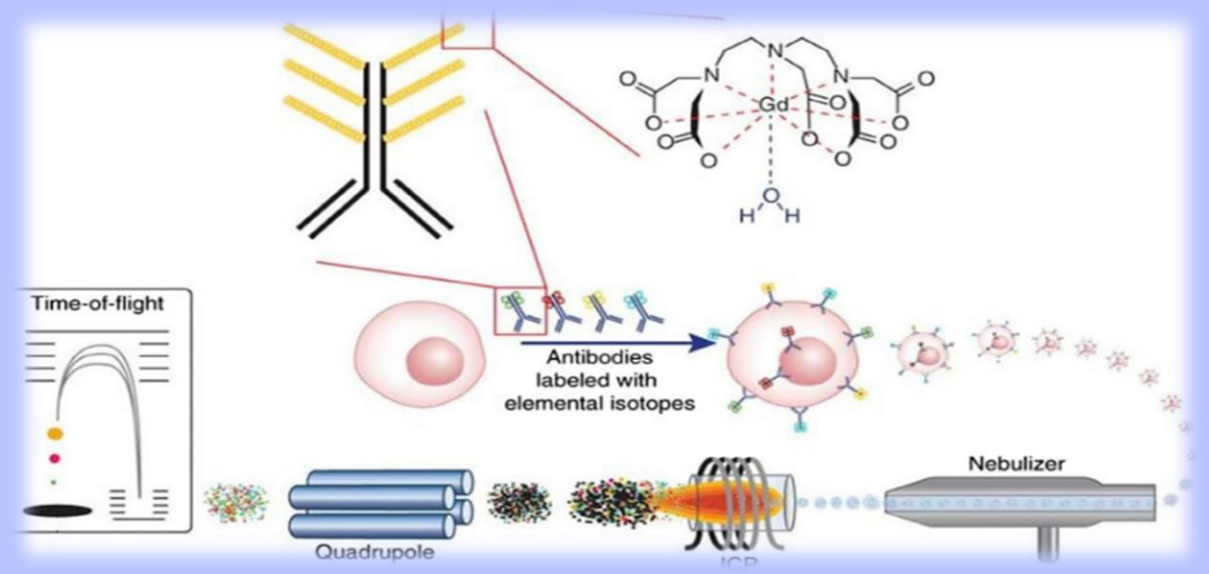


Disadvantages of single cell western blot

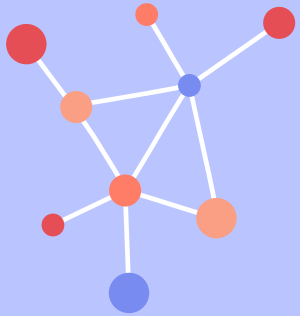
Despite its excellent protein separation efficiency, scWesterns may suffer from the limited detection sensitivity, as ~40, ~72, and up to 50% of the proteins are lost during cell-lysing, protein immobilization and antibody stripping, respectively.

04) Mass cytometry

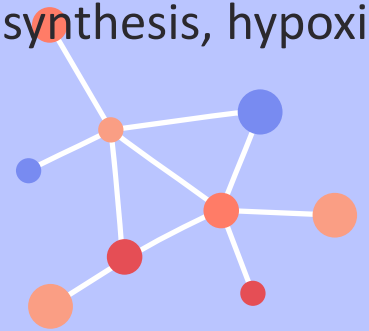
To allow single-cell proteomic analysis in a large population of cells, mass cytometry was developed .



Uses of mass cytometry



- Because of its high sample throughput and multiplexing capacity, along with its ability to characterize the various molecular states in the cells, mass cytometry is one of the most widely adopted single-cell proteomic technologies.
- Moreover, except for protein analysis, mass cytometry can also be applied for the study of posttranslational modification, proteolysis products, RNA transcripts, DNA synthesis, hypoxia states and enzymatic activity, and so on .



Mechanism of mass cytometry

- In this method, the protein targets in single cells are stained with antibodies conjugated to different metal isotopes. After sprayed as single droplets, individual cells are transported into plasma by the flow of argon gas, and subsequently vaporized, atomized, and ionized. The generated ions from each cell are then quantified by a time of flight mass spectrometer. The signals for each metal isotope are integrated, calibrated, and translated into the expression levels of protein targets in single cells. With an approximately a thousand cells analyzed per second, mass cytometry has the highest sample throughput among all the current single-cell proteomic assays. Additionally, mass cytometry enables over 40 varied proteins to be profiled in millions of individual cells in a given sample, which is critically required for the thorough characterization of the rare but functionally important cell types in a complex biological system.

Workflow of mass cytometry

Antibodies conjugated with varied metal isotopes are first applied to stain the protein targets. Subsequently, cells are nebulized into single-cell droplet, and passed through an inductively couple plasma (ICP) time-of-flight (TOF) mass spectrometer to obtain an elemental mass spectrum for every single cell rtant but rare cells could be missed.

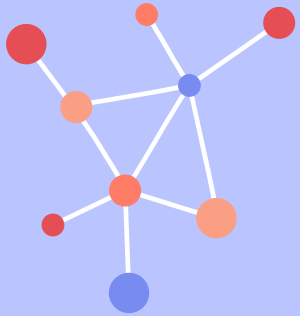
Disadvantages of mass cytometry

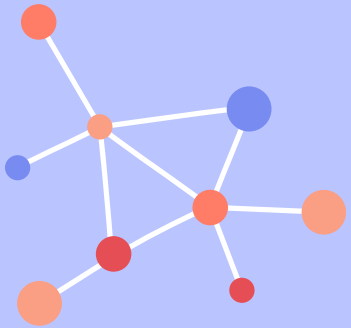
Despite its advantages, the method still suffers from some limitations:

1. For instance, some ionized metals can form oxide in the plasma, resulting in an interfering increased mass (M + 16) for the accurate data analysis.
2. Also, the enriched metal isotopes usually contain 1% of impurities, which could create confusing background signals . only 30–40% of the injected cells are quantified by mass cytometry.

As a result, protein expression profiles from important but rare cells could be missed.

Single Cells In Situ Proteomic Technology





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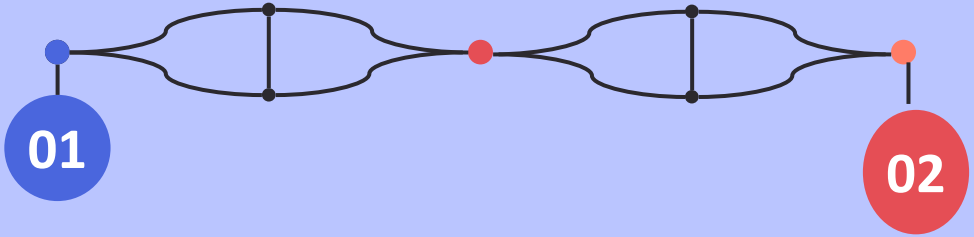
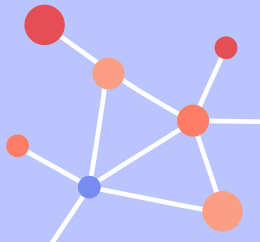


Reiterative immunofluorescence

Cleavable fluorescent streptavidin

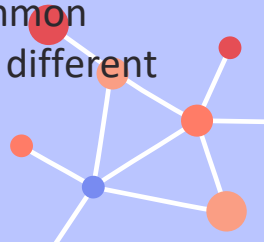
SINGLE CELL IN SITU PROTEOMIC TECHNOLOGY

Biological systems are complex organizations ,The precise locations of cells in a tissue and biomolecules in a cell can be crucial for the organization, regulation, and function of the biological systems. Therefore, to better understand the regulation of these complex biological systems and their malfunction in disease, we will present the recently developed single cell in situ proteomics technologies.



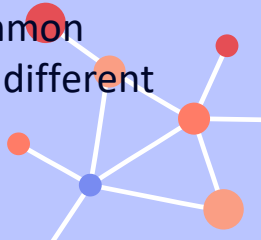
01) Reiterative immunofluorescence

To address the issues of low sample throughput and also to avoid the specialized and expensive instrument, reiterative immunofluorescence has been developed. This approach is composed of three major steps. First, antibodies conjugated with different fluorophores are used to stain their corresponding protein targets in the specimen. Second, the specimen is imaged in different fluorescence channels to quantify the abundances of the protein targets in their original cellular locations. Finally, the staining signals are erased before the start of the next immunofluorescence cycle. By repeating the three major steps of reiterative immunofluorescence, a large number of different proteins can be profiled in individual cells at the optical resolution. As a large imaging area consisting of millions of pixels can be captured within milliseconds to seconds, to profile 40 proteins in a common 1 cm2 tissue takes about 80–120 hr by most of the reiterative immunofluorescence technologies. With shorter assay time, reiterative immunofluorescence enables a larger number of cells to be profiled in a given sample, which facilitates the study of rare but important cells in a complex biological system. Moreover, reiterative immunofluorescence only requires a common fluorescent microscope as the instrument, which makes this approach widely applicable in different research and clinical laboratories.



02) Cleavable fluorescent streptavidin(CFS)

To address the issues of low sample throughput and also to avoid the specialized and expensive instrument, reiterative immunofluorescence has been developed. This approach is composed of three major steps. First, antibodies conjugated with different fluorophores are used to stain their corresponding protein targets in the specimen. Second, the specimen is imaged in different fluorescence channels to quantify the abundances of the protein targets in their original cellular locations. Finally, the staining signals are erased before the start of the next immunofluorescence cycle. By repeating the three major steps of reiterative immunofluorescence, a large number of different proteins can be profiled in individual cells at the optical resolution. As a large imaging area consisting of millions of pixels can be captured within milliseconds to seconds, to profile 40 proteins in a common 1 cm2 tissue takes about 80–120 hr by most of the reiterative immunofluorescence technologies. With shorter assay time, reiterative immunofluorescence enables a larger number of cells to be profiled in a given sample, which facilitates the study of rare but important cells in a complex biological system. Moreover, reiterative immunofluorescence only requires a common fluorescent microscope as the instrument, which makes this approach widely applicable in different research and clinical laboratories.



THANK YOU