Multiplexed Single-Cell Imaging: Past, Present and Future

Running title: Multiplexed Single-Cell Imaging

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Jia-Ren Lin from the Laboratory of Systems Pharmacology at Harvard Medical School, was awarded best poster at the annual Society of Biomolecular Imaging and Informatics (SBI2) meeting held in Boston, September 2016. His work focuses on single-cell imaging, especially on developing new methods for simultaneously detecting many antigens, named Cyclic Immunofluorescence (CycIF). This method could be applied in different stages of drug development, from discovery phase, preclinical research to clinical research. The current works and future directions of CycIF method are summarized in the following overview.

The need for high-dimensional single-cell measurements

Our lab, in common with many others in the field of cancer research and drug discovery, is highly interested in studying the molecular and cellular heterogeneity in human preclinical (e.g. cell lines) and clinical samples (e.g. tumor sections, blood). This is particularly important for a better understanding of the efficacy of drugs and the molecular origins of drug resistance. To this end, a comprehensive view of intercellular heterogeneity involving measurements at single-cell resolution is crucial. Multiplexed imaging is an ideal tool to probe not only high-dimensional single-cell data in heterogeneous samples, but also the encoded spatial information in such samples.

Multiplexed imaging, a decade ago

Early attempts toward multiplexed single-cell imaging included a "toponome" which used photobleaching fluorophores prior to applying the next round of antibodies.[1] A related method, array tomography, used serial sections to reconstruct "3D" multiplexed images to investigate, for example, brain synaptic "molecular architectures".[2] Although toponomics is a powerful method for high-content and highly multiplexed imaging, it has not been widely adopted in either basic or clinical research. This may be due to an unstable and ineffective photobleaching step. This process is highly antibody-specific and is typically done by scanning the sample using a microscope, a process that is too slow for large regions of interest. Thus the search for new multiplexed methods continued.

Current technology of multiplexed imaging

Imaging CyTOF and Multiplexed ion-beam imaging

Recently, several new technologies based on state-of-the-art mass cytometry have been developed for multiplexed imaging of tissue sections. Imaging CyTOF (Cytometry by Time of Flight) and multiplexed ion-beam imaging (MIBI) are two platforms that enable simultaneous probing of up to 100 targets in a single sample.[3,4] These methods have opened up new avenues for understanding heterogeneity in solid tumors, analogous to the use of mass cytometry for liquid tumors.[5] Furthermore, the data from these approaches have stimulated the development of a variety of statistical and computational tools for understanding multidimensional single-cell behaviors. However, the cost of instruments and reagents required to carry out such experiments

has proven prohibitive in many cases, and the scanning speed remains a significant throughput bottleneck.

CLARITY, SWITCH and Expansion microscopy

A more conventional way to achieve multi-color imaging is to strip antibodies and restrain samples. Many methods using this principle have been developed in recent years, but most of them are optimized for formalin-fixed paraffin-embedded (FFPE) sections with only a small number of antibodies, limited by the number of rounds of immunolabeling. The main problem lies in the stripping step; complete removal of antibodies often requires treatments deleterious to the sample. More recently, methods like CLARITY, SWITCH and expansion microscopy have combined chemical transformation of samples and antibody stripping to probe more than 20 different markers in modified tissue sections.[6–8] Despite the advantage of these "sample strengthening" approaches in removing auto-fluorescence, application of different processing steps might change antigenicity toward antibodies used in current clinical workflows.

Cyclic immunofluorescence and related approaches

In 2013, the GE global research center published a multiplexed imaging method called MxIF (brand name: Multiomyx).[9] This method combined novel chemical inactivation procedures with iterative staining and imaging to achieve highly multiplexed images of FFPE tissue sections. However, the method has not been widely used since its inception, due to the requirement for the end user to purchase several costly proprietary components. Two academic groups, including our own, have published "open-source" methods to accomplish high-dimensional analysis in tissue sections (Multi-Dimensional Microscopic Molecular Profiling (MMMP) by Pat Brown and colleagues at Stanford university) and in cultured cells (Cyclic Immunofluorescence (CycIF) by our group).[10,11] All three methods are based on the same principle: using chemical inactivation followed by re-immunolabeling with fluorophore-conjugated antibodies. Among the advantages of these cyclic immunolabeling methods are: easier implementation, no requirement for special instrumentation, and compatibility with other fluorescence-based assays. In addition, our CycIF approach can be easily applied to high-content and high-throughput screens at very low cost, so it should be highly useful method for drug discovery and preclinical research.

The origin of Cyclic Immunofluorescence

The path toward CycIF began when I joined the Laboratory of Systems Pharmacology (LSP) at Harvard Medical School. Inspired by other multiplexed imaging methods, I began to develop an efficient way to profile high-dimensional heterogeneity in cultured cells. Trained as a molecular biologist, I was familiar with the "stripping" technique used in Western blotting for probing multiple targets in the same sample. However, antibody stripping is often too aggressive to be applied to adherent cells in culture. Another Western blotting technique is the application of sodium azide to quench enzymatic activity of horseradish peroxidase (HRP) conjugated to secondary antibodies; it allows for re-application of antibodies from different species. This type of "chemical quenching" is the key to CycIF. With some luck, I found that an easy-to-make solution consisting of hydrogen peroxide and sodium hydroxide is highly effective at inactivating Alexa-based fluorophores without damaging the samples. We also developed different methods for combining CycIF with live-cell imaging and fluorescence *in situ* hybridization (FISH), allowing us to expand the measurements to include both time and transcription dimensions. The power of high-dimensional single-cell data has perhaps been most clearly demonstrated by mass cytometry, a technology from which we have inherited many analysis tools. CycIF data have also been analyzed using these tools as published in our original paper and follow-up studies. We are now applying CycIF to blood samples, where the "imaging cytometry" approach not only allows us to obtain multiparametric readings of liquid biopsies, but also enables us to immunoprofile clinical specimens.

The future of CycIF

We continue to optimize our protocols and update the list of antibodies compatible with CycIF; a list of these antibodies is available on our website (http://lincs.hms.harvard.edu/lin-natcommun-2015/). By promoting CycIF as an open-source multiplexed imaging platform, we allow a larger scientific community to access otherwise costly propriety methods; this may lead to the rapid development of new applications and tools for multiplexed imaging and analysis. In the meantime, we continue to explore further applications of CycIF in our group: from super-resolution imaging of molecular architectures to whole organism imaging for developmental studies. We have also extended our method to FFPE tissue sections from patients to achieve "multidimensional digital pathology". Such highly multiplexed molecular maps with spatial information will allow us to ask questions unaddressable by several contemporary technologies.

For example, we can now ask: “How many different cell types are in the samples and how are the cell types spatially organized?”, “How much heterogeneity can we detect before and after drug treatment?”, and “How do different subpopulations respond to different treatments?” In conclusion, we envision that the translation of the aforementioned experimental methodologies and analytic workflows from bench to bedside will permit more rapid drug development and biomarker discovery.

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