ACS chemical biology

Point of VIEW

Seeing Is Believing

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ABSTRACT Modern visualization techniques are affording a peek into complex cellular processes. A recent paper describes an automated fluorescence microscopy method to map the subcellular localization of up to 100 different proteins in the same sample.

In their thoughtful commentary, Kim Morrison and Greg Weiss (*1*) describe how the origins of modern chemical biology can be traced, in part, to the pioneering applications of dyes and chemical processes to resolve the form and function of tissues and cells. Visualizing the structures of cells and the components that bind to chemicals has been the source of inspiration for so many discoveries: Anna Atkins' use of the cyanotype process developed by John Herschel to image botanical specimens, the synthesis of the first example of aniline dyes by William Perkins and their subsequent use by Rudolf Virchow to uncover details of cellular structure and lineage, and Paul Ehrlich's crucial insights that led to the receptor concept, among many others. We've come a long way since these pioneering works. Now, we can visualize the physical locations and associations of individual proteins or complexes of proteins with fluorescent dyes conjugated to artificially created antibodies, thus achieving sensitivities, spatial resolutions, and specific information that Ehrlich and his contemporaries could only dream of.

In a world in which we are bombarded with huge protein data sets, we must remember that where proteins are expressed, in what forms, and what other proteins they interact with can provide the deepest insights into their functions. However, can we bridge the gap between the massive data analysis afforded by contemporary analytical techniques such as mass spectrometry and the rich but usually limited data provided by cellular imaging? In a recent article in *Nature Biotechnology*, Schubert and colleagues (*2*) describe a significant step in this direction, with an automated fluorescence microscopy method to map the subcellular localization of up to 100 different proteins in the same sample, an approach that could be used to unravel complete protein networks. This technology enables one to monitor when and where proteins are found together or excluded from a complex and how protein organization is altered in diseased cells. Further, they demonstrate the potential of this approach for facilitating the development of diagnostics and targeted drugs.

Currently available imaging technologies enable the detection of a limited number (~10) of different fluorophores in a sample (*3*, *4*). Schubert and colleagues (*2*) expand the number of proteins that can be simultaneously investigated by using one fluorophore attached to many different monoclonal antibodies (Figure 1). They visualize one protein after another in a fixed tissue or cell sample by sequentially adding, imaging, and photobleaching each antibody-conjugated fluorophore. The images are then superimposed to obtain a protein colocalization map. This technology, which the authors call multi-epitope ligand cartography (MELC) technology, has the advantage of not requiring a detailed analysis for separating the contributions from individual, simultaneously present fluorophores.

To estimate how many particular proteins are present at each position in the sample, the authors use appropriate threshold values to determine whether each pixel contains each protein epitope. This leads to the creation, for each pixel, of a vector they call

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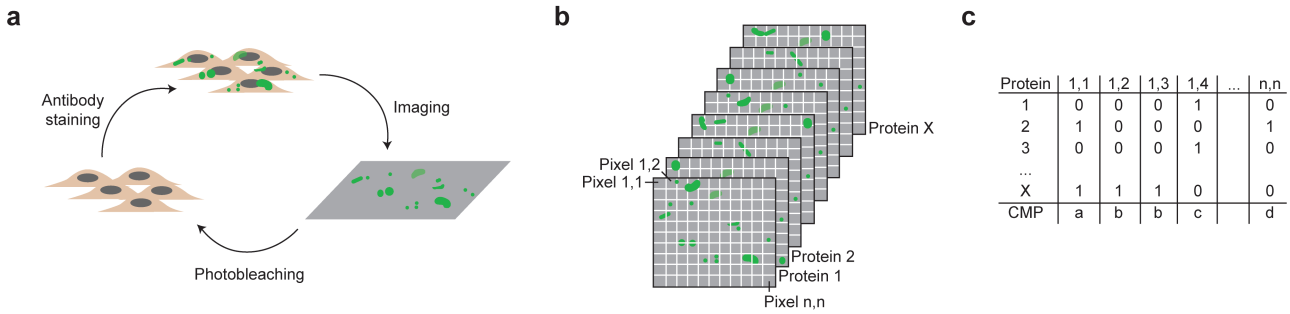

Figure 1. MELC technology. a) A fixed cell or tissue sample is subjected to sequential cycles of staining with a fluorescent monoclonal antibody, imaging, and photobleaching. b) The proteins are imaged one after another, and the images are superimposed to obtain a protein colocalization map. c) Each pixel can be represented as a vector of 0s and 1s to indicate whether it contains a particular protein. Each unique protein combination corresponds to a particular CMP. Adapted with permission from Macmillan, copyright 2006 (*12*).

combinatorial molecular phenotype (CMP), which represents the combination of proteins found at that pixel. This vector has a length corresponding to the number of proteins and is composed of 0s and 1s (1 if the signal for that protein is higher than the threshold value, 0 if it is lower). The authors show that although the protein distributions vary from cell to cell within a tissue, it is possible to distinguish between healthy and diseased tissues by comparing the distributions of CMPs in these tissues. For example, the abundance of a particular CMP motif in skin tissues can discriminate between tissues with psoriasis or atopic dermatitis and healthy tissues. The MELC technology thus provides a tool for the identification of candidate target proteins in disease.

Furthermore, to show that this methodology can also be used to decipher functional protein networks in a single cell, the authors monitored the colocalization and antilocalization of 23 different cell surface proteins in a spontaneously migrating rhabdomyosarcoma cell line called TE671. They found that four cell surface CMPs were present throughout the migration process and that the alanine-specific protease aminopeptidase N (APN) belonged to these four CMPs, an indication that this protein plays an important role in cellular migration. Indeed, after selective inhibition of APN activity, the cells did not enter the migratory state and the distribution of the CMPs was affected. Therefore, MELC can be used to identify key proteins in functionally relevant CMP motifs and to selectively inhibit them in order to analyze all functional protein networks.

With this new technology, the number of proteins that can be simultaneously investigated in a tissue or cell sample is limited only by the amount of available fluorescently tagged antibodies (or other fluorescent ligands). However, as usual, caveats exist: antibody labeling is generally highly specific, but parallel methods should be used to validate the accuracy of protein recognition. This is especially true in the case of diseased tissues, because antibodies have the tendency to non-specifically bind to dead cell components. Similarly, the large size of an antibody (typically 150 kDa) might prevent the recognition of neighboring proteins inside protein complexes because of steric hindrance. According to the authors, this limitation could be overcome by carefully determining the proper sequence of labeling cycles.

The MELC technology is a versatile method for unraveling the complexity of protein networks in fixed samples. Live cell imaging of the spatial and temporal dynamics of individual proteins and protein–protein interactions can be achieved with genetic fusion of reporter fluorescent proteins or other tagging technologies. For example, GFP variants or the use of small fluorescent molecules that can be covalently attached to genetically modified proteins can allow for the real-time analysis of protein localization and trafficking in living cells, as well as the monitoring of protein–protein interactions by FRET (*5–7*). Similarly, protein fragment complementation assays can monitor the changes in protein complex levels and localization in living cells in response to chemical, genetic, or environmental perturbations (*8, 9*). Obviously, these techniques all require modifications of the proteins under investigation and cannot be done with living tissues, certainly not on the scale achieved with MELC in single cells, nor can they directly capture subtle changes in protein states, such as post-translational modifications and their causal links to cellular perturbations (*10*). Thus, despite great efforts to develop genetically encoded reporters of protein function, the best mileage can still be found with good old antibodies.

Schubert and colleagues (*2*) describe efforts to develop MELC into an impressive industrial-level process. However, it is reasonable to imagine doing the same thing in your basic mom-and-pop operation. The central limitation of their approach is the

availability of specific antibodies. Universities and funding agencies, in collaboration with industry, should focus resources on generating these crucial reagents and coordinate efforts to make them available to the scientific community at a reasonable cost. Further, efforts to devise alternative affinity reagents, including single-chain antibodies and protein alternatives to antibodies, should continue to be supported (*11*). Imagine a world in which researchers could request a set of affinity reagents for proteins and their different states and have them synthesized on demand. In this world, visualizing the organization of cellular networks and how they evolve in time and space could become a standard tool for building models of cellular machinery. This might seem far-fetched at the moment, but as Schubert and colleagues (*2*) have shown us, seeing is believing.

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