# Illuminating the cell's biochemical activity architecture

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## Abstract

All cellular behaviors arise through the coordinated actions of numerous intracellular biochemical pathways. Over the past twenty years, efforts to probe intracellular biochemical processes have undergone a fundamental transformation brought about by advances in fluorescence imaging, such as the development of genetically encoded fluorescent reporters and new imaging technologies; the impact of these approaches on our understanding of the molecular underpinnings of biological function cannot be understated. In particular, the ability to obtain information on the spatiotemporal regulation of biochemical processes unfolding in real time in the native context of a living cell has crystallized the view, long a matter of speculation, that cells achieve specific biological outcomes through the imposition of spatial control over the distribution of various biomolecules, and their associated biochemical activities, within the cellular environment. Indeed, the compartmentalization of biochemical activities by cells is now known to be pervasive and to span a multitude of spatial scales, from the length of a cell to just a few enzymes. In this Perspective, part of this special issue on "Seeing into cells", we highlight several recent imaging studies that provide detailed insights into not just where molecules are but where molecules are *active* within cells, offering a glimpse into the emerging view of biochemical activity architecture as a complement to the physical architecture of a cell.

## Graphical Abstract
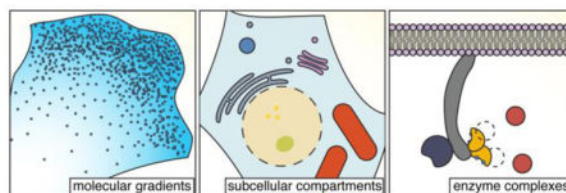


Global biochemical heterogeneity can often arise through local activity changes that are then reinforced by local and long-range feedback mechanisms, yielding biochemical activity gradients[1]. Approaches to directly visualize these spatial activity gradients have long included small molecule fluorescent indicators[2,3] and dye-labeled proteins[4,5], and the addition of genetically encoded fluorescent biosensors continues to provide important

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insights into the activity architecture of biochemical gradients. For example, in a recent study using FRET-based biosensors to image Ras, Rac, RhoA, and Cdc42 activity gradients[6], Yang and colleagues identified Cdc42 as the chemotactic "compass" that directs cell migration in neutrophils, with local Cdc42 activity elevations not only predicting cell turning in response to a graded stimulus but also preceding spontaneous symmetry breaking and displaying the properties of an excitable medium[7] in response to a uniform stimulus[6]. Similarly, by imaging cyclic AMP (cAMP) and protein kinase A (PKA) activity in cultured hippocampal neurons, Gorshkov et al observed that both cAMP accumulation and PKA activity were higher towards the distal region of developing axons in neurons grown for 5 days *in vitro* (DIV), but not in less mature, DIV3 cells[8]. Notably, disrupting interactions between PKA and A-kinase anchoring proteins (AKAPs) both accelerated axon growth and prompted the formation of an axon-directed cAMP gradient in DIV3 neurons, thus revealing the establishment of a developmentally timed cAMP gradient via AKAP/PKA-dependent negative feedback to control axon growth in these cells[8].

Physical compartmentation of the cell imposes additional layers of spatial control over biochemical processes. Membrane-bound organelles, for instance, form functionally specialized subcellular compartments with distinct regulatory landscapes, and imaging studies have revealed that a given biochemical pathway can exhibit distinct activity patterns in association with different organelles. For example, by targeting a novel, genetically encoded mTORC1 activity reporter to different subcellular regions (e.g., plasma membrane, cytosol, lysosome, and nucleus), Zhou and colleagues observed that although growth factor stimulation induced mTORC1 signaling throughout the cell, amino acids specifically stimulated lysosomal and nuclear mTORC1 activity[9]. A recent study using a FRET-based Rab1 biosensor capable of cycling between the Golgi membrane and cytosol similarly revealed that Rab1 is active in the cytosol but is inactivated at the Gogli[10]. The ability to visualize compartment-specific biochemical activities has also modified the long-accepted model of canonical G-protein-coupled receptor (GPCR) signaling, revealing that internalized GPCRs continue to engage in G-protein-dependent signaling from endosomes[11]. In a recent example, Jean-Alphonse et al imaged cAMP and PKA activity using cytosol- and nuclear-targeted FRET sensors and found that $\beta_2$AR activation promotes sustained cAMP signaling by endosomal parathyroid hormone receptor, leading to rapid and prolonged nuclear cAMP accumulation and PKA activity[12]. This nuclear PKA activity was not due to translocation of PKA from the cytosol, consistent with previous evidence that sustained, local cAMP production is required to activate nuclear-resident PKA[13].

Membrane microdomains introduce further spatial differences in biochemical activity that can be visualized using selectively targeted fluorescent biosensors, as was done in a recent study by Seong and colleagues, who used a novel FRET-based biosensor to monitor PDGFR activation. By appending various lipid-modification signals to their biosensor, these authors were able to show that, although PDGF stimulation induced uniform PDGFR activity throughout the plasma membrane, PDGFR activity was uniquely antagonized by integrin-mediated tension sensing within lipid rafts[14]. These submicron-scale subdivisions within the plasma membrane are thought to result from in-plane, liquid-liquid phase separation by subsets of membrane lipids[15] and to serve as organizing centers for various biochemical processes related to intracellular signaling[16]. However, membrane microdomains have long

been a topic of debate, and recent observations of phase separation by membrane proteins add another layer of complexity. For instance, Banjade and Rosen observed phase separation by fluorescently tagged Nephrin inserted into a model membrane[17], and Su and colleagues also visualized the formation of phase-separated domains of fluorescently tagged Linker for Activation of T cells (LAT) both *in vitro* and in live Jurkat cells[18]. Indeed, by visualizing phase-separated lipid domains in the plasma membranes of mouse B cells, Stone and colleagues recently observed that B cell receptors not only clustered within but also stabilized and expanded lipid raft-like domains[19], adding to questions about whether lipid rafts drive receptor clustering or *vice versa*[20].

Growing evidence indicates that liquid-liquid phase separation is not limited to membranes and is in fact responsible for producing discrete spatial domains of biochemical activity – sometimes called "membraneless organelles" – throughout the cell interior[21]. For example, Brangwynne et al previously used live-cell imaging of fluorescently tagged proteins to reveal that cytosolic P granules behave like phase-separated liquid droplets in *C. elegans* embryos[22], and other well-known ribonucleoprotein domains such as nucleoli and Cajal bodies are also considered to be phase-separated structures[21]. Zeng and coworkers also recently visualized the formation of phase-separated droplets by the postsynaptic density (PSD) proteins SynGAP and PSD-95[23]. Such phase separation events appear to be driven by multivalent interactions among the various components[21]: the polyphosphorylated C-terminal tails of Nephrin[24] and LAT[18] with cytosolic adaptor proteins; the SynGap homotrimer with multiple copies of PSD-95[23]; and RNA-binding proteins through intrinsically disordered regions (IDRs), also called low-complexity domains due to their highly repetitive amino acid sequences[25]. Intrinsic disorder and multivalent binding modalities are common among signaling and regulatory pathways[21,26], hinting at a larger role for phase separation, yet just how prevalent phase separation is in regulating intracellular biochemistry remains an open question. Furthermore, the switch-like concentration dependence of phase separation suggests an important role in all-or-none cell fate decisions, as well as other state-switching processes (e.g., actin polymerization[18]). It remains to be seen how phase separation fits in with other sources of nonlinear signaling dynamics[27–29].

Live-cell imaging approaches are also shedding light on the spatial regulation of biochemical activity by the assembly of protein complexes containing multiple enzymes. For example, by expressing fluorescently tagged proteins in living cells, An and colleagues previously found that the six metabolic enzymes that catalyze the ten-step pathway responsible for *de novo* purine biosynthesis all colocalize within a multi-enzyme complex – the "purinosome" – that dynamically forms visible cytosolic clusters directly associated with purine synthesis[30]. In a recent study, Kohnhorst et al similarly investigated the behavior of fluorescent protein-tagged glucose metabolic enzymes in living cells and observed the formation of a "glucosome" containing four enzymes that catalyze rate-limiting steps in glycolysis and gluconeogenesis[31]. These complexes also formed visible clusters that were distinct from other known cytosolic bodies and whose sizes varied dynamically in response to treatments that shifted glucose flux to favor specific pathways (e.g., the pentose phosphate pathway, serine biosynthesis), suggesting a mechanism for compartmentalizing metabolic processes and controlling cellular metabolic states[31]. Additional imaging studies have

revealed that purinosomes localize to the mitochondrial surface[32] and are regulated by GPCR[33] and mTOR[32] signaling; biosensor-based imaging may therefore be useful in revealing additional spatial links between signaling enzyme activity and purinosome function. Purinosomes and glucosomes also bear some resemblance to phase-separated bodies[34], yet whether these assemblies are driven by multivalent interactions among their protein components or by some other mechanism remains unclear.

Signaling enzyme activities are likewise spatially modulated by assembly into multi-protein complexes via scaffold proteins. PKA is well known to associate with diverse AKAPs[35], and ERK/MAPK pathway components also assemble onto various scaffold proteins[36]. Scaffolds anchor signaling enzymes to different subcellular locations, direct activity towards specific substrates, and recruit various regulatory proteins or other signaling enzymes to produce macromolecular signalosomes with unique signaling behaviors. Direct fusion of fluorescent biosensors to scaffold proteins has been extremely useful in revealing local activity dynamics near signalosomes and continues to offer new insights into how scaffolds modulate enzyme activity[37], as illustrated by Greenwald et al using a FRET-based protein kinase C (PKC) biosensor tethered to either the plasma membrane or to the C-terminus of AKAP7α, wherein AKAP-anchored PKC activity was kinetically enhanced compared with unanchored PKC[38]. Nevertheless, questions remain concerning whether and how AKAPs are able to spatially confine PKA activity given the classical model of PKA catalytic subunit (PKAcat) diffusion. Mo and colleagues were recently able to directly investigate this question in living cells using a novel super-resolution PKA activity reporter[39]. By targeting this sensor to the plasma membrane, they were able to observe discrete, ~350-nm-diameter puncta of high PKA activity in response to cAMP production. These PKA activity puncta were dependent on AKAP anchoring and also appeared to colocalize with AKAP79 clusters along the membrane, suggesting a mechanism whereby AKAP clusters increase the local concentration of PKA regulatory subunits, thereby enhancing capture of free PKAcat to locally confine PKA activity[39].

As alluded to above, all biochemical processes are ultimately governed by events occurring on molecular length-scales, and researchers are increasingly turning towards more advanced imaging modalities that can be used to directly visualize biochemical activity architectures with molecular precision. In their effort to visualize PKA activity microdomains, for instance, Mo and colleagues took advantage of their discovery of a novel fluorescence phenomenon, in which molecular proximity to the green fluorescent protein Dronpa influences the fluorescence fluctuation behavior of the red fluorescent protein TagRFP-T through an effect termed fluorescence fluctuation increase by contact (FLINC), to generate a new class of fluorescent biosensors for super-resolution activity imaging, including probes for PKA activity, ERK activity, and protein-protein interactions[39]. Targeted biochemical perturbation strategies are also crucial for elucidating the functional importance of spatial organization, with optogenetic systems being well suited for achieving both spatial and temporal precision[40]. Along these lines, Shin et al have developed an "optoDroplet" system that enables the local and reversible generation of IDR-mediated phase separations in living cells[41]. Thus, given the success of live-cell fluorescence imaging as a tool to probe the dynamic spatial control of biochemical activities within cells, ongoing technological

advances such as these promise to further transform our efforts in bringing the cell's biochemical activity architecture to light.

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