ACSNANO
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PERSPECTIVE

High Density or Urban Sprawl: What Works Best in Biology?

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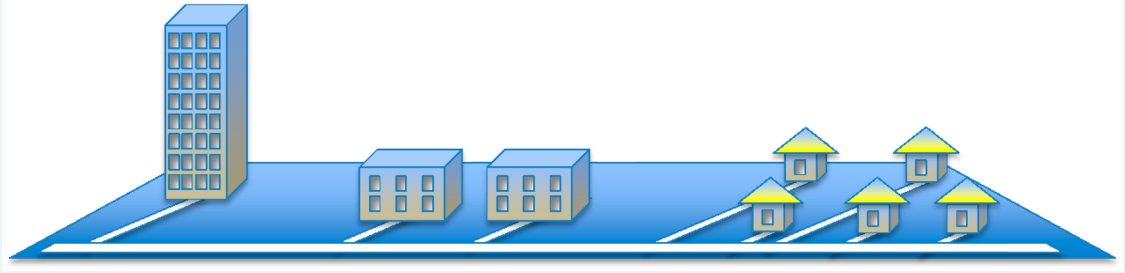

ABSTRACT: **With new approaches in imaging—from new tools or reagents to processing algorithms—come unique opportunities and challenges to our understanding of biological processes, structures, and dynamics. Although innovations in super-resolution imaging are affording novel perspectives into how molecules structurally associate and localize in response to, or in order to initiate, specific signaling events in the cell, questions arise as to how to interpret these observations in the context of biological function. Just as each neighborhood in a city has its own unique vibe, culture, and indeed density, recent work has shown that membrane receptor behavior and action is governed by their localization and association state. There is tremendous potential in developing strategies for tracking how the populations of these molecular neighborhoods change dynamically.**

Location, Location, Location: Just as the saying goes for real estate, the same adage applies in biology. In biology, the link between interaction and function is defined not only by the accessibility of a binding site, its geometry, and its conformational state but also by where and how these interactions take place in the broader, cellular context. How does the functional state of a protein depend on its association state and dynamics? What controls these parameters? How is a protein's function dependent on its location and disposition within the cellular milieu? For a membrane receptor, what is responsible for its activation (or deactivation) during storage in and transport on organelles to the cell surface? What are the physical, chemical, or electronic cues responsible for triggering key structural or conformational changes?

The analogies to real estate and city planning may not be that far-fetched. For a city, what are the appropriate mix and spatial distribution of single-family dwellings, townhomes, and high-density apartments? What determines their locations? If we were to draw a biological analogy, consider a membrane receptor: How does the clustering of receptors in a membrane determine their functional state and activation behavior? Perhaps it may be more important to focus on a slightly different question. Which is more important: a higher spatial density or the spatial density of a particular association state? Extending the metaphor slightly further and using a rather loose analogy to how housing density scales with one's proximity to public transit, what role does ligand binding play in driving receptor association states and complex formation? These are all critical questions that guide public policy in the context of housing but also underpin biological interactions in the cell. Understanding how these interactions dynamically change in response to external (and internal) cues is crucial to our interpretation of not only normal physiological response but also altered dynamics and functional characteristics in the context of disease.

Where this spatial density becomes even more contentious or complex, as it would for housing, is in considering how these specific interactions guide the large system—the organism, or equivalently, a city. Recognizing that cells are adaptive, dynamic machines that integrate signals from their environment to perform complex functions and to assemble into diverse architectures such as tissues, organs, and biofilms, we need to contextualize how signaling occurs, not only through orchestrated inter- and intramolecular interactions between cells but also within individual cells. We need to develop and to exploit strategies that enable us to explore the complexities of protein−protein and protein−ligand interactions fully, such as receptor self-association or ligand-initiated complex formation, which can often involve intricate, orchestrated structural and compositional reorganization and recruitment. This signaling is

DOI: 10.1021/acsnano.7b00061
ACS Nano XXXX, XXX, XXX−XXX

further complicated in systems where interactions take place at membrane interfaces. Rather than simply acting as a passive surface, the membrane itself plays an active role in facilitating specific interactions, inducing conformational changes, and driving complex association behaviors.[1] The structure−function relationships that underpin many membrane-active molecules are linked to the local composition and structure of the membrane itself.[2,3] Extracting the complex structural, compositional, orientational, and chemical interactions that control how molecules behave and interact at interfaces, both membrane and otherwise, has fundamental importance to structural and cell biology, biochemistry, and biophysics.

In the context of receptor signaling, the focus has largely been on discerning the linkages between ligand binding, receptor association state, functionality, and signaling.[4−7] In the past, strategies for assessing functionality or conformational and structural changes have relied on classic structural biology approaches (*i.e.*, nuclear magnetic resonance, X-ray crystallography), which provide highly detailed insights into local conformational states, both free- and ligand-bound, as well as the structural changes between the monomeric and dimeric forms of the receptors.[8,9] Interestingly, these structures have often provided intriguing perspectives on the key drivers for the receptor association state, for instance, whether ligand binding facilitates association or the role of other agents. This situation can be further complicated when one begins to examine other complexities for the functional behavior and activity, including the potential (or even requirement) for heterogeneous complex formation.

Extracting the complex structural, compositional, orientational, and chemical interactions that control how molecules behave and interact at interfaces, both membrane and otherwise, has fundamental importance to structural and cell biology, biochemistry, and biophysics.

Let us draw on the carcinoembryonic antigen (CEA) family to illustrate the extent of the complexities that one faces in interrogating and interpreting these very questions.[10−13] The CEA family includes the membrane-anchored, CEA-related cellular adhesion molecules (CEACAMs), which are known to modulate intercellular adhesion *via* both homo- and heterotypic binding. Structurally, the extracellular domain of all CEACAMS is made up of a single immunoglobulin variable domain (IgV)-like amino (N)-terminal domain and between zero and six immunoglobulin type 2 constant domains, with a single nonglycosylated β-sheet in the N-terminal domain mediating intercellular interactions.[13−16] Changes in CEACAM expression are known to play significant roles in cell growth and differentiation, with overexpression being associated with tumor formation.[17−19] How this overexpression is manifested in terms of CEACAM self-association and clustering remains poorly understood, including the role the surrounding membrane plays in mediating these effects. In that context, the membrane anchor of CEACAM is known to control the cellular response to binding, as well as CEACAM's distribution on the cell surface, at least in the context of known splice variants.[20] Adding to the complexity is the observation that some CEACAMs, notably CEACAM1, can exist as monomers or *cis*-homodimers on the cell surface.[21] This raises the intriguing question of how CEACAM signaling takes place: Does it involve recruitment of signaling molecules to the monomeric or dimeric state, and how does this impact intercellular interaction? In addition to heterotypic interactions between cells, different CEACAMs may form hetero-*cis*-dimers (or oligomers) on the cell.[22] One then starts to question how intercellular CEACAM association takes place: does it involve (1) dissociation of *cis*-dimers to enable *trans* homophilic binding in a monomer−monomer fashion, (2) monomer association to form *cis*-dimers that then drive homophilic intercellular association, or (3) a combination thereof that is somehow context-dependent? These same questions arise in the context of understanding transport of CEACAMs from intracellular storage vesicles to the cell surface.[20,23] Are they transported as monomers, dimers, or oligomers? Are they homogeneously distributed within the storage vesicle? What controls the distribution of CEACAMs once they have been delivered to the cell surface? Does clustering occur? Is the clustering random or directed? What is the makeup of these clusters? Recent work in our own laboratory has focused on addressing these questions using fluorescently labeled CEACAM1[16] (Figure 1).

These questions of spatial distribution, expression, association, and localization are not unique to the CEACAM system. They could be applied to any signal transduction pathway and

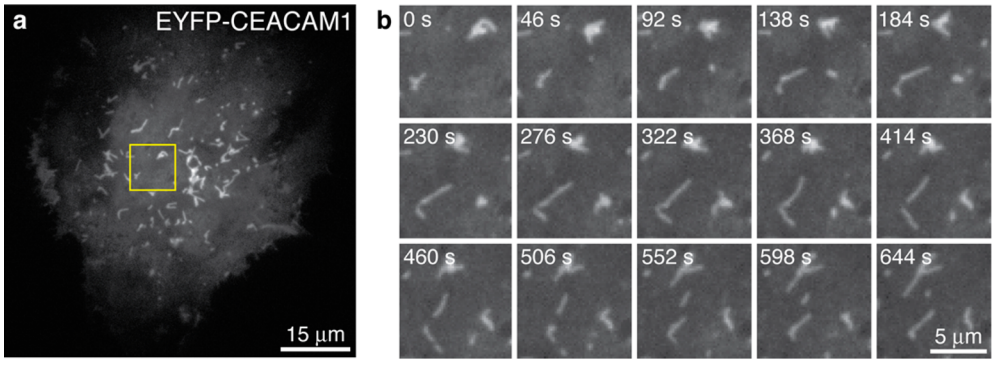

Figure 1. Time-lapse TIRF microscopy of a live HeLa cell transiently transfected with EYFP-CEACAM1. (a) Full cell image, t = 0 s. (b) Time-lapse image sequence of boxed 10 μm × 10 μm region of interest in (a). The time associated with each frame is indicated in the upper left corner of the images.

DOI: 10.1021/acsnano.7b00061
ACS Nano XXXX, XXX, XXX−XXX

certainly are critical to our understanding of signaling and cellular response. With that in mind, what has emerged has been a significant focus on understanding and mapping how these interactions take place at these interfaces and specifically tracking events and dynamics on the nanoscale. While single-molecule techniques have been used for decades, recent advances in optical and electron microscopies are now enabling researchers to ask key questions in biology directly, such as

(1) How many molecules are involved?
(2) How diverse are the interacting partners?
(3) Where do these interactions take place and how does that relate to function?
(4) What are the dynamics of these interactions?
(5) Are there critical conformational changes involved and, if so, what are they?

Efforts in super-resolution fluorescence spectroscopy and microscopy have been critical to the success of these approaches and most recently culminated in the awarding of the 2014 Nobel Prize in Chemistry to Betzig, Hell, and Moerner.[24−26] This award recognized their collective efforts in devising approaches and tools that enable mapping and measurement of single-molecule fluorescence. Although these innovative strategies focused largely on obtaining subdiffraction limit spatial localization, in principle, tracking dynamic changes in a fluorophore's position with subdiffraction limit resolution is more tractable because one is focused on positional variations over time.[27,28] In all of these approaches, however, the emphasis tends to be on measurements of individual molecules.

Within these high-resolution approaches remain a number of key central questions: how does localizing an individual molecule, either spatially, or dynamically, help one discern functional implications and/or context, and more importantly, what are the ties between function, organization, and structure?[29] This opens a significant challenge for super-resolution approaches—namely, linking organization with function and, in particular, the role of specific association states or oligomerization dynamics. This is a challenging and topical area of research, and while there has been much preliminary work in this area, it remains ripe for new developments and platform advances.[30−32] Moreover, there is keen interest in understanding the nature of cellular heterogeneity in these events and pathways and developing ways to extrapolate these insights.

In the January issue of *ACS Nano*, Zhang *et al.* report on a particularly powerful synergistic approach for addressing these very questions.[33] Using a technique they developed known as gSHRImP, they examined epidermal growth factor receptor (EGFR)-mediated mitogen-activated protein kinase (MAPK) activation to address questions about EGFR clustering, organization, and cellular localization, in an effort to understand cell-to-cell variations in response. Their underlying hypothesis was that EGFR clustering and organization within the cell, including localization to specific intracellular bodies, was partially responsible for these variations. Although EGFR clustering has certainly been tackled before by others, the strategy taken by this group exploits quantum dot blinking, which enabled them to super-resolve the location of individual QD-labeled EGFR molecules. From this information, the authors then extracted the number density of EGFR molecules per cluster and compiled statistics in intracluster distances between individual molecules. They developed a predictive Bayesian classifier model that linked both number density and intracluster distances with MAPK phosphorylation activity. Although this research provided a compelling example of how one can quantitatively determine nanoscale spatial distributions of clustered receptors, including the tentative assignment of oligomeric states (monomers, dimers, trimers, oligomers) through a fairly straightforward application of imaging, the authors themselves noted that, for all the super-resolution localization work they did, the predictive model itself was not necessarily significantly better than what might be obtained through the use of conventional diffraction-limited imaging. The context for this observation was a subtle difference in the predictive power of the model based on coupling the intracluster distances with the heterogeneity in the population of associated species within the clusters themselves.

Because their gSHRImP approach enables imaging all the QD-labeled EGFR molecules in the cell, the authors questioned whether subcellular localization could be playing a role. In order to answer that question, and with a strategy that could be correlated with their optical data, the authors turned to transmission electron microscopy and, using gold-labeled anti-EGFR antibodies, proceeded to classify EGFR cluster size, structure, and density with subcellular location. Remarkably, the authors resolved a significant distinction between the structure of EGFR clusters found on the limiting membrane of multivesicular bodies (MVB) and those located on intraluminal vesicles (ILVs) within the MVBs. This distinction enabled them to propose a compelling model that MAPK signaling through EGFR activation was a consequence of larger EGFR clusters, with lower molecular packing density, located on the limited membrane of MVBs. The localization of higher density, predominately dimeric, EGFR clusters to ILVs within the MVBs prevents their interaction with MAPK. These additional insights helped reconcile the differences in their predictive model, confirming the importance of the EGFR association state, distribution, and localization on MAPK activation.

FUTURE OUTLOOK

The work by Zhang *et al.* is an exciting and sophisticated approach for correlating and characterizing receptor association, clustering, and subcellular localization with function. However, a number of exciting avenues remain to be explored, including the potential for (and challenges with) live cell imaging. First, we can consider labeling strategies that enable nanoscale localization and characterization. In addition to the use of quantum dots, techniques such as photoactivated localization microscopy and direct stochastic optical reconstruction microscopy take full advantage of innovative activation (and quenching) approaches to improve spatial resolution[34] with real potential for live cell imaging.[35,36] However, as with all localization tools, and implicit in the present work by Zhang *et al.*, is tacit recognition that the localization is based on the tag itself, which may be located distal to the actual site of interest, depending on the nature of the tether.[37] Arguably, this is quite important if one is using a conventional antibody label and describing clustering and packing densities in terms of the label's localization, not directly that of the actual molecule of interest. Certainly the size of the antibody plus that of the label has to be considered when describing a model of potential interactions or association states. Even the introduction of single-domain antibody or nanobody-based labels for single-molecule microscopy[38] and conformational mapping of EGFR structural dynamics upon ligand binding[39] leaves open the question of spatial localization.

DOI: 10.1021/acsnano.7b00061
ACS Nano XXXX, XXX, XXX−XXX

The possibility of confirming function with localization offers intriguing opportunities for future research. In the case of the work by Zhang *et al.*, cell-population-based phosphorylation analysis was mapped against super-resolved clustering data. In the context of the CEACAM system described above, the relevant metric is the spatial−temporal monomer−dimer−oligomer distribution both within and between cells. Conventionally, one may apply live cell fluorescence resonance energy transfer (FRET) analysis to track these distributions; however, while the FRET signal does report on the proximity of the donor−acceptor pairings, it does not necessarily afford super-resolved spatial localization within the cell. This information can be important, especially if receptor clustering and association state are convolved on length scales below the diffraction limit. Recent efforts, however, are showing promise for coupling super-resolution and single-molecule FRET in live cells,[40] while the potential of coupling FRET signals with fluctuation-based approaches might represent another encouraging avenue for super-resolution live cell imaging.[41,42] Another challenge is that of subcellular localization. Many of the current super-resolution platforms rely on evanescent wave or total internal reflection fluorescence (TIRF) microscopy, which because of its rapidly decaying *z*-illumination profile provides excellent insights into basal membrane structures and dynamics; however, it has its own inherent technical challenges and cannot provide detailed insights deeper into the cell.[43−45] Although this limitation is now being addressed by a number of recent innovations in structured illumination and spinning disk confocal microscopies, it is important to recognize that there is approximately an order of magnitude difference (~150 nm *vs* ~20 nm) in resolution between these live cell approaches and the fixed cell localization strategies described earlier.[46,47] Interestingly, recent developments in single-cell super-resolution light sheet microscopy are now pushing spatial resolution down to ~50 nm in live cells.[48]

These outstanding advances in labeling technologies, imaging tools, and innovative analytical approaches represent a veritable boon in versatile and powerful strategies to address what have been long-standing and compelling questions in cell biology and biophysics. The critical driver for all of these tools has been the revolutionary developments in single-molecule approaches, which at first indirectly enabled single-molecule measurements but increasingly are now enabling direct interrogation and localization. The future lies in the ability to perform these experiments in living systems with comparable resolution and on relevant time scales and ideally with direct confirmation of function.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge funding support from the Canadian Institutes of Health Research (MOP-123320).

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DOI: 10.1021/acsnano.7b00061
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