Membrane Protein Clusters at Nanoscale Resolution: More Than Pretty Pictures

Fluorescence microscopy is powerful for analyzing the composition and dynamics of cellular elements, but studying precise molecule patterns is precluded due to diffraction limited resolution. This barrier has been lifted now through several superresolution microscopy techniques. They revealed that proteins assemble in defined groups (clusters). A new challenge thus appears for the biologist: to find out whether clusters are molecular machines, stabilizers of defined protein conformations, or simply protein reservoirs.

Since the creation of the first microscopes in the 17th century, a number of technical revolutions have contributed to maintaining light microscopy at the forefront of biological research. The initial studies have relied on natural color or density and were thus sufficient only for a broad analysis of tissue structure. Histological stains, along with fixation procedures and sectioning, have been applied later to the study of both cell and organelle structure (as in the classical brain structure studies of Golgi and Cajal). Finally, fluorescence microscopy, with its higher sensitivity, and with its equally high specificity [in the context of immuno-affinity labeling and green-fluorescent-protein (GFP) expression], pushed the limit of microscopy observations to the level of small organelles.

Recent Techniques for Super-Resolution Light Microscopy

The theoretical description of the diffraction of light (Abbe, 1873) prompts the conclusion that even “infinitely” small light sources appear in the microscope as disks with a diameter of approximately half of the light wavelength. Such modulation of point light sources by microscopic lenses leads to systematic blurring of the recorded image [which is referred to as the point spread function (PSF) of the imaging system]. The size of the PSF determines the resolution of the microscope. As a result of diffraction, resolution in light microscopy is limited laterally to ~200 nm and axially to ~500 nm.

This serious limitation has only recently been circumvented by realizing several concepts of super-resolution light microscopy. In brief, these concepts are based on 1) applying in STED (stimulated emission depeletion) microscopy a smaller effective PSF, 2) using in 4Pi-microscopy an axially narrower standing wave/PSF, 3) determining in PALM (photoactivated light microscopy)/STORM (stochastic optical reconstruction microscopy) positions of single molecules with higher precision than diffraction-limited resolution, and 4) collecting in SIM (structured illumination microscopy) information from frequency space outside the observable range.

**STED Microscopy**

STED microscopy was the first high-resolution microscopy concept applied more widely for the study of biological questions. As performed in classical confocal laser scanning microscopy, a conventional laser beam is used to stimulate the fluorescent molecules in what is a normal excited spot (of several hundred nanometers in diameter). A second toroidal (doughnut shaped) beam is used to quench the fluorophores by stimulated emission (37). By overlapping the two beams, fluorescence is allowed only from the center of the spot, which thus generates a much smaller exciting focal spot (FIGURE 1A). Thus, in practical terms, a smaller effective PSF is generated that causes less blurring and, therefore, results in higher resolution. Theoretically, the gain in resolution is not physically limited: it only depends on the power of the quenching laser beam (the higher the power, the smaller the imaging spot becomes). In practice, up to ~10-fold increases in resolution in one dimension have been achieved. Whereas first applications have provided higher resolution in the lateral plane, the technique has also been recently developed to increase resolution in all three dimensions [isoSTED (28)].

Many applications have demonstrated the usefulness of STED microscopy (see also FIGURE 1, B AND C). The strength of the method relies in the direct recording of super-resolved signals without the necessity of subsequent image processing, which may modify the image information. However, STED microscopy is a scanning microscopy technique, and as resolution increases, scanning of more and smaller pixels is required (e.g., 100-fold more pixels need to be scanned if the pixels become smaller by 10-fold in one dimension). Hence, the cost of higher resolution...
is longer scanning times, which limit, in addition to high bleaching, the suitability for live cell imaging. Despite these limitations, live, real-time STED microscopy has been recently shown to be in principle applicable (36).

**4Pi Microscopy**

4Pi microscopy generates a several-fold increase only in axial resolution by using two objectives placed opposite to each other for illumination and/or detection of the same point in the preparation (which is thus sandwiched between two coverslips). By interference of the wave fronts of the opposed lenses, a standing wave is generated with an approximately fivefold smaller central maximum in the axial direction compared with the PSF of a conventional confocal microscope. Hence, resolution increases axially from 500 to ~100 nm (8). 4Pi microscopy is a very interesting technique from a theoretical point of view. However, practical applications are difficult since cells have to be placed between two coverslips with distances in the range of 100 μm, making solution exchange, for example, difficult. In addition, the two smaller side maxima of the standing wave generate “ghost images” that cannot be reliably filtered out when too many objects are present in the sample. 4Pi-microscopy has been the first commercially available super-resolution technique, but it is not widely distributed due to practical constraints and the somewhat limited applicability (also since increases in lateral resolution are easier to apply to biological questions compared with increases in axial resolution).

**PALM/STORM**

Although the ~200-nm limit of light microscopic resolution prevents separation of two objects at distances lower than 200 nm, centers of individual objects can be readily determined with nanometer precision. Hence, in principle, cells could be imaged at nanoscale resolution, provided the position of each of their fluorescent labels (hundreds of thousands) could be determined one by one. In addition to increased resolution, spatial information from individual molecules within larger supramolecular structures would be gained.

What sounds at first glance impossible has been realized by the application of photoswitchable dyes. The principle has been published by two different groups independently from each other, who have named the techniques PALM (5) and STORM (24). As photoswitchable dyes for PALM and STORM, photoactivatable proteins or Cy3-Cy5 dye pairs were used, respectively. Unless fluorophores are not inactivated from the beginning, recordings start by switching “off” all fluorophores, and then subsets of fluorophores are switched “on” with a brief laser pulse. The laser pulse is weak so that only few molecules are stochastically switched “on,” resulting in such a low density of activated molecules that overlap within diffraction limited

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**FIGURE 1. STED microscopy**

A: as in a conventional confocal microscope, in a STED microscope fluorescence is excited by a laser beam (EXC beam) that is focused to a spot (EXC spot) used for scanning of the sample. The size of the spot (half-width of intensity) is limited by diffraction and depends on the wavelength. In practical terms, laterally it cannot be smaller than several hundred nanometers, thus directly determining the resolution of the microscope. To make the spot smaller (increase the resolution), a STED microscope uses a second, phase-modulated doughnut-shaped laser beam (STED beam) to de-excite the excited fluorophores before they can emit fluorescent light (green). De-excitation is not complete in the center of the EXC spot, because intensity in the center of the STED spot is much lower than in the periphery. For this reason, central fluorophores are left excited and eventually emit fluorescent light. As a result, the sample is scanned with a several-fold smaller (than the diffraction limit) excitation spot (green spot and graph in A), increasing resolution accordingly.

B and C: comparison of resolution in the confocal (left) and the STED channels (right). B: the image shows single synaptic vesicles visualized in primary cultured hippocampal neurons; the vesicles distribute along the axon, with the higher density areas representing synapses. Scale bar, 500 nm. C: dual color STED resolving the synaptic proteins synaptophysin (red) and syntaxin (green) in neurons. Spherical objects at left are fluorescent beads stained with the dyes on the surface. Images have been adopted/modified from Ref. 37 (A and B) (adapted by permission from Macmillan Publishers Ltd.) and Ref. 20 (C) (adapted by permission from Wiley-VCH Verlag GmbH & Co.).
FIGURE 2. PALM/STORM

A: principle of operation of PALM on a sample stained with photoactivatable fluorescent protein (PA-FP). AA and AB, a subset of PA-FP are activated with a brief flash of light using the 405-nm laser line and then imaged using a 561-nm laser line until bleaching of most PA-FP molecules (AC). AC and AD, the cycle (activation, and then imaging until bleaching) is repeated until no more inactivated molecules are available. AE and AF, all recorded signals are used to build up one image; an increase in resolution is only obtained if, before summing up, the location of each molecule is determined. To this end, the expected image (determined by the PSF of the microscope; AG, middle) is fitted to the recorded image (AG, left), resulting in the position of the molecule that is determined with a certain uncertainty (AG, right). AA’-AD’ were obtained from the corresponding images AA-AD by fitting the PSF to the recorded signals, and AE’ and AF’ represent the summed-up image.

B: focal adhesions visualized in a FoLu cell expressing dEos-labelled Vinculin. Image recorded with total internal reflection (TIRF) microscopy (top) and PALM (bottom). Total internal reflection microscopy allows the imaging of samples within ~50–300 nm from a glass cover-slip, with conventional diffraction-limited lateral resolution.

C: relative distributions of transferrin receptor (labeled with PamCherry, red) and clathrin light chain (labeled with PAGFP, green) visualized by TIRF-microscopy (left) and PALM (middle; right, magnified view). Scale bars, 2 μm (left and middle) and 0.5 μm (right). Images have been adopted/modified from Ref. 5 (A and B) (reprinted with permission from AAAS) and Ref. 35 (C) (reprinted by permission from Macmillan Publishers Ltd.).
resolution is very unlikely (FIGURE 2A). Hence, under these conditions, several molecules are imaged, which are individually resolved due to their large spatial separation. Imaging of the “on” fluorophores is performed until all activated molecules are bleached (PALM) or switched again to the dark state (STORM). The process is repeated many times until up to several hundreds of thousands of molecules are imaged. The signal obtained from all emitted and detected photons from one molecule is fitted to the PSF of the microscope, allowing for mathematical determination of the molecule location. From the positions of all molecules, an image is reconstructed, generating a 10- to 20-nm super-resolution image.

This method is the only high-resolution method that provides images composed of single molecule positions. It is relatively easy to implement into a conventional microscope, so that several custom-made setups are currently in use. Multi-color and fluorescent protein imaging is in principle easier to realize than with STED microscopy, but live imaging is presently impossible, unless the biological question allows for determining only the position of a subset of molecules that participate in a slow process (e.g., assuming that the position of only 1,000 molecules from a set of 100,000 needs to be determined at any time point). A disadvantage is the relatively indirect approach of data extraction. As signals are fitted to the PSF of the microscope, the final image depends very much on the settings of the image processing software. Another restriction can be that the method does not detect all fluorophores (PALM) or detects the same molecule several times (STORM), so that it is difficult to determine the precise number of molecules present.

SIM

The main principle of SIM is the use of a structured pattern of illumination light for excitation. Due to the use of patterned illumination, interference generates moiré fringes in the image (see also FIGURE 3A), which can be used to back-calculate the structure of the sample in a frequency domain not resolvable by diffraction limited resolution.

Structured illumination (SIM) allows resolutions of ~100 nm in each the x-, y-, and z-direction when biological material is used (12, 26). Theoretically, under extreme illumination conditions (when saturated structured illumination is applied, allowing for a nonlinear dependency of the fluorescence emission rate on the illumination intensity), the resolution limit should be unlimited, and resolution below 50 nm has been demonstrated using fluorescent beads (11). However, at least for the near future, only linear dependency appears to be applicable to biological samples, allowing at maximum a twofold increase in resolution compared with diffraction limited imaging. In any case, it is clear that, from all super-resolution methods, SIM is the least powerful regarding the gained resolu-

**FIGURE 3. SIM**

A: the principle of resolution enhancement through structured illumination. When the sample structure (left) is illuminated through a known pattern (middle), a new pattern (moiré fringes) appears (right). The original sample structure can be calculated from the observed moiré fringes. B: simplified structured illumination set-up. Laser light is sent onto the sample through a linear phase grating; three beams (diffraction orders -1, 0, and +1) are generated, which interfere and generate an illumination intensity pattern in both the lateral and axial directions. The fluorescence arising from the sample illuminated with such a pattern is collected by the CCD camera. C: illumination pattern produced as described in B. D: comparison between multi-color images of nuclei, in both confocal (left) and structured illumination microscopy (right). Red, nuclear pore complexes (NPC); green, Lamin B; blue, DAPI. Scale bars are 5 μm for overview and 1 μm for boxed areas. Images have been adopted/modified from Ref. 11 (A) (with permission from National Academy of Sciences), Ref. 12 (B and C) (with permission from Elsevier), and Ref. 26 (D) (with permission from AAAS).
Advancements Through Super-Resolution Light Microscopy: Seeing Clusters

Traditionally, electron and light microscopic approaches are used for the study of cellular structures. Electron microscopy provides ultrastructural information at resolutions up to 1–2 nm, taking advantage of heterogenous cellular protein densities for contrast generation. In addition, specific proteins can be visualized by immunogold labeling, although antigenicity on the surface of electron microscopic samples is very low. Electron microscopy is not a high-throughput technique; even the tomographic 3D reconstruction of one small cellular structure (e.g., the Golgi complex) requires strong experimental efforts (see, e.g., Ref. 18). The most limiting constraint regards the imaging of live cells that is impossible to perform with electron microscopes.

In contrast, light microscopes allow for dynamic studies of live cells and for quick optical sectioning and 3D reconstruction. With few exceptions, cellular organelles have no intrinsic features that would generate any contrast, except when they are labeled with fluorescence dyes. Although also antigenicity is expected to be in principle limited (see below), proteins can be readily visualized at high signal-to-noise, also by the fluorescent protein technology, which even allows for the study of protein dynamics or protein-protein interactions in vivo. Apart from photobleaching, in fluorescence microscopy a strong limitation is diffraction limited resolution that prevents resolving organelles, filaments, other cellular structures, or even single molecules if present at high densities (as mentioned above, low resolution does not per se preclude the detection of objects much smaller than the resolution but separation of small objects with short distances).

Hence, both electron and light microscopy provide essential advantages that, if combined in super-resolution light microscopic methods, generate a strong tool for analyzing cellular structures and dynamics. Presently, STED microscopy, PALM/STORM, and SIM are just beginning to explore these possibilities, and technical developments in the near future should attenuate some of the current limitations. However, it is already clear that one result persistently obtained in basically all super-resolution investigations is the clustering of proteins that had not been recognized by conventional microscopy or electron microscopy (compare conventional resolution and superresolution in FIGURE 1C AND FIGURE 2, B AND C). The discovery is at a first glance not surprising; after all, many studies applying light microscopy or biochemistry have found membrane proteins, for example, not being homogenously distributed, and it has been hypothesized for more than a decade that membranes are compartmentalized, containing specialized membrane domains (for example resembling the cholesterol and sphingolipid-rich “rafts” originally proposed in 1997 by Simons and Ikonen (33)) and various other protein assemblies.

So far, high-resolution microscopy has revealed an impressive array of proteins to be found in small clusters (for examples, see FIGURE 1C AND FIGURE 2, B AND C). To cite only a few of the studies, they include synaptic vesicle proteins [synaptotagmin (37)], plasma membrane SNARE fusion proteins (32), lysosomal and endosomal membrane proteins (5, 9), clathrin-coated pits (2), focal adhesion proteins (5), several mitochondrial proteins (5, 7, 29), as well as components of the nuclear lamina, chromatin, or nuclear “speckles” enriched in RNA splicing factors (6, 26). Moreover, donut-shaped structures or half-moons have been observed in cases where conventional microscopy images only diffraction limited fuzzy objects (6, 15).

Although nothing speaks against these proteins being clustered, there are other examples for which differently shaped signals would have been expected. For instance, the organization of filamentous cytoskeletal elements is well understood, and the strands (branched or not) they form are immediately recognizable in techniques such as scanning electron microscopy or atomic force microscopy. As expected, actin and microtubules still appear as continuous filaments under SIM imaging (12), with the microtubules giving only a small suggestion of non-uniformity. However, under the higher resolution of STORM (2, 14), the microtubules are strongly non-uniformly labeled and appear as interrupted, inhomogeneous structures. The same is the case under STED optics (22), and neurofilaments have also been observed as punctate, discontinuous structures in STED (6). Another
example of the fluorescence labeling conforming little with the expected structure is found for clathrin-coated pits: instead of the highly symmetric structure observed in electron microscopy, a circular structure, with relatively poor symmetry and inhomogeneous labeling, was found (14).

The clustering of cytoskeletal elements could have various causes beyond the purpose of this review; however, these observations may also be explained by the fact that these experiments have relied on immunoaffinity labeling. First, antibodies are relatively bulky structures (~5–10 nm), may not penetrate well through the detergent-permeabilized cells, and thus may not bind to all available epitopes. Second, epitopes may be masked by various proteins binding to the elements of interest (such as the plethora of transport proteins and cargo organelles binding to cytoskeletal elements). Third, antibody labeling typically is performed on fixed and permeabilized tissue; chemical fixation may affect some of the epitopes, causing relatively poor (and therefore discontinuous) detection. Even worse, these effects may become increasingly negative with the use of multiple antibodies for multiple epitopes, due to steric hindrance.

Thus one concern in antibody-based labeling is that insufficient epitope binding may result in a punctuate staining, thus pretending “protein clusters.” Clustered appearance of proteins can also be induced when unixed membranes are stained by diluted antibodies that induce protein cross-linking (a primary antibody binds to two individual membrane proteins and one secondary antibody to two primary antibodies), a mechanism that can be in part suppressed by using high antibody concentrations (17). Although these effects may be avoided by fixation before antibody addition, fixation followed by subsequent permeabilization induces another concern, since inaccurately performed permeabilization can grossly perturb the membrane structure.

One may conclude from such observations that antibody-based detection is unreliable. However, reality is not as grim. Antibody staining can be compared with the GFP-based imaging of the same protein to determine whether antibodies recognize only an unrepresentative subset of proteins. Also, in early endosomes, several neuronal proteins have been found clustered in presence of cholesterol (by antibody detection), with cholesterol depletion dispersing the clusters (9). Thus fixation/antibody staining were unable to cause the formation of clusters. Importantly, discontinuous arrangements of proteins have been observed also with PALM, which relies on the expression of fluorescent proteins (5), not only in fixed but also in cryopreserved preparations. More recently, paxillin was found clustered also in living cells (30). Of course, one may still argue that the concentration of overexpressed proteins may be low and the mosaic of native and overexpressed proteins could lead to a punctuate distribution (with the overexpressed proteins scattered randomly, in distinct spots, in the otherwise randomly distributed native protein population). However, this is not a strong argument, at least in view of a recent detailed analysis of clustering of proteins involved in bacterial chemotaxis (10), which showed that overexpressed proteins formed clusters in a genetic background lacking the native untagged proteins.

Thus one may safely conclude that, although controls are inescapable, protein clustering as observed under super-resolution imaging is a real phenomenon, and super-resolution light microscopy is currently the only technique that allows one to determine the density and size of clusters and to obtain an estimate on the number of molecules inside or outside of clusters.

If protein clustering is not artifactual, how is it achieved? The answers are probably complex, with many mechanisms already proposed, and with many mechanisms still to be identified. The preference for certain lipid phases can induce the formation of clusters. For example, GPI-anchored proteins have been found to associate with membrane rafts (33); also, the SNARE molecule SNAP25 (which lacks transmembrane domains) forms clusters that only depend on its palmitoyl chains, presumably integrating in the proper lipid phases, and not on protein-protein interactions (13, 25). In a more complex description of membrane partitioning, the actin cytoskeleton may form fences in the plasma membrane, on which transmembrane proteins are anchored (16). Clearly, more than one mechanism can participate in the formation of one cluster. For example, syntaxin 1 clusters depend on lipids as cholesterol (17) and phosphatidylinositol 4,5-bisphosphate (21) and on protein-protein interactions (31).

Why Clusters?

As outlined above, clustering of proteins is a principle in nature that can now be quantitatively investigated. However, there is no general explanation for what reason, if any, proteins form clusters. The simplest answer to the issue is that it probably depends on each protein individually. However, some distinguishable roles of clusters can be already predicted from initial studies that have explored these possibilities (FIGURE 4).

For instance, a long debated question is whether synaptic vesicles can be directly recycled or whether their components, after fusing with the presynaptic membrane, are diluted in the plasma membrane and taken out one by one by complex
endocytotic pathways that finally allow the reassembly of vesicles in intracellular compartments. By using the synaptic vesicle protein synaptotagmin as synaptic vesicle marker, STED microscopy has shown that synaptotagmin patches of a defined size appear on the surface of hippocampal neurons (37). Hence, this finding provides support for the idea that synaptic vesicle components remain clustered after fusion to be directly retrieved as a membrane patch containing selectively synaptic vesicle components. In this case, clustering would have the function to avoid lateral exchange and therefore preserve the identity of a supramolecular ensemble that constantly cycles between an intracellular vesicle (i.e., being the vesicle) and a plasma membrane patch (FIGURE 4A).

Intuitively, one would also expect a role of clustering for increasing the local protein concentrations to accelerate reaction kinetics or to exceed locally the threshold of signaling components (FIGURE 4B). Numerous types of membrane channels may need cluster to generate a stronger signal locally; for example, calcium channels may cluster near active zones to generate strong presynaptic calcium influxes [as observed by STED microscopy (19)]. Also, the clustering of postsynaptic ligand-gated channels (such as nicotinic acetylcholine receptors or glutamate receptors) is well known from many preparations [even at the confocal level; see, for example, the review by Slater (34) or Ref. 27 for glutamate receptors] and serves to cause a strong local depolarization, which would in turn trigger an action potential.

However, it is possible that, for proteins that do not just open a pore but have to undergo strong lateral rearrangements, clustering leads to such strong molecule crowding that the biochemical reactions mediated by the clustered proteins cannot take place due to steric hindrance. Such an example has been described for the membrane fusion protein syntaxin 1 that in the plasma membrane forms dense clusters of ~75 molecules crowded in an area with a diameter of 50–60 nm that exchange syntaxin molecules with each other (32). Only a small fraction of syntaxin is free, and further analysis has predicted that the function of syntaxin clustering is to preserve its reactivity in the cluster, since otherwise it would form in an uncontrolled manner complexes with its partner SNAREs in the plasma membrane (1). In this case, clustering serves as a molecule buffer that would allow having always a defined concentration of reactive molecules available (FIGURE 4C). Whereas the number of 75 syntaxin molecules per cluster has been predicted by a combination of approaches including STED microscopy, a more recent study has documented that estimates of the number of molecules per cluster can be directly obtained by PALM (23). In this case, imaging of single fluorescent protein-tagged syntaxin molecules has yielded 30–40 molecules in an area covered by a 50-nm spot. Taking into account that PALM can only provide a lower estimate of molecules, and also that unlabeled endogenous syntaxin is present in the clusters, this value is remarkably similar to the previously published one.

**FIGURE 4. Possible roles of clustering**

A: in biological membranes, clustering of multiple proteins into stable entities would avoid loss of components from functional supramolecular complexes. As a result, clustering would preserve the identity and function of supramolecular ensembles. In extreme cases, the integrity of the clusters may be maintained even during membrane trafficking events including exocytosis and endocytosis, as suggested by super-resolution microscopic studies of the synaptic vesicle protein synaptotagmin; after synaptic vesicle fusion, it still appears to be organized in plasmalemmal clusters (37). B: clustering of (for example) enzymes would generate local activity spots, provided the active domain (red) is not hindered or shielded by the clustering process. By summing up the activities of the individual enzymes, signal thresholds/product concentration levels would exceed the ones required for triggering or maintaining a biological process.

C: clustering may also lead to shielding or inactivation of a functional protein domain (red). In an equilibrium between clustered (inactive) and free molecules, clustering would function as a molecule buffer generating a defined concentration of active molecules. For syntaxin 1, it has been suggested that buffering its reactivity is required, since otherwise it would form unproductive cis-SNARE complexes with its partner SNAREs in the plasma membrane (1). In extreme cases, all molecules may be present in clusters to completely block activity. This has been suggested for synaptobrevin during transport in endosomes as a mechanism that prevents synaptobrevin from forming unproductive complexes with endosomal fusion proteins (3, 4).
A different example of the molecule buffer has been observed when synaptobrevin (which functions in vesicle fusion to the plasma membrane) is transported in endosomes. Since synaptobrevin has no role in endosome function, it is maintained in tight clusters, a mechanism that prevents it from making complexes with the endosomal fusion proteins and disturbing their activity (3, 4).

In summary, the examples show clearly how high-resolution light microscopy has opened an avenue to new biological concepts regarding functional lateral organization of proteins into supramolecular architectures.

The Future: Stoichiometric Biology?

Finding proteins localized to certain organelles in conventional imaging has indicated the area where they reside. Now their organization becomes known—or, to be more correct, the organization of the exact places where they reside. As an example, SNAP25 and syntaxin 1, proteins functioning in the vesicle fusion to the plasma membrane, are found “all over” the membrane, including areas where no vesicles could be found, so areas where they could not actually function (22). Also, although they are molecular partners with a common function, syntaxin 1 and SNAP25 are found in clusters that do not correlate particularly well (13).

The cluster of proteins provides a reserve pool of molecules from which single molecules may be extracted for function. Whether the reserve of molecules is ever used may depend on the specific protein and on the challenges the specific system is functioning under. However, for some proteins expressed at much higher levels than their interaction partners (with proteins from the SNARE family providing several examples), the reserve may actually never be used. Clearly, identifying the role of free molecules vs. clusters will lead to a much deeper understanding of the complex molecular networks that control biological function. However, another complication may be that at this point it becomes increasingly clear that not all proteins of a specific type, even if they are not engaged in any heterologous complexes, are equal.

Thus simply investigating the location of proteins may not give much information on function, after all; the average kid’s toy spends most of its lifetime in a drawer (or thrown under the bed) without that being the intended function. Those few and far between instances where protein function actually takes place may be difficult to follow if one concentrates solely on the clusters. Determining the amount of free molecules, that of molecules found in clusters as well as their exchange rates, is a requirement for understanding function. A further level of complexity is thus needed: a computation of molecular organization and function in which the molecular assemblies are quantified and the number of molecules in the different structures or states are determined (for an approach in this direction, see Ref. 32), which one may term stoichiometric biology. That, however, means that microscopy needs to part to some extent with that structure that caught most attention in the initial studies—the protein cluster.

S. O. Rizzoli acknowledges the support of a starting grant from the European Research Council (ERC FP7 NANOMAP).

T. Lang was supported by a grant of the Deutsche Forschungsgemeinschaft (LA1272/2-3).

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