Meeting Report – Visualizing signaling nanoplatforms at a higher spatiotemporal resolution

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The International Symposium entitled 'Visualizing signaling nanoplatforms at a higher spatiotemporal resolution' sponsored by the Institució Catalana de Recerca i Estudis Avançats (ICREA) was held on 29–31 May 2013 at the ICFO-Institute of Photonic Sciences, in Barcelona, Spain. The meeting brought together a multidisciplinary group of international leaders in the fields of super-resolution imaging (nanoscopy) and cell membrane biology, and served as a forum to further our understanding of the fundamental mechanisms that govern nanostructures and protein–function relationships at the cell membrane.

Held in the modern facilities of the ICFO-Institute of Photonic Sciences, in the beautiful beach town of Castelldefels (Barcelona), Spain, the ICREA Symposium was co-organized by Maria Garcia-Parajo and Melike Lakadamyali (both ICFO, Barcelona, Spain), Alessandra Cambi (Radboud University, Nijmegen, The Netherlands) and Diane Lidke (University of New Mexico, Albuquerque, NM). The meeting brought together international experts and young scientists in an intimate setting to discuss the latest advances in our understanding of the nanolandscape and spatiotemporal organization of the cell membrane. The 3-day program featured a total of 30 talks by invited speakers and selected contributions from submitted abstracts. In addition, numerous poster presentations and laboratory tours of different research facilities at ICFO provided a stimulating and lively environment with plenty of time for discussion. Each day focused on a specific topic; the first day on 'super-resolution techniques: from methods to data analysis', with 'lipids and protein nanoislands' and 'Signaling nanoplatforms' on days 2 and 3, respectively.

The Symposium kicked off on Wednesday 29 May with opening remarks by Maria Garcia-Parajo, who set the stage for the meeting by highlighting the issues the Symposium aimed to address, such as the role of lipids, proteins and actin cytoskeleton in templating the cell membrane, in particular asking how and why so many receptors appear to be 'nanoclustered' in their resting state. Other key aims of the meeting were to discuss how signaling is initiated at the cell membrane and the emerging role of the cytoskeleton in regulating order and function, as well as raising the question as to whether we have sufficient biochemical and biophysical tools at hand to interrogate the cell membrane at a high spatiotemporal resolution.

From cutting-edge optical tools to fluorescent probes and image analysis

The advent of novel optical techniques that are able to surpass the diffraction limit of light in combination with single-molecule dynamics approaches is driving exciting recent discoveries in cell membrane biology. The meeting thus placed a special emphasis on these techniques, with contributions from the experts who invented them. Stefan Hell (Max Planck Institute for Biophysical Chemistry, Germany) explained the concept behind and the technological advances in stimulated emission depletion microscopy (STED). He gave numerous examples of biological (and non-biological) applications of STED and also compared and contrasted the focused-light approach for breaking the diffraction limit to localization-based approaches. Samuel Hess (University of Maine, Orono, MN) then introduced the concept of localization-based super-resolution microscopy and in particular talked about fluorescence photoactivation localization microscopy (fPALM), including recent advances such as multi-color fPALM imaging, and its application to study the role of membrane organization in influenza virus biology. Finally, the technique of stochastic optical reconstruction microscopy (STORM) was presented by Xiaowei Zhuang (Harvard University and HHMI, Cambridge, MA), who gave an overview of a range of exciting technological advances, including 3D imaging using astigmatism, live-cell imaging with fast acquisition times and an additional twofold improvement in the 3D spatial resolution using a dual objective geometry.

One theme that emerged from these talks was the key importance of improved photoswitchable probes for all nanoscopy approaches. For example, Stefan Hell highlighted the development of novel photoswitchable fluorescent proteins that are highly photostable and undergo many photoswitching cycles, thereby allowing super-resolution imaging with focused light at low-light intensities. He encouraged the audience to seek for new molecular states with favorable characteristics for super-resolution approaches. Xiaowei Zhuang described a new way of 'caging' fluorophores into dark states using sodium borohydride; upon uncaging with UV light, these fluorophores emit a large number of photons that allows an extremely high localization precision (Vaughan et al., 2012). Last but not least, Markus Sauer (Julius-Maximilians University Würzburg, Germany) gave an overview of the different mechanisms that can be exploited to put fluorescent dyes into long-lived dark states, such as the semi-reduced radical anions or fully-reduced leuco dyes as used in direct STORM (dSTORM). He also stressed the importance of the photophysical properties of fluorophores, such as photon budget and duty cycle for achieving high resolution both in terms of localization precision and label density.

Novel methods that had been developed specifically for accessing dynamic information with high spatiotemporal resolution are another important area of recent technological advances. For example, Stefan Hell mentioned an approach that relies on parallelizing the STED-related concept RESOLFT using a light pattern

containing a hundred thousand 'doughnuts' that are simultaneously scanned to record large field of views within seconds. Melike Lakadamyali (ICFO, Barcelona, Spain) presented her work on combining live-cell and super-resolution nanoscopy in a correlative way to place intracellular dynamics into the context of subcellular ultrastructure, and summarized results on the impact of the 3D microtubule network architecture on cargo transport dynamics at microtubule intersections, revealed by using this method (Bálint et al., 2013). Keith Lidke (University of New Mexico, Albuquerque, NM) presented an exciting and new high-speed hyperspectral imaging method his laboratory had developed to extend high-resolution single-particle tracking of quantum dots to many colors. This method uses a confocal illumination and detection scheme with a fast camera coupled to a prism spectrometer to split the emission into many spectrally distinct channels. This approach enables the tracking of up to eight spectrally distinct quantum dots simultaneously, and therefore holds great promise to study the dynamics–function relationship of important receptors on the cell surface with high spatiotemporal resolution (Cutler et al., 2013). The selected talk from Arnd Pralle (University of Buffalo, NY) introduced the concept of thermal noise imaging using an optical trap to measure the motion of molecules in small regions of the membrane. Interestingly, this approach also allows gathering sufficient data to 'image' the membrane, as it creates high-resolution maps of local diffusion, local attraction potentials and nanoscale membrane stiffness, with the latter apparently being modulated by cholesterol content.

The urgent need for developing new data analysis methods for the accurate quantification of super-resolution images was underscored in two selected talks. Bernd Rieger (Technical University of Delft, The Netherlands) demonstrated how Fourier ring correlation analysis can be used to determine the achieved spatial resolution, along with the number of fluorophores, in a super-resolution image (Nieuwenhuizen et al., 2013). Carlo Manzo from Maria Garcia-Parajo's group (IFCO, Barcelona, Spain) presented a new method that employs Bayesian analysis to locate the molecular position of individual fluorescence

markers and to reveal the stoichiometry of highly dense molecular aggregates within the point-spread function (PSF) of STED images.

Finally, Maxime Dahan's talk (Institute Curie, Paris, France) was a reminder that tools for manipulating signaling nanoplatforms must be developed in parallel to visualization tools. He presented his most recent manipulation method, which uses magnetic nanoparticles that can be displaced by magnetic forces inside the cytoplasm of living cells and used to trigger signal transduction at the plasma membrane (Etoc et al., 2013).

Membrane organization – the (un)usual suspects

A vast amount of recent literature implicates certain lipids, cholesterol, proteins and the cortical cytoskeleton in the nanoscale compartmentalization of the cell membrane. These 'usual suspects' were heavily discussed during the entire meeting, as well as some other components that also appear to organize the cell membrane and to influence cell function. To start, Ken Jacobson (University of North Carolina, Chapel Hill, NC) provided a comprehensive historical perspective of lipid rafts, outlining how their definition has been continuously evolving since the 2006 Keystone Symposium on lipid rafts and cell function (Pike, 2006). Originally being defined as small (10–200 nm), heterogeneous and highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes, Ken Jacobson pointed out that recent evidence provided by nanoscopy approaches now shows that lipid rafts are in the range of 25 nm or smaller and have lifetimes shorter than 10 ms. Mario Brameshuber from Gerhard Schutz's group (Vienna University of Technology, Austria), however, provided evidence that challenged this idea of dynamic behavior by using an elegant approach, the so-called TOCCSL (thinning out clusters while conserving the stoichiometry of labelling) (Brameshuber and Schütz, 2012). He showed that GPI-anchored proteins, thought to be raft constituents, could be found as dimers on the cell membrane, where instead of being highly dynamic, they form stable small nanoplatforms with lifetimes that exceeded the observation time of their experiments (~2.2 seconds). Similarly, using thermal

noise imaging, Arnd Pralle also showed that GPI-anchored proteins form stable nanodomains that exhibited slow diffusion and were stiffer than the surrounding membrane. The stability and almost stationary character of GPI-anchored protein nanodomains was also emphasized by Satyajit Mayor (National Center for Biological Sciences, Bangalore, India), who showed data that suggest a role for new cortical actin structures (he called them asters) in templating the cell membrane by actively driving the formation of 'hotspots' of stationary GPI-anchored and actin-binding proteins (Gowrishankar et al., 2012).

Aside from lipid rafts, several groups in the field have postulated the existence of protein islands on the cell membrane. Indeed, using immunoelectron microscopy of native membrane sheets ('rip-flips'), Bridget Wilson (University of New Mexico, Albuquerque, NM) had shown previously that almost all proteins concentrate in specific regions of the cell membrane, that is, protein islands, whether or not they partition into rafts (Lillemeier et al., 2006). In her talk, she now discussed how modeling combined with single-molecule dynamics approaches could provide insights into the impact of protein islands and nanodomains on signaling in order to address the dynamics of receptor interactions and role of protein co-confinement. She showed spatial stochastic simulations of EGFR–ERBB1 suggesting that domain co-confinement could promote related interactions between individual pairs of receptors and increase the probability of multiple docking events with signaling partners. One unexpected result was that under low expression levels and/or sub-saturating ligand conditions, membrane domains can actually act to segregate receptors and reduce signaling events.

Finally, lipid–lipid interactions are yet another way of compartmentalizing the cell membrane. Alf Honigmann from Stefan Hell's group (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) presented his elegant work from a collaboration with Reinhard Jahn's group that elucidates the molecular interplay between phosphatidylinositol (4,5)-bisphosphate clusters, the SNARE protein syntaxin-1 and the vesicular Ca^{2+} sensor synaptotagmin-1, which regulates the recruitment of synaptic vesicles to the plasma membrane for exocytosis

(Honigmann et al., 2013). For his stimulating presentation, he was awarded the short-talk prize.

The meeting also placed interest on other organizers of the cell membrane. For instance, Ken Jacobson showed traveling waves of cortical actin and demonstrated that compression and subsequent dilation of the coupled plasma-membrane–cortex layer can generate rapid cell shape changes (Kapustina et al., 2013). He suggested that this process, together with the glycocalyx, might be thus far underestimated factors that are involved in the nanoscale remodeling of the organization and dynamics of the plasma membrane. The selected talk of Christoph Feest from Facundo Batista's group (London Research Institute, UK) beautifully illustrated how the combined action of actin and the tetraspanin network can organize receptor nanoclusters to regulate signaling mediated by the B cell receptor. Using a combination of dSTORM and single-dye tracking, he showed that B cell receptors and the co-receptor CD19 exist as spatially distinct preformed nanoclusters in resting B cells. Although the actin cytoskeleton regulates the B cell receptor, interestingly, CD19 mobility was restricted by the tetraspanin CD81, indicating that both CD19 and CD81 could form scaffolds to support B cell receptor signaling (Mattila et al., 2013). The importance of tetraspanins as local organizers of the cell membrane was also highlighted in the talk of Maria Yañez-Mo (Instituto de Investigacion Sanitaria Princesa, Madrid, Spain). Using a combination of biochemical and microscopy cross-correlation analysis approaches, she showed that tetraspanin-enriched microdomains have a role in the compartmentalization of Rac GTPase at the plasma membrane and at exosomes, providing a molecular mechanism for the regulation of cell motility by tetraspanins (Tejera et al., 2013). Her talk also emphasized the need for super-resolution microscopy approaches to study tetraspanin networks within the plasma membrane. Considering the heterogeneous and dynamic character of these fascinating protein networks, approaches such as multiplex imaging at high spatiotemporal resolution might be attractive new tools for the tetraspanin field.

Other membrane structures that received considerable attention in the meeting were clathrin, cortical endosomes and caveolae. Bridget Wilson showed impressive transmission electron microscopy (TEM) images of clathrin-coated pits that reside close to or inside protein islands, and Joe Grove from Mark Marsh's group (University College London, UK) presented microscopic evidence for the existence of a distinct population of large flat clathrin structures that are morphologically and dynamically distinct from clathrin-coated pits and could potentially act as organizing signaling platforms on the plasma membrane. Thomas Weidemann from Petra Schwille's group (Max Planck Institute of Biochemistry, Martinsried, Germany) presented on a new class of cortical endosomes that reside very close to the plasma membrane; they are highly stable, contain early endosomal markers and potentially function to concentrate proteins such as the Interleukin-4 receptor to induce their oligomerization and downstream activation of receptor signaling. Finally, Miguel del Pozo (Centro de Investigaciones Cardiovasculares, Madrid, Spain) focused on the intriguing interplay between actin fibers and caveolar domain organization and demonstrated that caveolae trafficking responds to mechanical forces (Echarri et al., 2012). With an elegant combination of biochemical and cell biological approaches, his group identified the formin mDia1 as the actin polymerization regulator downstream of Abl kinases that controls the stress-fiber-linked pool of caveolin-1, demonstrating that caveolar domain organization and trafficking are tightly coupled to adhesive and stress fiber regulatory pathways (Echarri et al., 2012).

Signaling nanoplatforms – new paradigms in signal regulation

Presentations by Jay Groves (University of California and HHMI, Berkeley, CA) and Katharina Gaus (University of New South Wales, Sydney, Australia) focused on understanding the regulation of key events in T cell receptor (TCR) signaling. Using a number of biophysical techniques, Jay Groves provided evidence that a small number of localized and transient events can control cell decisions. For example, early work from his group that used patterned surfaces to partition available pMHC ligands showed that T cell

signaling could be initiated by TCR clusters, engaging as few as four ligands (Manz et al., 2011). TCR–ligand engagement also leads to an increase in binding of ZAP70 to TCRs, as revealed by single-molecule measurements of ZAP70 dwell times. Upon activation, TCR–ZAP70 signaling is propagated through the transmembrane scaffolding protein LAT, which activates the Ras-MAPK pathway. Through single-molecule studies of the enzyme activity of Ras molecules, Groves showed that, although there is only a small change in the average activity, a subpopulation of Ras molecules show a large increase, and these statistical outliers might be responsible for dictating the physiological response. These studies convincingly demonstrated the power of single-molecule measurements and emphasized the need to pay attention to the distribution of behaviors rather than focusing on the average.

Katharina Gaus presented the use of super-resolution imaging to understand how the activity of Lck, the upstream signaling molecule that phosphorylates TCR, is regulated. Her group has used PALM to generate cluster maps of Lck and found that Lck clustering does not require lipid domains, but is actually controlled by conformational states that promote self-association and activity (Rossy et al., 2013). Their results from PALM imaging also led them to suggest a new mechanism for the propagation of the TCR signal through LAT. Rather than relying on the reorganization of molecules in the plasma membrane, she proposed a new hypothesis whereby signaling molecules from pre-existing vesicular pools are transported to the sites of activation on the membrane. Indeed, her group and collaborators showed that LAT-containing subsynaptic vesicles are recruited to TCR-activation sites, which is dependent on the v-SNARE VAMP7 (Larghi et al., 2013). Finally, she presented a 3D mapping of the membrane using super-resolution imaging that revealed membrane undulations of >100 nm, clearly emphasizing the need to better understand the topographical landscape when membrane protein behavior is considered.

Three selected talks focused on understanding the functional consequences of membrane protein interactions and clustering. Elena Seiradake from Edith Y.

Jones' group (Oxford University, UK) presented studies linking the X-ray crystal structures for the ephrin receptors (EPH2 and EPH4) with their clustering behavior on intact cells. She used localization microscopy to determine that stimulated EPHA2 forms many large clusters containing over 20 molecules, whereas EPH4 does not form large clusters. This organizational difference could be attributed to differences in the ectodomain structure between the receptors as revealed by crystallography. Sandra de Keijzer from Alessandra Cambi's group (Nijmegen Centre for Molecular Life Sciences, The Netherlands) shared unpublished data regarding the cross-talk between two distinct but related GPCRs that are involved in prostanoid sensing. Using single-particle tracking, she showed that both receptors influence each other's lateral mobility within the plasma membrane and differentially activate downstream G-proteins. Finally, Samantha Schwartz from Diane Lidke's laboratory (University of New Mexico, NM) continued the theme of recruitment of single-molecule adaptor proteins by visualization SYK recruitment during FcεRI signaling. Similar to ZAP70, SYK also undergoes transient interactions with the membrane receptor and showed that the dwell time of these interactions increased upon FcεRI activation.

Cortical cytoskeleton templates – asters, corrals and arcs

A number of studies have suggested that membrane-proximal actin filaments can organize membrane proteins. In the classical model, actin restricts the motion of membrane proteins, either directly or through alignment of actin-binding proteins, acting much like a fence or corral (Tomishige et al., 1998; Andrews et al., 2008). Throughout the symposium there was a reoccurring theme of new roles for the cortical cytoskeleton in membrane organization. For example, Mayor's presentation of the existence of elusive sub-membranous actin asters that facilitate protein clustering (Gowrishankar et al., 2012) fuelled lively and stimulating discussions, and continuing application of super-resolution imaging will help to provide unambiguous evidence for the existence of such nanoscale actin filaments. Related unpublished studies, also from the Mayor laboratory, were presented in the short talk from Garima Singhal (National Centre for Biological Sciences,

Bangalore, India) who showed that the nicotinic acetylcholine receptor (nAChR) exhibits an organization at the supra-molecular scale; it appears in the form of optically resolvable dynamic micron-scale clusters, which in turn are comprised of nanoscale clusters, as revealed by STED, that are sensitive to perturbations of actin and actin-binding proteins. Ligation of the receptor modulates the global cellular actin dynamics and thus also impacts on other actin-coupled membrane molecules, possibly through the dynamic assembly of nanoscale actin filaments. Further evidence for control of protein organization by actin came from Samuel Hess' work on the membrane organization of the influenza virus hemagglutinin (HA) protein and its relationship with cortical actin, which they investigated by fPALM. Their results demonstrate the colocalization of HA with actin-rich membrane regions and its restricted mobility therein. Interestingly, the actin-binding protein cofilin was excluded from these regions, suggesting that HA clusters or neighboring proteins locally influence the cortical actin structures (Gudheti et al., 2013). However, whether these actin-rich membrane regions correspond to clustered actin asters remains to be established. In any case, it is clear that cortical actin can give rise to structures beyond the corrals or the fences, which was also emphasized during Xiaowei Zhuang's presentation of their application of 3D and multi-color STORM imaging to study molecular architecture in axons. They discovered that, in neuronal axons, actin and spectrin show alternating, highly organized, periodic, ring-like structures that are wrapped around the axon circumference, which in turn dictate the periodic distribution of sodium channels at the membrane (Xu et al., 2013).

A comprehensive and elegant study presented by Sergio Grinstein (Hospital for Sick Children, Toronto, Canada) demonstrated the interplay between Fcγ receptor mobility and the recruitment of signaling SYK molecules with subsequent actin remodeling that regulates the spatiotemporal amplification of local signaling cascades at sites of phagocytosis (Heit et al., 2013).

Finally, two short presentations from Thierry Rose's group (Institute Pasteur, Paris, France) provided nice examples of

combining the different super-resolution microscopy methods (STED, localization microscopy) with electron microscopy in a comprehensive approach to elucidate signal transduction during cytokine-induced cytoskeleton dynamics in human T cells via interleukin-7 (IL-7) receptors, and proposed a previously unappreciated role for microtubules in signal transduction (Tamarit et al., 2013). Thierry Rose and Bianca Tamarit showed that upon activation with IL-7, IL-7 receptors are compartmentalized into cholesterol-enriched membrane microdomains, from which the formation of an actin-microfilament meshwork is induced; here, anchored microtubules radially grow from the receptor domains towards the nuclear membrane. These radial microtubules allow phosphorylated STAT5 to glide across the cytoplasm and reach the nucleus within 2 minutes after IL-7 stimulation, with the microtubules disappearing at later time points after the signal has subsided (Tamarit et al., 2013).

The meeting was concluded by an exciting lecture given by Jennifer Lippincott-Schwartz (NIH, Bethesda, MD) who reminded us of how dynamic the actin meshwork is just underneath the membrane in migrating cells. Her previous work used PALM and speckle microscopy to identify the myosin-dependent retraction of actin at the leading edge of the cell, which creates actin arcs that run parallel to the cell edge, with focal adhesions dictating the location of arc formation (Burnette et al., 2011). Using fast two-color structured illumination microscopy (SIM), her group has now been able to image, for the first time, the shape of a live migrating cell and to show how the distribution of actin and myosin orchestrates cell motility. From these super-resolution images, it was apparent that myosin II is associated with the contraction of the dorsal actin arcs, which then apply force to the actin stress fibers that connect the focal adhesions to the top of the cell and create tension that shapes the cell. This could not have been determined by 2D or fixed cell imaging. The audience was mesmerized by the amazing movies illustrating actin and myosin dynamics. In her animated talk, Lippincott-Schwartz used an actual camping tent (mounted on stage for demonstration) to demonstrate the effect of the tension that is exerted on

the stress fibers during actin contractility on the architecture of the tent.

Many challenges lie ahead in this field that is at the crossroad between nanoscopy and membrane biology. We believe that interdisciplinary meetings, such as this one, will be crucial in overcoming the current challenges and to foster technological developments, as well as to discuss the emerging biological principles that regulate membrane organization and signal transduction.

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