Membrane nanodiscs/single-molecule analysis

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Lightly counting membrane proteins in native nanodiscs

Koushambi Mitra & Yamuna Krishnan

To determine the physiologically relevant oligomeric form of membrane proteins is extremely challenging. Now an elegant method of counting the oligomers in membrane proteins in near-native states is presented, using photobleaching and nanodiscs formed directly from cellular membranes.

Nearly a third of all proteins within a cell are associated with membranes. These proteins perform diverse functions as receptors, transporters, ion channels and enzymes. Many of these membrane proteins are produced as monomers that oligomerize in specific stoichiometries to form the functional unit. For example, the tetramerization of potassium (K^+) channels governs every facet of their biology, from ion conduction to tuning their responsiveness. And although the oligomerization of soluble proteins can be studied by several methods, both at single-molecule resolution and in bulk, analysing the oligomerization of membrane proteins is particularly challenging.

This is because membrane proteins co-evolved with the lipids in their native membranes, and their functionality is often intimately coupled to the molecular composition of their surrounding native membrane[1]. For example, some K^+ channels are activated by phosphoinositol -4,5-bisphosphate (PIP2), but others are inactivated by it[2]. In fact, lipid composition controls the oligomerization equilibrium of transporters, such as in the Cl^-/H^+ antiporter, CLC-ec1 (ref. 3). Therefore, methods that determine the physiologically relevant oligomeric form of membrane proteins must do so under conditions that are as native as possible, preserving the local membrane around the protein oligomer and yet retaining high spatial resolution. Until now, it has been extremely challenging to fulfil these criteria. Writing in *Nature Nanotechnology*, Walker and colleagues now describe an elegant method that they call Native-nanoBleach to quantify the oligomeric distribution of membrane proteins in an almost native state with 10-nanometre spatial resolution[4].

Prior to this report, membrane protein oligomerization has been studied by diffraction-limited methods such as fluorescence correlation spectroscopy, fluorescence cross-correlation spectroscopy or subunit counting by photobleaching fluorescently tagged proteins of interest. Alternatively, they can be studied in fixed cells amenable to super-resolution nanoscopic imaging. The state-of-the-art method, MINFLUX, can be applied to protein assemblies in living cells with 1- to 6-nanometre spatial resolution[5]. However, the specialized hardware required and the limited sizes of single-particle datasets restrict its wider adoption and the ability to pinpoint rare events in native membranes, respectively.

The present method from Walker and colleagues builds on two orthogonal developments, one chemical and the other biophysical. The chemical development enables stable and active membrane proteins to be isolated. This was first achieved, albeit inefficiently, with amphipathic polymers that sliced biological membranes into monodisperse, lipid bilayer disks around 10 nanometres in diameter[6]. These polymers produce native lipid nanodiscs by acting like chemical cookie/biscuit cutters on biological membranes analogous to the dough. The biophysical development enables the oligomeric status of a protein to be determined using photobleaching. This was achieved by expressing cell surface membrane proteins fused to green fluorescent protein (GFP) in the oocytes of the South African clawed frog, *Xenopus laevis*[7]. Because the number of GFP units in a complex equals the number of photobleaching steps required to go from full intensity to a dark state, this process yields the protein stoichiometry within the complex. Walker and colleagues have insightfully optimized and integrated these methodologies with total internal reflection microscopy and greatly expanded our ability to study the oligomerization of membrane proteins to include almost any cellular membrane.

Briefly, Walker et al. tag their protein of interest with GFP, disrupt cells without using detergents, isolate cell membranes, and add the nanodisc-forming polymer. Thereafter, they purify the native nanodiscs containing the GFP-tagged protein using fluorescence-based size exclusion chromatography. The fluorescent nanodiscs are then immobilized on glass substrates displaying a GFP-nanobody and observed by total-internal-reflection fluorescence microscopy, where the number of photobleaching steps per nanodisc reveals the oligomerization status therein (Fig. 1). The ensemble reveals the relative proportion of

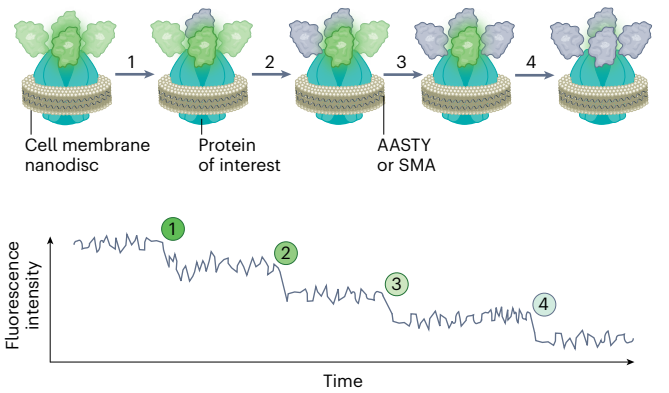

Fig. 1 | Protein counting using the Native-nanoBleach method. Schematic of photobleaching events on a tetrameric membrane protein of interest (blue) fused to green fluorescent protein (green), performed on nanodiscs of native cell membrane. The number of photobleaching events yields the protein stoichiometry in individual nanodiscs. AASTY, poly(acrylic acid-co-styrene); SMA, styrene maleic anhydride polymer. Figure partly created with BioRender.com.

monomers, dimers, trimers and tetramers of the protein of interest. They validated the method on well defined oligomeric plasma membrane proteins, namely transporters such as SemiSWEET (a dimer) and AmtB (a trimer), which transport sugar and ammonium respectively, and the potassium ion channel KcsA (a tetramer).

We note that the Native-nanoBleach method resolves a long-standing debate on the oligomeric status of the oncogenic GTPase, KRas. Functional assays in cells have suggested that it works as a dimer, while in vitro studies on supported bilayers indicate that it is predominantly monomeric. Native-nanoBleach on wild-type KRas reveals a mixture of monomeric (54%), dimeric (40%) and higher-order oligomeric (about 5%) populations. The equilibrium could be shifted to favour dimers or oligomers by either mutating KRas at G12 and Q61 or by pharmacologically treating cells with the KRas inhibitor BI-2852. A similar population distribution was observed for both wild-type KRas and the G12V variant expressed at endogenous levels in pancreatic ductal adenocarcinoma cells. The power of the Native-nanoBleach method was also revealed by applying it to proteins in organelle membranes, which have not previously been accessible. Using Native-nanoBleach, the authors show that the mitochondrial outer membrane protein, OMP25, is monomeric.

Native-nanoBleach is an elegant and valuable addition to the burgeoning toolkit for organelles, which has spurred the growth of the organellar membrane protein field. Many membrane proteins shuttle between the plasma membrane and organelles. It will be interesting to see whether Native-nanoBleach can be used to decipher location-specific structural differences in their organization. If this method can be applied to systems beyond tetramers, it could reveal oligomerization equilibria associated with multimeric membrane proteins and deepen our understanding of their biogenesis. In that spirit, expanding Native-nanoBleach to include native nanodiscs of different diameters would allow larger complexes with higher stoichiometries to be studied or, conversely, even higher spatial resolution to be achieved. The robustness, low sample requirements, broad applicability and relative simplicity of Native-nanoBleach in terms of instrumentation will democratize a previously niche expertise.

Koushambi Mitra [1,2,3] **& Yamuna Krishnan** [1,2,3] ✉

[1]Department of Chemistry, The University of Chicago, Chicago, IL, USA. [2]Neuroscience Institute, The University of Chicago, Chicago, IL, USA. [3]Institute of Biophysical Dynamics, The University of Chicago, Chicago, IL, USA.

✉e-mail: yamuna@uchicago.edu

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Competing interests

The authors declare no competing interests.