HHS Public Access

Author manuscript

Biochemistry. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:

Biochemistry. 2018 May 01; 57(17): 2432–2436. doi:10.1021/acs.biochem.7b01173.

Optogenetic Reconstitution for Determining the Form and Function of Membraneless Organelles

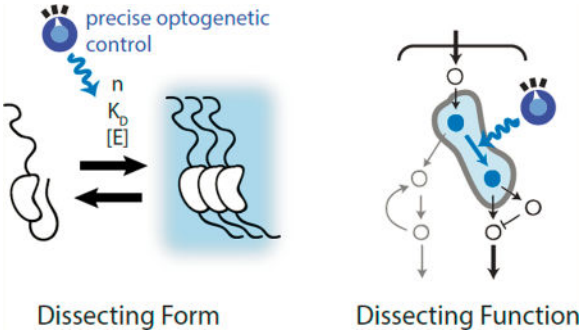
Elliot Dine and **Jared E. Toettcher**[*,iD]

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, United States

Abstract

It has recently become clear that large-scale macromolecular self-assembly is a rule, rather than an exception, of intracellular organization. A growing number of proteins and RNAs have been shown to self-assemble into micrometer-scale clusters that exhibit either liquid-like or gel-like properties. Given their proposed roles in intracellular regulation, embryo development, and human disease, it is becoming increasingly important to understand how these membraneless organelles form and to map their functional consequences for the cell. Recently developed optogenetic systems make it possible to acutely control cluster assembly and disassembly in live cells, driving the separation of proteins of interest into liquid droplets, hydrogels, or solid aggregates. Here we propose that these approaches, as well as their evolution into the next generation of optogenetic biophysical tools, will allow biologists to determine how the self-assembly of membraneless organelles modulates diverse biochemical processes.

Graphical abstract



Cell biologists have long observed the presence of subcellular structures that are not encapsulated by a lipid bilayer. Indeed, observations made as early as 1946[1] led to the suggestion that the nucleolus may be “a separated phase out of a saturated solution”, conjecture that was confirmed by elegant experiments 65 years later.[2] Since that time,

[*]**Corresponding Author**. Lewis Thomas Laboratory, Room 140, Washington Road, Princeton, NJ 08544. Phone: 609-258-9243. Fax: 609-258-1894. toettcher@princeton.edu.

ORCID

Jared E. Toettcher: 0000-0002-1546-4030

The authors declare no competing financial interest.

membraneless organelles like the nucleolus—micrometerscale collections of proteins, RNAs, or their combination—have been shown to be ubiquitous in cell biology. They participate in a huge variety of cellular processes, including carbon fixation,[3] transcriptional regulation,[4] spatial patterning during embryo development,[5] and immune-cell activation.[6] The recent advent of live-cell imaging and fluorescence microscopy has uncovered an even more surprising feature: many membraneless organelles are highly dynamic structures. They not only assemble and disassemble with changes in cell state or the external environment but also can rapidly exchange their constituent components with the surrounding solution.[7] The biophysical mechanisms and functional consequences of these myriad dynamic changes remain poorly understood.

In recent years, biophysicists have performed pioneering *in vitro* experiments to elucidate the biomolecular properties that give rise to the formation of membraneless organelles and specify their phase (e.g., whether they form as liquid droplets, hydrogels, or solid aggregates).[8] These experiments led to the hypothesis that weak, multivalent interactions lead to the formation of macromolecular liquid droplets, structures that are defined by the fast movement of subunits within a droplet and exchange with the surrounding diffuse phase. These droplets may mature into solid aggregates based on the concentration of their constituents,[8,9] salt content,[10] post-translational modifications,[11] or specific mutations[12] in the protein sequence. Two classes of proteins have been noted to display the characteristic multivalent yet weak interactions necessary for phase separation. The first class comprises low-complexity sequences (LCSs) that fail to fold into defined secondary structures [“intrinsically disordered regions” (IDRs)].[13] Alternatively, phase separation may be encoded by repeated arrays of modular protein–protein interaction domains,[14] such as the interactions between SH2 domains and their cognate phosphotyrosine residues (pTYRs)[6] or SH3 domains and proline-rich motifs (PRMs).[15]

Yet while these successful experiments recreating phase separations in a test tube have offered us a deeper understanding of the underlying biophysics driving the self-assembly of membraneless organelles, there is still much to learn. Which enzymes and substrates must be localized to a membraneless organelle to result in a functional cellular outcome? How does this localization change affect biochemical pathway function? Do the biophysical details matter – that is, would a liquid-like and gel-like assembly lead to similar cellular responses? Moreover, the formation of membraneless organelles is often exquisitely regulated, occurring at exactly the right place (cells in a tissue or subcellular locations) or at the right time (external condition) to participate in a cellular response. How do cells regulate phase separation with such high spatiotemporal precision?

A satisfying answer to each of these questions requires the development of protein-based tools with a number of properties that, at first blush, appear to be notoriously difficult to engineer. To test whether colocalization of particular constituents is sufficient to drive a cellular response or biochemical change, we must be able to tag any proteins of interest with a localization sequence that confers membership to a phase-separated structure. To study how spatial and temporal regulation affects organelle function, one should induce phase separation at a desired time or spatial location within the cell. Dissecting how biochemical parameters (e.g., protein concentration, binding affinity, and multivalency) govern

biophysical properties *in vivo* requires the ability to independently vary each of these properties while holding others constant. Finally, to be relevant to their true biological context, each of these perturbations should be achieved in a live cell.

Optogenetic approaches are ideally suited for such “control freak” experiments, those with seemingly impossible requirements for biochemical specificity and spatiotemporal control. Light can be acutely delivered and focused with micrometer-scale spatial precision, enabling the selective activation of proteins at a particular time or location within a cell or tissue. Light stimulation is also highly specific for an engineered pathway, as cells typically express few naturally light sensitive proteins that regulate a process of interest (although there are of course exceptions to this rule). Finally, the intensity or schedule of light delivery can be easily tuned, which in many cases can be used to precisely and independently control specific biochemical properties, such as the concentration of an active protein or even the duration of formation of a complex between a photosensitive protein and its binding partner.[16]

OPTOGENETIC APPROACHES TO STUDYING THE FORM OF MEMBRANELESS ORGANELLES

How then might one combine optogenetics and phase separation into a unified experimental framework? As a first step toward this goal, we and the Brangwynne laboratory turned to the *Arabidopsis thaliana* Cry2 protein, which was already known to form dynamic, homotypic clusters when stimulated with blue light.[17] However, the light-dependent clustering of the photolyase homology region (PHR) of Cry2 is highly context-dependent, occurring most readily in scenarios that tend to enhance protein–protein association (e.g., on the plasma membrane or when fused to proteins that already tend to cluster).[18,19] We reasoned that fusing Cry2 to intrinsically disordered regions of proteins that are known to phase-separate at high concentrations could potentiate light-dependent clustering, leading to the formation of IDR–Cry2 “optoDroplets”.[20] Indeed we found that, upon light stimulation, optoDroplets formed clusters within seconds and dissociated within 5–10 min in the dark. These clusters displayed hallmark properties of liquid-like protein droplets: they formed clusters that quickly relaxed to a spherical shape after fusing with one another, and FRAP assays revealed that individual molecules rapidly exchanged between droplets and the surrounding cytosol. Swapping Cry2 for a mutant variant with an increased homotypic affinity (so-called Cry2olig)[21] led to the rapid, light-induced formation of solid aggregates.

OptoDroplets enabled us to tune one biophysical parameter with high quantitative precision in live cells. By altering the intensity of blue light or the frequency of light pulses, we could acutely alter the concentration of photoactivated IDR–Cry2 proteins. We found that the concentration of “active” (that is, photostimulated) IDR–Cry2 proteins was sufficient to quantitatively describe their phase separation: dim blue light, delivered to a strongly expressing cell, drove phase separation like bright light stimulation of a weakly expressing cell. (Photoconversion of IDR–Cry2 proteins is also functionally analogous to post-translational modification, a process that is believed to be a general mechanism by which cells spatiotemporally regulate condensation.) Thus, a single parameter, the extent to which

the concentration of light-stimulated cytosolic monomers surpassed a “saturation concentration”, was sufficient to describe this simplified system.

Nevertheless, other biophysical properties remained out of reach. While light can be used to tune the optoDroplet concentration, it cannot help determine the role played by the other two key biophysical parameters, multivalency and interaction strength (Figure 1). This challenge arises because we still lack a good structural understanding of how homooligomerization proceeds for both the Cry2 protein and IDR constituents, leaving the number of binding sites, their affinities, and the overall multivalency of the resulting fusion proteins poorly defined. In contrast, these properties are easier to manipulate in the other archetypal class of phase-separating proteins, repeated arrays of modular interaction domains. Given the diverse heterodimer-forming tools from optogenetics[16,22] and chemical biology,[23] one might imagine engineering an array of light-switchable dimerizers to build a controllable aggregation system in which multivalency and interaction strength could be quantitatively controlled.

Indeed, a recent report from the Inoue lab establishes just such a system.[24] These researchers began by using the FKBP–FRB chemical dimerization system to build what they term iPOLYMERs, repeated sequences of FRB or FKBP domains whose interaction could be induced by the addition of a rapamycin analogue. They found both *in vitro* and *in vivo* that phase separation occurs only when the combined number of FKBP and FRB domains is greater than five (Figure 1b). Nevertheless, the rapamycin-induced interaction between FKBP and FRB domains is of nanomolar affinity and poorly reversible. The authors thus proceeded to replace their initial chemical dimerization approach with the light-induced iLID/SspB heterodimerization module,[22] constructing the iPOLYMER-LI system. This tool opens the door to reversible spatiotemporal control (the iLID–SspB interaction is reversed upon incubation in the dark for ~2 min). Moreover, the use of other light-induced dimerizers[25] or point mutations to the iLID–SspB binding interface could also be used to fine-tune interaction strength (Figure 1b). Together, these systems can help complete the biophysical picture of how intracellular protein phase separation is controlled *in vivo*.

OPTOGENETIC APPROACHES TO DETERMINING THE FUNCTIONS OF MEMBRANELESS ORGANELLES

The benefits that are provided by these “*in vivo* biochemical reconstitution” systems do not end with determining how membraneless organelles form, but also in delineating their function (Figure 2a). In principle, colocalization to a phase-separated compartment could drastically increase reaction rates by concentrating proteins in a pathway (e.g., a kinase and its substrate), preventing interaction with undesired cellular partners (e.g., an inhibitory phosphatase), or lowering the barrier to forming a multiprotein complex. On the other hand, such compartments could trap a protein away from its interacting partners. Reactions of the first type may require a liquid-like state, enabling productive binding of proteins within the compartment and exchange with the surrounding cytosol, whereas for the second type, a rigid hydrogel may be sufficient. Optogenetic phase separation allows one to acutely control when, where, and with what constituents membraneless organelles form and can even be

used to induce the formation of different phases (e.g., liquid-like optoDroplets vs iPOLYMER hydrogels or Cry2olig aggregates). These tools could be invaluable for dissecting how each candidate mechanism contributes to the regulation of those cellular processes in which membraneless organelles have been observed.

Some work to link phase separation to biochemical function has already occurred. Actin nucleation by the Nck–WASP–Arp2/3 complex was one of the first biochemical signaling systems shown to be driven by liquid–liquid phase separation.[15] This simple biochemical system has since proved to be ideal for optogenetic reconstitution. By fusing Cry2Olig to either the SH3 domain of Nck or the VCA domain in WASP, Chandra Tucker's group was able to induce actin recruitment in cell lines upon blue light stimulation.[21] However, they found that clustering did not drive cytoskeletal protrusion, only retraction, suggesting that Nck–Cry2Olig clusters may not be functionally organized in the same manner as after extracellular stimulation. Interestingly, both Cry2Olig and IDR–Cry2Olig fusion proteins form solid, static assemblies, rather than dynamic liquid droplets, upon light stimulation.[20,21] Future studies in which Nck and WASP are optically driven into a liquid-like state may help to determine if such a state is sufficient to reconstitute functional cytoskeletal organization.

So far, we have focused on how optogenetic reconstitution can teach us about membraneless organelles in the many cases in which they naturally arise. We would be remiss if we did not mention the promise that these optogenetic platforms hold for constructing wholly synthetic organelles. The reduced search space for biomolecular interaction afforded by the dynamic, multivalent protein–protein interactions and high local concentration in a liquid droplet could dramatically accelerate the flux through an engineered signaling or metabolic pathway. This logic is analogous to the synthetic protein or DNA scaffolds that have already been successfully applied in a wide range of synthetic biology applications, from rewiring the logic of cell fate decisions[26] to the production of desired metabolites.[27,28] Light-controlled membraneless organelles potentially offer two further advantages: they can be implemented as modular protein tags (avoiding the challenge of designing scaffold proteins that precisely orient multiple substrates), and they can be acutely switched on and off. Meeting another need for the synthetic biology community, the biochemical rate enhancement in membraneless organelles offers the potential ability to acutely alter the strength of the edges in a biochemical network (e.g., the sensitivity with which a downstream effector is trigger by an upstream activator), not just the ability to turn on or off particular nodes by directly toggling the activity of an intracellular protein[29] (Figure 2b).

Throughout this Perspective, we have proposed using optogenetic tools to further our understanding of the biophysics underlying phase transitions and the biochemical parameters they regulate, yet there are a number of examples of membraneless organelles that do not seem to regulate any biochemical reactions at all but rather seem to contribute to the asymmetric localization of cellular components. For instance, in the developing *Caenorhabditis elegans* embryo, P granules act as liquid droplets that dissolve and condense along the anterior–posterior axis, providing a sharp boundary of protein localization without directed transport.[5] The dramatic spatial asymmetries formed by protein and mRNA granules during embryogenesis[5,30,31] suggest that phase separation may play a special role

in establishing or maintaining spatial patterns. We hope that the advent of light-gated tools to control protein phase will broaden our understanding of how biophysical processes act on length scales much larger than their constituent proteins and longer than individual molecular interactions. In this manner, we might discover why cells use membraneless organelles to control not just a handful of biochemical reactions, but such a wide array of processes that are fundamental to a cell's life, death, and development.

Acknowledgments

Funding

J.E.T. was supported by National Institutes of Health (NIH) Grant DP2EB024247, and E.D. was supported by NIH Training Grant T32GM007388.

References

1. Ehrenberg L. Influence of temperature on the nucleolus and its coacervate nature. Hereditas. 1946; 32:407–418. [PubMed: 20998141]
2. Brangwynne CP, Mitchison TJ, Hyman AA. Active liquid-like behavior of nucleoli determines their size and shape in Xenopus laevis oocytes. Proc. Natl. Acad. Sci. U. S. A. 2011; 108:4334–4339. [PubMed: 21368180]
3. Freeman Rosenzweig ES, Xu B, Kuhn Cuellar L, Martinez-Sanchez A, Schaffer M, Strauss M, Cartwright HN, Ronceray P, Plitzko JM, Forster F, Wingreen NS, Engel BD, Mackinder LCM, Jonikas MC. The Eukaryotic CO2-Concentrating Organelle Is Liquid-like and Exhibits Dynamic Reorganization. Cell. 2017; 171:148–162. [PubMed: 28938114]
4. Larson AG, Elnatan D, Keenen MM, Trnka MJ, Johnston JB, Burlingame AL, Agard DA, Redding S, Narlikar GJ. Liquid droplet formation by HP1alpha suggests a role for phase separation in heterochromatin. Nature. 2017; 547:236–240. [PubMed: 28636604]
5. Brangwynne CP, Eckmann CR, Courson DS, Rybarska A, Hoege C, Gharakhani J, Julicher F, Hyman AA. Germline P granules are liquid droplets that localize by controlled dissolution/condensation. Science. 2009; 324:1729–1732. [PubMed: 19460965]
6. Su X, Ditlev JA, Hui E, Xing W, Banjade S, Okrut J, King DS, Taunton J, Rosen MK, Vale RD. Phase separation of signaling molecules promotes T cell receptor signal transduction. Science. 2016; 352:595–599. [PubMed: 27056844]
7. Kroschwald S, Maharana S, Mateju D, Malinovska L, Nuske E, Poser I, Richter D, Alberti S. Promiscuous interactions and protein disaggregases determine the material state of stress-inducible RNP granules. eLife. 2015; 4:e06807. No. [PubMed: 26238190]
8. Kato M, Han TW, Xie S, Shi K, Du X, Wu LC, Mirzaei H, Goldsmith EJ, Longgood J, Pei J, Grishin NV, Frantz DE, Schneider JW, Chen S, Li L, Sawaya MR, Eisenberg D, Tycko R, McKnight SL. Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. Cell. 2012; 149:753–767. [PubMed: 22579281]
9. Elbaum-Garfinkle S, Kim Y, Szczepaniak K, Chen CC, Eckmann CR, Myong S, Brangwynne CP. The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. Proc. Natl. Acad. Sci. U. S. A. 2015; 112:7189–7194. [PubMed: 26015579]
10. Lin Y, Protter DS, Rosen MK, Parker R. Formation and Maturation of Phase-Separated Liquid Droplets by RNA-Binding Proteins. Mol. Cell. 2015; 60:208–219. [PubMed: 26412307]
11. Nott TJ, Petsalaki E, Farber P, Jervis D, Fussner E, Plochowietz A, Craggs TD, Bazett-Jones DP, Pawson T, Forman-Kay JD, Baldwin AJ. Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. Mol. Cell. 2015; 57:936–947. [PubMed: 25747659]
12. Patel A, Lee HO, Jawerth L, Maharana S, Jahnel M, Hein MY, Stoynov S, Mahamid J, Saha S, Franzmann TM, Pozniakovski A, Poser I, Maghelli N, Royer LA, Weigert M, Myers EW, Grill S,

Drechsel D, Hyman AA, Alberti S. A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. Cell. 2015; 162:1066–1077. [PubMed: 26317470]

13. Pak CW, Kosno M, Holehouse AS, Padrick SB, Mittal A, Ali R, Yunus AA, Liu DR, Pappu RV, Rosen MK. Sequence Determinants of Intracellular Phase Separation by Complex Coacervation of a Disordered Protein. Mol. Cell. 2016; 63:72–85. [PubMed: 27392146]
14. Bhattacharyya RP, Remenyi A, Yeh BJ, Lim WA. Domains, motifs, and scaffolds: the role of modular interactions in the evolution and wiring of cell signaling circuits. Annu. Rev. Biochem. 2006; 75:655–680. [PubMed: 16756506]
15. Li P, Banjade S, Cheng HC, Kim S, Chen B, Guo L, Llaguno M, Hollingsworth JV, King DS, Banani SF, Russo PS, Jiang QX, Nixon BT, Rosen MK. Phase transitions in the assembly of multivalent signalling proteins. Nature. 2012; 483:336–340. [PubMed: 22398450]
16. Levskaya A, Weiner OD, Lim WA, Voigt CA. Spatiotemporal control of cell signalling using a light-switchable protein interaction. Nature. 2009; 461:997–1001. [PubMed: 19749742]
17. Zuo ZC, Meng YY, Yu XH, Zhang ZL, Feng DS, Sun SF, Liu B, Lin CT. A study of the blue-light-dependent phosphorylation, degradation, and photobody formation of Arabidopsis CRY2. Mol. Plant. 2012; 5:726–733. [PubMed: 22311776]
18. Kennedy MJ, Hughes RM, Peteya LA, Schwartz JW, Ehlers MD, Tucker CL. Rapid blue-light-mediated induction of protein interactions in living cells. Nat. Methods. 2010; 7:973–975. [PubMed: 21037589]
19. Bugaj LJ, Choksi AT, Mesuda CK, Kane RS, Schaffer DV. Optogenetic protein clustering and signaling activation in mammalian cells. Nat. Methods. 2013; 10:249–252. [PubMed: 23377377]
20. Shin Y, Berry J, Pannucci N, Haataja MP, Toettcher JE, Brangwynne CP. Spatiotemporal Control of Intracellular Phase Transitions Using Light-Activated optoDroplets. Cell. 2017; 168:159–171. [PubMed: 28041848]
21. Taslimi A, Vrana JD, Chen D, Borinskaya S, Mayer BJ, Kennedy MJ, Tucker CL. An optimized optogenetic clustering tool for probing protein interaction and function. Nat. Commun. 2014; 5:4925. [PubMed: 25233328]
22. Guntas G, Hallett RA, Zimmerman SP, Williams T, Yumerefendi H, Bear JE, Kuhlman B. Engineering an improved light-induced dimer (iLID) for controlling the localization and activity of signaling proteins. Proc. Natl. Acad. Sci. U. S. A. 2015; 112:112–117. [PubMed: 25535392]
23. Spencer DM, Wandless TJ, Schreiber SL, Crabtree GR. Controlling signal transduction with synthetic ligands. Science. 1993; 262:1019–1024. [PubMed: 7694365]
24. Nakamura H, Lee AA, Afshar AS, Watanabe S, Rho E, Razavi S, Suarez A, Lin YC, Tanigawa M, Huang B, DeRose R, Bobb D, Hong W, Gabelli SB, Goutsias J, Inoue T. Intracellular production of hydrogels and synthetic RNA granules by multivalent molecular interactions. Nat. Mater. 2017; 17:79–89. [PubMed: 29115293]
25. Tischer D, Weiner OD. Illuminating cell signalling with optogenetic tools. Nat. Rev. Mol. Cell Biol. 2014; 15:551–558. [PubMed: 25027655]
26. Park SH, Zarrinpar A, Lim WA. Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms. Science. 2003; 299:1061–1064. [PubMed: 12511654]
27. Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal AV, Prather KL, Keasling JD. Synthetic protein scaffolds provide modular control over metabolic flux. Nat. Biotechnol. 2009; 27:753–759. [PubMed: 19648908]
28. Castellana M, Wilson MZ, Xu Y, Joshi P, Cristea IM, Rabinowitz JD, Gitai Z, Wingreen NS. Enzyme clustering accelerates processing of intermediates through metabolic channeling. Nat. Biotechnol. 2014; 32:1011–1018. [PubMed: 25262299]
29. Dagliyan O, Tarnawski M, Chu PH, Shirvanyants D, Schlichting I, Dokholyan NV, Hahn KM. Engineering extrinsic disorder to control protein activity in living cells. Science. 2016; 354:1441–1444. [PubMed: 27980211]
30. Melton DA, Ruiz i Altaba A, Yisraeli J, Sokol S. Localization of mRNA and axis formation during Xenopus embryogenesis 144, 16–29. Ciba Found. Symp. 2007:16–36.
31. Berleth T, Burri M, Thoma G, Bopp D, Richstein S, Frigerio G, Noll M, Nusslein-Volhard C. The role of localization of bicoid RNA in organizing the anterior pattern of the Drosophila embryo. EMBO J. 1988; 7:1749–1756. [PubMed: 2901954]

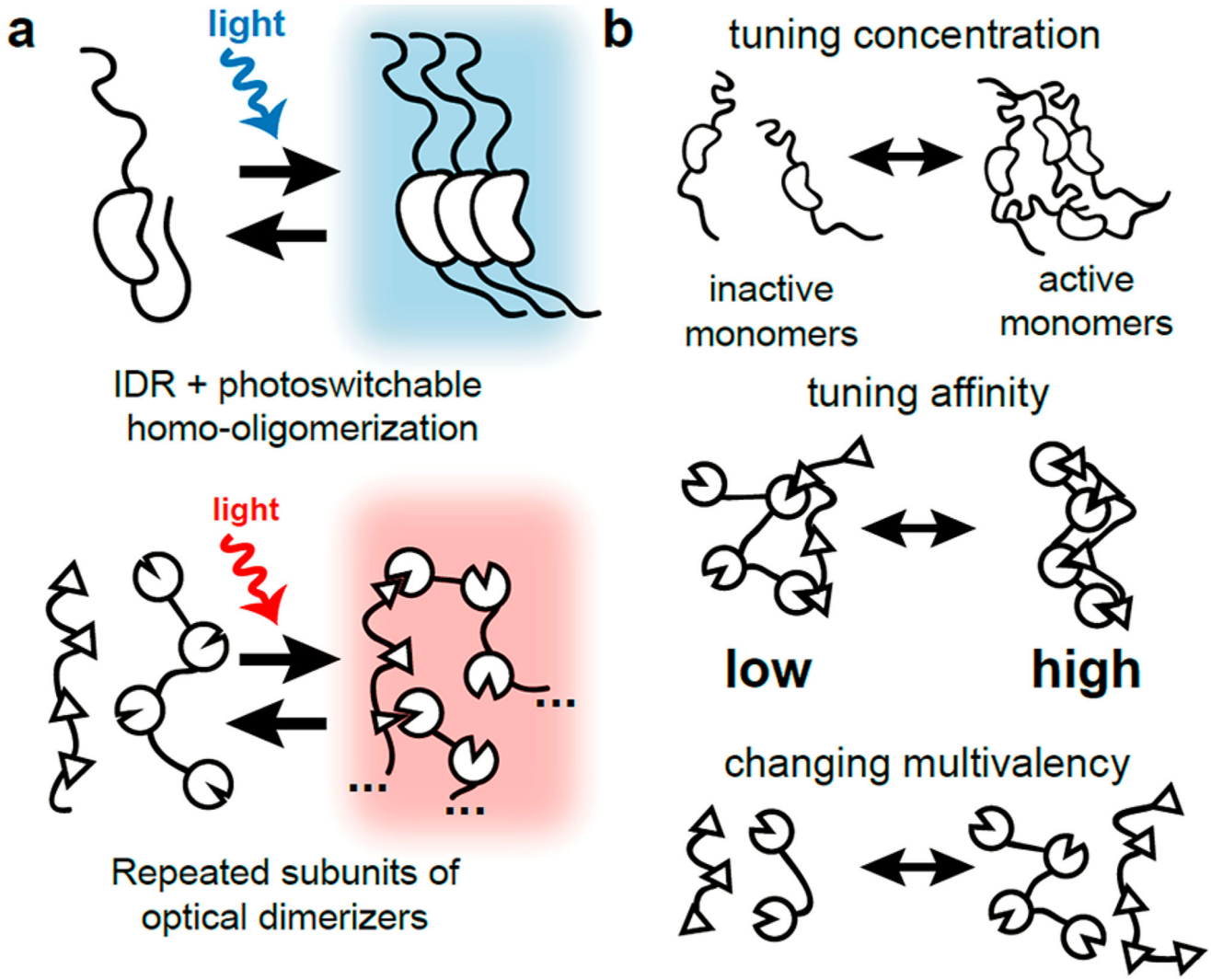


Figure 1.

Precise input control to dissect the form of membraneless organelles. (a) Two main strategies have been adopted for regulating phase separation with light. A photoswitchable oligomerization domain can be fused to an intrinsically disordered protein sequence to drive homotypic, light-induced aggregation (top), and repeated protein domains that undergo light-induced heterodimerization (bottom). (b) Precise optogenetic control enables the experimentalist to acutely and spatially control three distinct biochemical properties: the concentration of photoactive monomers (top), the number of domains that are simultaneously exposed for binding (middle), and the affinity between individual heterodimerization pairs (bottom).

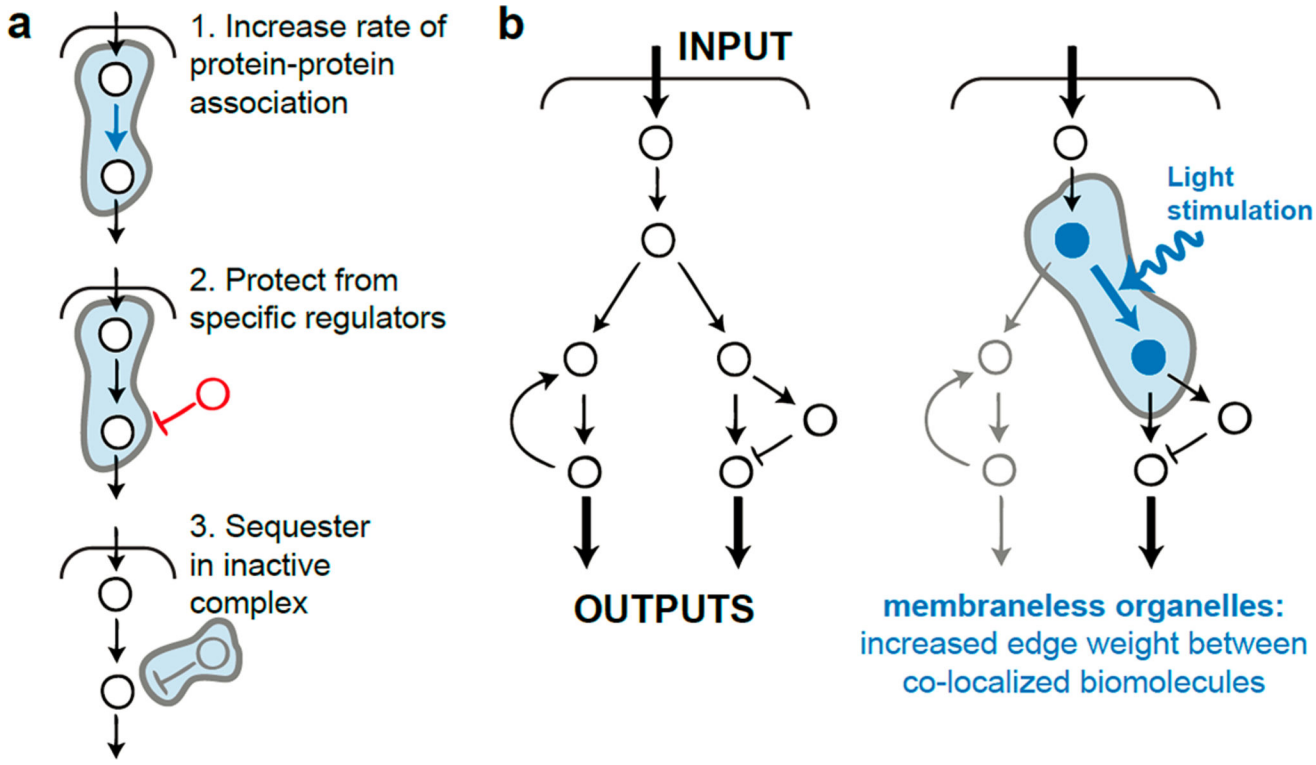


Figure 2.

Applying optogenetic tools to dissect the function of membraneless organelles. (a) Relocating proteins to phase-separated organelles can potentially alter three biochemical properties. It can concentrate interacting components within the droplet to enhance the extent and rate of interaction (top). It can exclude components that participate in undesired reactions, such as a phosphatase from a kinase–substrate reaction (middle). Finally, undesired interactors can be sequestered within the separated phase and prevented from interacting within the pathway (bottom). (b) Membraneless organelles as a synthetic biology platform. Unlike prior optogenetic approaches, phase separation offers the possibility of a tunable, reversible strategy for modulating the edges (e.g., reaction rates), not just the nodes (e.g., protein activity states), in a biochemical pathway.