REVIEW

Chemical Physics in Living Cells—using Light to Visualize and Control Intracellular Signal Transduction†

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(Dated: Received on June 26, 2018; Accepted on July 16, 2018)

Cells are crowded microenvironments filled with macromolecules undergoing constant physical and chemical interactions. The physicochemical makeup of the cells affects various cellular responses, determines cell-cell interactions and influences cell decisions. Chemical and physical properties differ between cells and within cells. Moreover, these properties are subject to dynamic changes in response to environmental signals, which often demand adjustments in the chemical or physical states of intracellular molecules. Indeed, cellular responses such as gene expression rely on the faithful relay of information from the outside to the inside of the cell, a process termed signal transduction. The signal often traverses a complex path across subcellular spaces with variable physical chemistry, sometimes even influencing it. Understanding the molecular states of such signaling molecules and their intracellular environments is vital to our understanding of the cell. Exploring such intricate spaces is possible today largely because of experimental and theoretical tools. Here, we focus on one tool that is commonly used in chemical physics studies—light. We summarize recent work which uses light to both visualize the cellular environment and also control intracellular processes along the axis of signal transduction. We highlight recent accomplishments in optical microscopy and optogenetics, an emerging experimental strategy which utilizes light to control the molecular processes in live cells. We believe that optogenetics lends unprecedented spatiotemporal precision to the manipulation of physicochemical properties in biological contexts. We hope to use this work to demonstrate new opportunities for chemical physicists who are interested in pursuing biological and biomedical questions.

Key words: Optogenetics, Signal transduction, Optical microscopy, Super-resolution imaging, Protein-protein interactions, Receptor, Cytoskeletal track, Cargo trafficking, Gene transcription and translation

I. INTRODUCTION

A. Why should we care about chemical physics in cells?

Compared with single-cell organisms such as bacteria, an adult human has approximately 40 trillion cells. Both the size of a cell and the intricacy of the structures within it increase in proportion to the complexity of the living entities. Complexity can be defined by the ability to process information as well as to diversify responses dynamically while preserving fundamental functions. Structural complexity provides an infrastructure for achieving multiplexed functionality in high-order organisms.

With increasing complexity, the size and diversity of the repertoire of macromolecule are larger, which results in problems: (i) the number of potential non-specific interactions between macromolecules goes up leading to steric hindrance and (ii) catalysis is sub-optimal in the same environment for all chemical reactions. Even in the primitive cells, the organization of space is essential for their sustenance. Cells with more sophisticated functions solve these issues by compartmentalization of their intracellular space into organelles where conditions are optimized to suit certain classes of reactions tailored to perform subsets of cellular functions. Favorable interactions of macromolecules are maximized in such organelles leading to effective management of functions. Compartments differ regarding their physicochemical properties including composition, pH, and viscosity, to

†Part of the special issue for celebration of "the 60th Anniversary of University of Science and Technology of China and the 30th Anniversary of Chinese Journal of Chemical Physics".
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DOI:10.1063/1674-0068/31/cjcp1806152

name a few.

Such a compartmentalized design in cells has a profound impact on fundamental cellular machinery in that cellular functions are modularized. This design also empowers cells to make complex responses by integrating the outputs from these different modules, a capacity that helps cell survival and its adaptiveness under changing environmental conditions. In addition, such modularized functional design facilitates cell-cell communication, which is crucial for cell fate determination during early embryonic development.

Curiously, although multicellular organisms have distinct cell types with different cell shape, function, and localization, they share an identical genomic "blueprint". How exactly does one cell use its master blueprint to produce trillions of variegated off-spring cells? And how these trillions of cells are organized into an entity and perform high-level functions like thinking and breathing remain a miracle in biological sciences. Answers to these questions involve signal transduction, a cellular mechanism that links the extracellular space with the cell interior. Briefly speaking, extracellular signals, which can be small chemical and biological molecules, or even physical stimulation (heat, light), can trigger changes of the physical and chemical state of signaling molecules and elicit distinct cellular responses. Defective signal transduction can, therefore, elicit undesirable cellular responses such as uncontrolled cell proliferation which underlies many forms of cancers. Thus, understanding the physicochemical properties of a cell provides insights into the operating principles underlying fundamental cell functions in health and disease.

B. Cells are more than "water sacs" of macromolecules

Although water makes up for the bulk of almost all known cells, cells are by no means simply sacs of molecules in water. The cell is often regarded as an ideal dilute solution of molecules, but its properties are quite far from such an ideality. Divergent properties such as anomalous diffusion and crowding-induced phase separation are often seen [1]. Diffusion of nutrients is a limiting factor in cell size determination, and hence the cells tend to limit their volume and maximize their surface area. The limitation in volume results in macromolecular crowding. Indeed, the macromolecule concentrations within the cell have been estimated to be as high as 500 g/L [2]. Such a tight packing of molecules within the cell impacts the kinetics and equilibrium of reactions as well as the transport rates of molecules. Additionally, it leads to the volume exclusion effect which requires that a significant free energy cost must be met to make room for additional molecules [1]. While most organelles within eukaryotic cells are delimited by membranes, an entire class of non-membrane-bound compartments is known to exist. These include the nucleolus, nuclear speckles, paraspeckles, Cajal bodies, PML bodies, germ granules, P bodies, and stress granules [3]. Formation of these bodies is better reasoned using colloid chemistry as a matter of phase separation induced by molecular crowding. These considerations have led to the conclusion that simplistic models for ideal dilute solutions cannot be applied to cells and that the cell is more than simply a sac of macromolecules. Therefore, biochemical processes occurring within cells differ from those happening in standardized dilute solutions and hence experimental approaches to determining these alterations have an immense impact on our comprehension of the cell.

C. Core components and information flow during signal transduction

Once an extracellular signal is available, cells employ a series of components to sense, process, and integrate information carried by this signal. Core components of signal transduction can be categorized into the following: receptor, cytoskeleton, cargo, and transcription factor.

Receptor: Here we refer to the receptors spanning the plasma membrane, where they serve as antennas to receive signals. Often the interaction between the signal (*e.g.*, ligand) and the receptor involves direct physical interactions (binding) between them. This binding can induce allosteric conformational changes in the receptor and activate it. The activated receptor then initiates downstream signaling pathways. Thus the receptor serves as a "switch" for signal transduction. There are several types of receptors, such as receptor tyrosine kinase (RTK) and G-protein coupled receptor (GPCR). These receptors are the first gates of signal transduction, and their activation state determines cellular responses such as cell survival, proliferation, and differentiation.

Not surprisingly, any factor that causes abnormality in receptor function could potentially promote disease. For instance, mutation or upregulation of receptors has often been found in cancers. Upregulation in the form of increased receptor density on the plasma membrane leads to inadvertent receptor collisions, resulting in an increased baseline activity of these receptors. When this activity exceeds the activation threshold of the receptor, cells will switch on downstream responses even in the absence of ligand (signal). Thus, upregulation of receptors can often lead to uncontrolled cell proliferation. On the other hand, some gain-of-function mutations of receptors may reduce the threshold of ligand so that less ligand is needed to activate the downstream pathway, which also causes an altered cellular response.

Cargo: Here, cargoes refer to the information carriers. There are two types of cargoes, the first being membrane-wrapped vesicles such as endosomes, lysosomes, and multivesicular body or MVB. The second type is membrane-less cargoes, often referred to as gran-

DOI:10.1063/1674-0068/31/cjcp1806152

ules, which are aggregates of different cytosolic components such as a messenger RNA and their binding proteins. Both types of cargoes can be regarded as a unit of information carriers. They can recruit transport machinery such as motor proteins and physically translocate to different intracellular compartments. Cargo-mediated translocation is an important property because most of the signaling transduction ends up regulating gene transcription, which occurs in the nucleus.

Cytoskeletal tracks: Cytoskeletal components such as microtubules and actin help support and shape cellular morphology while also serve as tracks for intracellular cargo transport. Motor proteins such as kinesins and myosins associate with both the cargoes and the cytoskeletal tracks on their own or with the help of adaptor proteins. Using energy from ATP hydrolysis, motor proteins such as kinesins and dyneins carry the cargo and "walk" along microtubules, thereby constituting an important machinery to translocate organelles and vesicles within the crowded cell environment directionally.

Transcription factor: Signal transduction often terminates in the regulation of gene expression. Transcription factors are a class of proteins that carry out this function. Transcription factor activation typically involves either its translocation from the cytoplasm to the nucleus or its conjunction with an activated nuclear-located co-factor. In either case, an activated transcription factor often binds specific genes in the genome and initiates their transcription. The generated messenger RNA transcripts are exported to the cytoplasm where they generate polypeptides after the process of translation.

D. Progress in technology development in chemical physics pushes forward biological sciences

To study the physicochemical properties and processes in a complex environment as the cell, typically two approaches are employed: a bottom-up approach where reactions are modeled for simplistic environments *in vitro* or *in silico* and layers of complexity gradually imposed upon it to create a rational model; alternatively, a top-down treatment wherein one begins by probing into the crowded environment of the cells and tries to elucidate the complexity therein. While the former approach is practically simpler, it can instruct only at a very general level with limited considerations [1]. The top-down method is the method of choice for life scientists who are concerned with the effects of macromolecular crowding on biochemical and cell biological processes. This method commonly involves the expression or injection of fluorophores or radio-isotopic probes into the cell to serve as tracers which can be monitored by optical spectroscopy and microscopy.

Technological advances in physical sciences push the frontiers of biomedical research. For instance, our comprehension of the brain has greatly benefited from developments in optical and electronic microscopy. It is beyond the scope of one review to encompass all aspects of technology enhancements in physical sciences and relate their contributions to biological sciences. Here, we will focus on only one form of the physical tool—light. In fact, we will only discuss emerging applications of visible light within the wide electromagnetic spectrum. Light is a powerful tool that is blessed with multiple attractive features, making it a unique asset for biological studies. Light can be manipulated conveniently both in space and time—typical far-field optics allows for focusing of light to a diffraction limited spot measuring merely several hundred nanometers. Light has minimal invasiveness to biological systems because it can be controlled remotely. Indeed, light has long been used as an imaging tool which allows for the visualization of many cellular structures and functions.

Very recently, light has found a new role in the biological sciences to control cellular functions in live cells and multicellular organisms. In this review, we will first discuss several recent works where the light was used as an investigative tool to probe into the cellular microenvironments. In the second part of the review, we present relevant studies to show how light has served as a perturbation tool at several levels commencing at the plasma membrane and flowing through the cytoskeleton, to cargo transport, and terminating at the axis of transcription-translation, with the idea of following the direction of information flow from the extracellular space to the cell interior (FIG. 1). We will present advances in probes, experimental strategies, and new applications and highlight work from the past five years.

II. USE LIGHT TO VISUALIZE CELLULAR STRUCTURES AND FUNCTIONS

A. Challenges in biological imaging advance optical microscopy techniques

Biological imaging deals with cells, tissues, or even whole-body biological samples. These samples are made of materials that strongly absorb, scatter, and autofluoresce visible light when under excitation. In addition, macromolecules do not assume a homogeneous distribution in the same manner as an analyte in a buffer. Here, we will first discuss how these features of biological samples have motivated the invention of new techniques in optical microscopy. Then, in the framework of signal transduction, we will discuss cases where advances in imaging help discover and elucidate the structures and functions of biological macromolecules inside cells.

1. Deep tissue fluorescence imaging

A single layer of cells does not absorb light so strongly as to impede visualization. However, most biological systems are three-dimensional entities. Thickness becomes a serious issue for deep tissue imaging with

DOI:10.1063/1674-0068/31/cjcp1806152

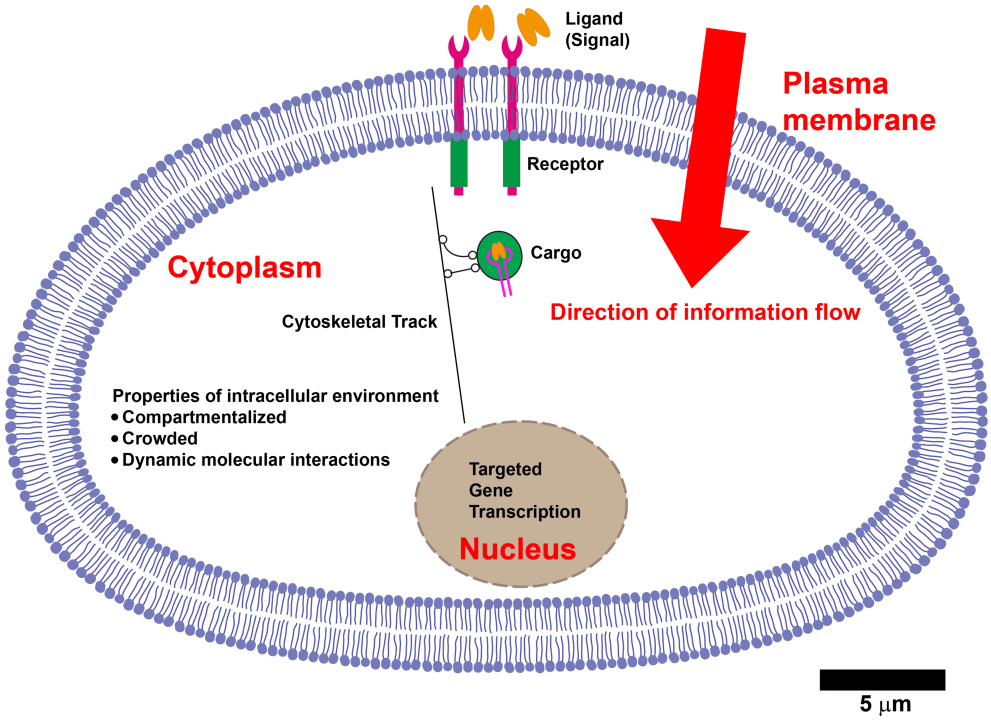

FIG. 1 Scheme of information flow within a typical mammalian cell, which is a highly compartmentalized and crowded environment filled with dynamic interactions between different macromolecules. Signals (here depicted by ligands) interact with receptors on the plasma membrane and initiate the formations of cargoes carrying the activated signaling complex, which moves along the cytoskeletal tracks towards the nucleus to regulate gene transcription. The size of a typical mammalian cell is about 20 μm in diameter. Receptors and cargoes are not drawn to scale.

the commonly used epi-illumination fluorescence microscopy, which excites the sample with wide-field excitation light. Because biological tissue is filled with endogenous chromophores such as NADH and FAD-containing proteins, strong absorption and scattering of excitation light occurs (FIG. 2). Fluorescence signals from areas off-focus commingle with the signals from the focal plane and reach the detector. In other words, the desirable signal (from the focal plane) is contaminated by the undesirable signal (from the off-focus plane), leading to distortion of images.

This challenge motivated researchers to manipulate the excitation scheme to reduce the background. The total-internal-reflection microscopy (TIRF) directs a coherent laser beam to the interface of the substrate and the aqueous buffer to generate an evanescent wave. Because the intensity of this evanescent wave decays exponentially as it travels away from the interface in the z-axis, it only excites a thin layer (approximately 200 nm) of the sample and significantly reduces the background (FIG. 2). Alternatively, confocal microscopy focuses a collimated laser beam into a diffraction-limited spot and uses a pinhole to block the off-focus signal, taking advantage of the differences in the divergences of the off-focus signals and the in-focus light [4].

However, because of the absorption and scattering by tissues, visible light that is often used as the excitation light source gets attenuated by them. For visible light, this penetration depth is only about 200 microns. One way to circumvent this issue is to use multi-photon excitation. The absorption spectra of biological tissues indicate that absorption is weak in the infrared (IR) range, which results in its higher penetrability. However, the energy carried by one photon in the IR range is not sufficient to excite the chromophores from their electronic ground state to the excited states. However, two such IR photons carry enough energy to excite the chromophore provided both can be concurrently absorbed. Gopert-Mayer discovered that molecules do have such a two-photon absorption cross-section, although it is so small that a very high intensity of excitation light is needed (*i.e.*, two photons need to simultaneously hit one molecule to excite it). Fortunately, currently available instrumentation such as the pulsed laser does generate light with an intensity sufficient to achieve this goal. Thus, two-photon excitation fluorescence microscopy can excite deeper tissues compared with one-photon excitation [5].

Although deep tissue samples can be excited via two-photon excitation, their imaging presents another challenge. Fluorescence signals emerging from excited molecules in the tissue are often in the visible range. These signals must travel through deep tissue, which inevitably distorts the wavefront of the emitted light

DOI:10.1063/1674-0068/31/cjcp1806152

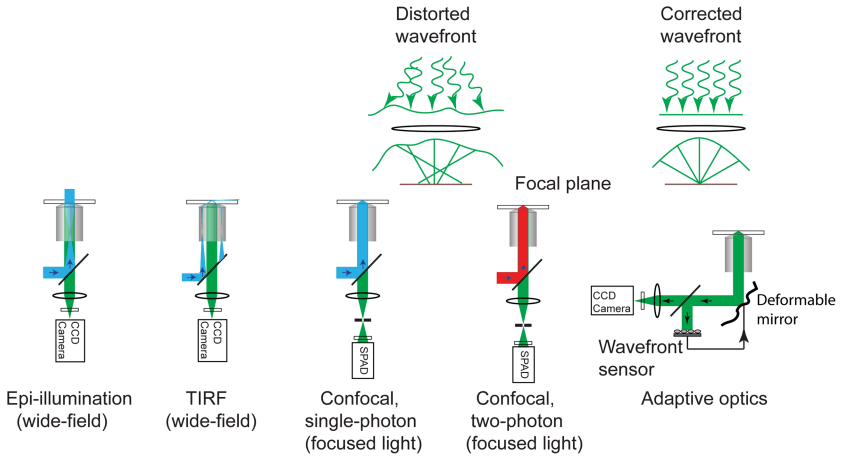

FIG. 2 Fluorescence microscopy commonly used in biological sciences. Epi-illumination provides a wide-field region of interest but suffers from the off-focus signal when used to image thicker biological tissues. Total internal reflection fluorescence (TIRF) microscopy uses the evanescent field to excite a thin layer (200 nm) of the sample, but suffers from the limited penetration depth. One-photon confocal microscopy uses a pinhole to block off-focus signal but the penetration depth is still limited. Two-photon confocal microscopy increases the penetration depth of excitation light. All these microscopy techniques suffer from the aberration of thick biological tissues. Adaptive optics uses a deformable mirror, whose shape is modulated by input from a wavefront sensor, to restore the distorted wavefront and achieve better image quality. CCD: charge-coupled device. SPAD: single-photon avalanche diode.

and blurs the image, a phenomenon that is referred to as aberration. To address this issue, researchers use the strategy of adaptive optics to correct this wavefront distortion. The basic idea is to calibrate the distortion and compensate the phase difference of all distorted light using a wavefront modulator [6, 7]. This strategy significantly improved image quality in deep tissue imaging.

Importantly, besides using fluorescence intensity as the contrasting parameter, one can also use another parameter such as fluorescence lifetime [8] or the energy transfer efficiency between two fluorophores (Forster resonance energy transfer or FRET) [9, 10] to reconstruct an image.

2. Single-molecule microscopy

Single-molecule microscopy detects the signal one molecule at a time, thereby enabling measurements of the probability distribution for microscopic properties. On the other hand, bulk experiments measure the mean value. Single-molecule sensitivity was first realized by detection of absorption [11] and fluorescence [12] at low temperature. Later, room-temperature single molecule detection was accomplished both inside the solution [13] and on the surface [14]. Since then, great progress has been made in bringing single-molecule measurement to the study of biological systems [15–20].

3. Super-resolution imaging

The diffraction limit poses a challenge for utilizing far-field optics in cells. The size of individual macromolecules, such as proteins and nucleic acids, is typically on the order of one to several nanometers, which is two orders of magnitude smaller than the typical diffraction limit. Thus, detailed visualization of molecular structures has been largely dependent on high-resolution electron microscopy. Although electron microscopy works well for fixed, thin-sliced biological samples, it is not always suitable for biological imaging, especially when live cells are to be studied. In addition, electron microscopy lacks the molecular specificity required to target certain types of macromolecules. To address these challenges, researchers have been pushing the limits of far-field optics.

The first strategy is based on modulating the photophysics of molecular probes. The idea is that the localization resolution of the peak position of a Gaussian beam can be more precise than the half-width maximum of the Gaussian beam itself [21, 22]. The localization precision is inversely proportional to the square root of the number of photons that can be collected, *i.e.*, the brighter the molecular probe, the better the spatial resolution. Successful realization of this strategy is to ensure that only one molecule is allowed to emit photons within the diffraction-limited area. Otherwise, the point spread function is not Gaussian, and peak fitting will generate artifacts. Practically, this condition is achieved by the optimization of both the labeling density and excitation light intensity.

The other strategy for super-resolution imaging is based on the manipulation of the spatial profile of the excitation light. By using a spatially structured excitation profile, structured illumination microscopy (SIM) can increase the resolution twofold [23]. Stim-

DOI:10.1063/1674-0068/31/cjcp1806152

TABLE I Super-resolution imaging techniques.

Acronym	Full description	Reference
SIM	Structured illumination microscopy	[23]
STED	Stimulated emission depletion	[24]
FIONA	Fluorescence imaging with 1-nm accuracy	[25]
(F)-PALM	(Fluorescence)-photoactivated localization microscopy	[26, 27]
STORM	Stochastic optical reconstruction microscopy	[28]
PAINT	Points accumulation for imaging nanoscale topography	[29]
NALMS	Nanometer localized multiple single molecule fluorescence	[30]
SHREC	Single-molecule high-resolution colocalization	[31]
SHRImp	Single-molecule high-resolution imaging with photobleaching	[32]
dSTORM	Direct stochastic optical reconstruction microscopy	[33]
GSDIM	Ground state depletion microscopy followed by individual molecule return	[34]
RPM	Reversible photobleaching microscopy	[35]
RESOLFT	Reversible saturable optical fluorescence transitions	[36]

ulated emission depletion (STED) microscopy overlays a donut-shaped depleting beam with the primary point spread function of the excitation beam [24]. Depleting beam effectively "quenches" the molecular emission at the periphery of the primary Gaussian beam so that no fluorescence is detected in that area. Only the unquenched, central area is effective for excitation and emission. Increasing the power of the quenching beam leads to a "thicker" donut and enhances resolution. This modality of super-resolution imaging is not constrained by the simultaneous emission of more than one molecule. A list of commonly used super-resolution imaging techniques has been listed in Table I.

B. Multiplexed fluorescence imaging

Despite the abundance of fluorescent dyes and fluorophore proteins, achieving multiplexing in fluorescence microscopy has remained a challenge due to the spectral overlaps between these fluorophores, which limits the number of molecules being visualized at the same time in a given cell. Fortunately, the simultaneous study of proteins is not inherently impossible since they can be teased apart individually with highly selective antibodies. However, their chromatic discrimination is not feasible now since only a handful of fluorophores with non-overlapping spectra are available for their conjugation. One solution is to sample a few proteins at a time with a set of fluorophore-conjugated antibodies and repeat this sampling process to improve the amount of information gathered from cells or tissues. Key here is to ensure that the fluorophores once used are permanently 'erased' prior to the subsequent samplings that employ the same set of fluorophores which are now conjugated to different antibodies. Gerdes *et al.* [37] and Lin *et al.* [38, 39] could repeatedly inactivate dyes using alkaline oxidation to achieve multiplexing in paraformaldehyde-fixed sample tissues and single cells, and they named their corresponding techniques multiplexed fluorescence copy method (MXiF) and cyclic immunofluorescence (CycIF), respectively.

Recently, a new alternative to fluorophore inactivation has emerged. Mondal *et al.* [40] employed cleavable fluorescent antibodies (CFAs) where fluorophores were conjugated to antibodies via a newly developed azide-based linker, which could be rapidly cleaved with a mild reducing agent. Another set of CFAs was then used in a 'rinse and repeat' fashion. Since the fluorophores are cleaved and washed off, this method bypasses harsh chemical treatments required to inactivate fluorophores while also speeding up sample processing times. These methods have enhanced the extent of cellular and sub-cellular morphological, positional and quantitative information gained from microscopic analyses of cells. These approaches hold promise in clinical diagnostics while serving as enabling tools for life scientists in general. However, these methods are currently limited to fixed cells. Expanding these tools into live cells will be a challenge. It is possible to envision a multiplexing method where cell-impermeant dye-antibody conjugates can be used to probe the surfaces of cells without compromising their integrity.

C. Visualizing cellular environment

1. Probing intracellular pH

One of the main advantages of using light to probe cell function lies in its ability to visualize intact cells non-invasively at a high resolution. As discussed earlier, the pH within cells is not a constant number across compartments and can dynamically change as a result of physiological processes as well as due to pathological conditions. Accurate measurements of intracellular pH values are therefore useful and even diagnostic. Conven-

DOI:10.1063/1674-0068/31/cjcp1806152

tional measurements of pH in intact cells utilized microelectrodes, optical microscopy, and NMR spectroscopy. Fluorescence-based methods are preferred because of their high sensitivity and superior spatiotemporal resolution. Single-cell level information can be assessed by microscopy and a high-throughput sampling rendered possible by flow cytometry. These methods use ratiometric fluorescent probes, the emission spectra of which red or blue shift in response to protonation or deprotonation events resulting from the alterations of pH in their local environment. Most sensors suffer from a limited dynamic range of the pH they can sense. Chen *et al.* developed a tetraphenylethene-cyanine (TPE-Cy) dye which can sense a range of pH from 4.7 to 8.0, with acidic and basic regions evoking intense red and blue fluorescence, respectively [41]. This dye also showed an aggregation-induced emission property, thereby circumventing the problem of crowding-induced quenching. Also, this sensor permitted the confocal imaging and flow cytometric analysis of pH. Lee *et al.* [42] reasoned that the narrow range of existing pH sensors stems from their use of a single functional group. They synthesized a chameleon probe which combined Fluorescein and Rhodamine, two fluorophores with distinct emission properties—the former shows high green fluorescence in basic or neutral pH, while the latter fluoresces red in acidic environments. This combination facilitated the measurement of a dynamic pH range of 3.2−10 with reliable linear measurement in the range 4−8 via confocal microscopy.

2. Probing viscosity inside a cell

Cellular viscosity is an important physical parameter which influences the transport rates of materials within cells. Measurement of intracellular viscosity has been possible based on three types of fluorescent biosensors. Fluorescent molecular rotors are associated with twisted intra-molecular charge transfer (TICT) properties and consist of electron donor and acceptor moieties which can rotate relative to each other. Low viscosity permits charge transfer and minimizes emissions, whereas high viscosity limits TICT and enhances emissions. In the case of aggregation induced emission probes [43], high viscosity restricts their intramolecular motions resulting in a concomitant increase in emission and also in their fluorescence lifetime in some cases. The third class of probes is photo-induced electron transfer (PET) probes [44]. Probes in this category sandwich a spacer between the fluorophore and the receptor, where factors affecting charge transfer modulate the emission intensity and lifetime of the fluorophore. In the case of viscosity measurements, the primary factor affecting PET is the molecular conformational changes stemming from local viscosity.

Liu *et al.* [45] introduced an additional modality by combining a ratiometric rotor with two-photon confocal imaging to achieve deep tissue imaging with improved axial resolution, reduced phototoxicity, and increased light penetration depth. Their carbazole-based cyanine dye exhibited two emission peaks in the blue and red region which varied with viscosity, thereby permitting ratiometric measurements of viscosity in live cells and tissues. A borondipyrromethene(BODIPY)-hemicyanine based ratiometric rotor which can sense viscosity and also can act as a chemosensor for intracellular hypochlorite has been developed [46].

Fluorescence lifetime imaging provides an alternative to ratiometric measurements because fluorescence lifetime is concentration-independent, which can be beneficial. Battisti *et al.* [47] utilized a hyperconjugated pseudostilbene rotor which was non-emitting in low-viscosity solvents, but displayed increased emission and, more importantly, increased fluorescence lifetime with increasing viscosity, which allowed for intracellular FLIM on cells. Using an efficient technique called phasor analysis for fluorescence lifetimes, the researchers could examine mitochondrial processes such as de-energization after subjecting the cells to experimental oxidative stress. BODIPY-based rotors targeting specific subcellular compartments have been developed. Including double-positive charges to prevent endocytosis and promote plasma membrane retention [48] or appending a moderately alkaline morpholine moiety to target the rotor to lysosomes [49] were some of the strategies employed to accumulate the rotors in specific intracellular regions.

The earlier mentioned TPE-Cy from Chen *et al.* [41] is an AIE probe which could also be used to measure viscosity [43] in addition to pH, making it a versatile probe for exploring cell microenvironments. The dye showed good aggregation-induced emission property and elicited increases in both fluorescence intensity and lifetimes, making it suitable for FLIM. In addition, it was conducive for two-photon imaging, which greatly limited the background interference in FLIM. Liu *et al.* [44] pioneered the use of PET probes for viscosity measurements. They developed a PET probe, which was suitable for ratiometric fluorescent imaging, FLIM, and two-photon microscopy. The probe exhibited increases in both fluorescence intensity and lifetime with increasing viscosity. These effects were attributed to both PET and TICT reductions in viscous environments. A quantitative map of intracellular viscosity revealed that the lysosomes and mitochondria were the most viscous compartments.

3. Probing phase separation inside cells

Unlike an ideal dilute solution, the cell can exhibit behaviors of viscoelastic fluids (actomyosin cytoskeleton), liquid crystals (meiotic spindle), hydrogels (nuclear pore complex), and liquids [50]. One consequence of the macromolecular crowding in cells is phase sep-

DOI:10.1063/1674-0068/31/cjcp1806152

aration or the formation of liquid droplets. There are a number of such structures where the local concentrations of certain proteins, nucleic acids or other macromolecules are higher than the surrounding bulk. In the case of non-membrane-bound compartments, such droplets facilitate catalysis while exchanging materials freely with the surrounding phase. In other cases, droplet formation serves to sequester components to stop cytoplasmic reactions. The crowded intracellular milieu favors phase separation particularly for proteins containing RNA-binding domains and low complexity/intrinsically disordered regions [51, 52]. The mobility of the proteins within the droplet phase and between the bulk and the droplet phases is commonly assessed using a technique called fluorescence recovery after photobleaching (FRAP) [3]. In this technique, the target protein is tagged with a fluorophore and allowed to accumulate into droplets; the droplets are then subjected to intense laser light to photobleach it partly or completely, following which the system is allowed to recover by diffusion of the unbleached surrounding molecules into or within the droplets. The kinetics of fluorescence recovery then provides useful information about the dynamics of the tagged protein and parameters such as viscosity indirectly. Single molecule FRET (smFRET) and FLIM methods have also been applied to study the conformational dynamics of intrinsically disordered proteins within cells [53]. These methods hold promise for studying phase transitioned structures within cells.

4. Visualizing cytoskeletal tracks in cells

Cytoskeletal tracks such as microtubules and actin filaments are nanometer structures that not only play roles in shaping cell morphology but also provide an infrastructure for supporting intracellular trafficking. Resolving these ultra-structures with high molecular specificity remains a challenge in bioimaging. With recent improvements in fluorophores, instrumentation, and image analysis algorithms, it is now possible to use super-resolution imaging to resolve these ultra-structures in cells. Structured illumination has been used to resolve the microtubule- and actin-cytoskeleton with a lateral resolution of 125 nm and an axial resolution of 280 nm in fixed mammalian cells. Microtubules were labeled with fluorescent antibodies and actins with rhodamine-phalloidin [54]. Three-dimensional STORM microscopy has also been used to resolve the structure of microtubules with a resolution of 30 nm (lateral) and 60 nm (axial) [21]. Intracellular structures have also been imaged with PALM-based microscopy [26] and an interferometric PALM [55]. Using STORM-based super-resolution imaging, Xu and colleagues found that actin formed ring-like structures that wrapped around the circumferences of axons and were evenly spaced along the axonal shaft with a periodicity of 180−190 nm [56].

5. Tracking of cargo transport one at a time

A neuronal cell can extend a meter-long process (axon) that is 10^4 times longer than the size of its cell body. If material transport within this cell were based on diffusion, it would take years to move a biological molecule along an axon projecting from our spinal cord to the tips of our hands! Furthermore, diffusion lacks direction, and therefore vast amounts of materials need to be synthesized to ensure that the target region gets an adequate amount. Therefore the use of diffusion in cellular contexts is limited and relying on it is impractical. How do long, polarized cells such as the neuron solve this problem? They utilize active, directed axonal transport mechanisms to move cargoes at an average speed that is about 1000 times faster than that of diffusion. Single-molecule fluorescence microscopy allows for real-time tracking of neurotrophin transport in live neuronal cells [57]. In early studies, the measured trajectory length was limited by the photobleaching of the organic fluorophore. This scenario has been significantly improved with the production of more photostable probes such as semiconductor nanocrystals (quantum dots), which have enabled continuous tracking of cargoes along neuronal processes for several minutes over hundreds of microns [58, 59]. The first prototype of a compartmentalized culturing device that separates the cell body from the distant neurite greatly helps in the visualization of the transport directionality [60]. Improved quality has been achieved by replacing Teflon with polydimethylsiloxane (PDMS), a transparent and highly biocompatible material [61–63].

III. USE LIGHT TO CONTROL CELLULAR FUNCTIONS

Besides serving as a tool to "visualize" molecular structures and functions, light can also be used to control molecular and cellular processes in live cells. The recently emerged field of optogenetics demonstrates such a modality.

A. Optogenetics utilizes light to control cellular processes

By combining synthetic light-sensitive ion channels [64] or channelrhodopsins [65, 66] with light illumination, researchers were able to interrogate neuronal firing, which led to the coining of the term "optogenetics" [67]. By controlling neuronal firing, optogenetics has provided a new way to investigate neuronal circuits in health and disease [68, 69]. Currently, optogenetics has been used in basic and preclinical research on a wide spectrum of topics such as Parkinson's disease [70], sleep [71], cardiac function [72, 73], neuropsychiatric diseases [74], epilepsy [75], and sight-restoring therapy [76, 77]. Because there are extensive reviews on this topic, we will not expound on it in this work.

DOI:10.1063/1674-0068/31/cjcp1806152

The power of light to control cell functions has gone beyond the modulation of the membrane potential of excitable cells [78]. Light has also been used to control many intracellular signaling processes and has initiated the field of optobiology [79–85]. In optobiology, light can be used as a manipulative device to modulate functionality of signaling components with high spatial and temporal resolution.

The core components of optobiology are a family of photoactivatable proteins, which respond to visible or infrared light stimulation by modulating their inter- or intra-molecular interactions [86] (FIG. 3(A)). When fused to specific signaling components, photoactivatable proteins allow for light-controlled activation or inactivation of target signal transduction (FIG. 3(B)). Commonly used photoactivable proteins include blue light-sensitive LOV [84, 87–92], CRY2-CIBN [93], Dronpa [94, 95], green light sensitive cobalamin (vitamin B12) binding domains (CBDs) [96] and their derivatives, and red-IR light controllable PhyB-PIF6 [83] together with Rhodopseudomonas palustris bacterial bathy phytochrome, BphP1 [97, 98], Their structure, light-mediated mechanism of action, as well as ways to interrogate specific signaling molecules have been reviewed [85, 86, 95, 99–107].

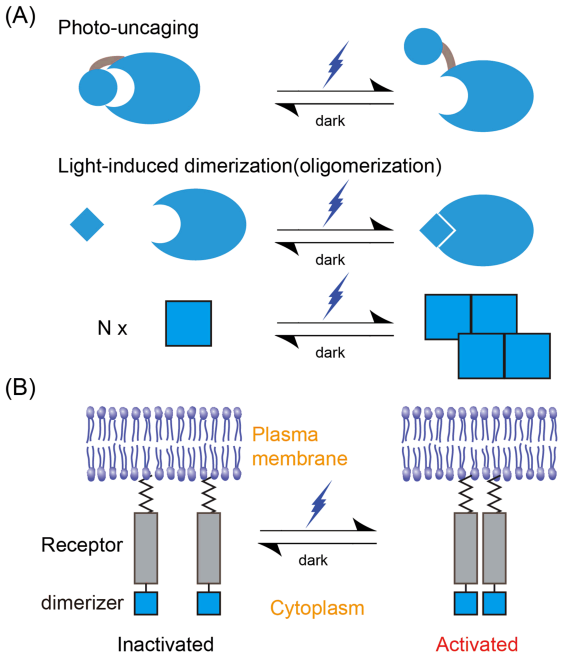

FIG. 3 (A) Typical modalities of photoactivatable proteins used in non-neuronal optogenetics. Upon light stimulation, the intracellular domain can change its conformation so that a "caged" active site can be exposed (the photo-uncaging mode). Alternatively, the inter-molecular interaction can be modulated (the light-induced dimerization or oligomerization mode) so that target molecules can be translocated to distinct subcellular localizations to achieve specific signaling output. (B) One scenario of light-mediated receptor activation via protein dimerization.

B. Using light to perturb cellular microenvironments

Light has been used in conjunction with the tools mentioned above to manipulate the cell microenvironment in addition to carrying out a range of other tasks. For instance, acidification of synaptic vesicles and lysosomes has been optogenetically achieved by targeting the yellow light-driven proton pump Arch3 to those compartments [108]. This tool termed "pHoenix" can be applied to other organelles by using appropriate targeting signals, thereby enabling perturbation of their pH.

In addition, the property of phase separation has been optogenetically recapitulated. Shin *et al.* [109] fused the self-associating CRY2 protein to intrinsically disordered regions of proteins which had a propensity to phase-separate. Blue light-induced the clustering of these IDR-CRY2 to form "optoDroplets" which exhibited liquid droplet like properties as evidenced by FRAP. Using a stronger clustering mutant of CRY2 termed CRY2olig resulted in the formation of a gel-like state followed by irreversible protein aggregation. Protein concentration-dependence for phase separation was demonstrated, and the concentration itself was tunable using light intensity and frequency. While concentration is one parameter in phase separation, others such as valency and interaction strength remain to be optogenetically investigated [110]. With the current repertoire of optogenetic tools, these parameters can be explored in a straightforward manner. Very recently, a system called "Corelets" was developed by Nakamura *et al.* [111] to study phase separation with rapid dynamics. This system was designed to take into to account the contributions of nucleation as well as the homotypic interactions of IDRs toward phase separation. Their system employed a high-valency protein core consisting of 24 human ferritin heavy chain (FTH1) subunits. FTH was fused to the photoactivatable heterodimerizer iLID and nuclear-localized, while sspB, the interacting partner for iLID, was fused to various IDRs and IDR-containing proteins. Blue-light stimulation recruited multiple IDRs to the ferritin core nucleus favoring a cohesive interaction between IDRs. This biomimetic system recapitulates the formation of membrane-less organelles with potential applications in synthetic biology and organelle engineering. In the following subsections, we present recent studies where light was used to control cellular processes in the framework of information flow which follows the axis of receptor-cytoskeleton-cargo-transcription.

C. Control receptor activity

Receptor tyrosine kinase and GPCR are two types of receptors that use distinct signaling mechanisms to

DOI:10.1063/1674-0068/31/cjcp1806152

transmit extracellular signals into the cell interior.

1. Optogenetic control of RTK

Growth factor receptors such as those for epidermal growth factor and neurotrophins are typical RTKs. Receptor tyrosine kinases form a large family of receptors which receive extracellular stimuli and activate intracellular signaling cascades. They are typically activated by their ligand-induced dimerization at the plasma membrane, following which they autophosphorylate and cross-phosphorylate intracellular domain tyrosine residues, after which some of those tyrosines become docking points for proteins mediating intracellular signaling cascades. Since the role of the ligand is to promote and stabilize dimer formation, it is dispensable for receptor activity if dimerization can be promoted by other means.

Light-activated dimerization provides attractive advantages such as spatiotemporal control as well as reversibility and tunability. Grusch *et al.* [92] constructed optoRTKs by fusing the optogenetic LOV protein from *V. frigida* to the intracellular domains of RTKs after removing their ligand-responsive extracellular domains. Blue-light induced dimerization of LOV resulted in RTK dimerization and its subsequent activation. Robust activation of several receptors was achieved using light with physiological responses mimicking those achieved by natural ligands. Similarly, the Heo group used the CRY2 protein to self-associate and activated fibroblast growth factor receptor (FGFR1) at the plasma membrane under blue light [112]. They could control cell polarity and migration using this approach. The designs discussed so far required engineering RTKs with photoactivatable domains and exogenously applying them in cells, which could lead to overexpression artifacts and in this regard, controlling endogenous receptors is a safer approach. Bugaj *et al.* [113] developed an optogenetic method to activate endogenous RTKs using CRY2. A receptor-targeting binding motif was fused to CRY2, which permitted its specific but weak affinity, non-covalent interactions with the target RTK. Blue light illumination resulted in CRY2 clustering which promoted increased avidity with its target RTK, consequently clustering and activating the RTK in the process. This approach, termed clustering indirectly using cryptochrome 2 (CLICR) enabled the activation of several RTKs.

2. Optogenetic control of GPCR

GPCRs are membrane-spanning transmembrane helices which associate intracellularly with membrane-attached heterotrimeric G proteins, the principal types being Gi/o, Gq and Gs. Receptor activation promotes guanine nucleotide exchange on the Gα-subunit, which subsequently dissociates from the G$\beta\gamma$ heterodimer. The α and $\beta\gamma$ subunits then elicit different signaling responses. Gα's inherent GTPase activity restores it to the inactive state, following which it re-associates with G$\beta\gamma$ and forms the inactive heterotrimer. Karunarathne *et al.* [114] adapted non-rhodopsin opsins to achieve asymmetric control of GPCR responses within cells using light. Opsins were spectrally selected to allow for fluorescent imaging without perturbing their light-induced activity. A blue-opsin, bOpsin was selected and tested for its ability to activate the endogenous Gi/o activity and consequently the production of the phosphoinositide PIP3 in a localized region of the plasma membrane which was under light stimulation. Mimicking biological neurotransmitter gradients, activation of this optogenetic system enabled neurite initiation in hippocampal neurons in a spatiotemporally controlled manner. Opsin systems activating the other two major G proteins, namely Gq and Gs, were also developed in this work. In another study, O'Neill and Gautam [115] used an optogenetic approach to inhibit G protein signaling in a localized fashion within cells. They used two approaches, one to accelerate Gα GTP hydrolysis using a GTPase accelerating protein RGS4 and the other to sequester and inhibit the G$\beta\gamma$ subunits using a kinase GRK2ct. The first approach involved localized blue light-dependent recruitment of an RGS4-CRY2 fusion to a membrane-anchored CIBN, while the second approach similarly recruited GRK2ct-CRY2 to the same membrane-anchored unit. The authors used this to mimic a chemotactic gradient and elicit macrophage movement in the direction opposing the light-mediated inhibition. These approaches provide a means for dissecting GPCR activities at various subcellular locations.

D. Control the dynamics of the cytoskeleton

Signal transduction often results in cells generating mechanical forces to drive cytoskeletal rearrangements which manifest in movements at the cellular or subcellular levels. The precise spatiotemporal resolution offered by light can be tapped to generate some of these movements at will, thereby enabling perturbations and investigations which are otherwise difficult to achieve. The cytoskeletal protein actin plays a major role in providing the framework for dynamic structures such as lamellipodia and filopodia in the moving edges of the cell. Hughes and Lawrence [116] presented an optogenetic design to direct cell motility by perturbing filamentous actin dynamics at the lamellipodia. They engineered the F-actin-severing enzyme, cofilin, to possess a higher concentration threshold by weakening both its actin affinity and actin-severing ability, thereby rendering its activity contingent on a higher local concentration of it on its substrate namely F-actin. This mutated cofilin was fused to CRY2 and used in conjunction with the CRY2-partner CIB fused to F-actin. Locally ap-

DOI:10.1063/1674-0068/31/cjcp1806152

plied blue light resulted in local recruitment of cofilin, which prompted rapid lamellipodia formation and expansion. Multiple light pulses generated larger and directed cell movements.

While this approach modulated existing actin polymerization by altering the process dynamics, it has also been possible to initiate actin polymerization using the clustering capabilities of CRY2. Tucker's group [93] screened a CRY2 point mutant E490G termed CRY2olig, which could greatly enhance blue light-induced clustering. Fusing the Verprolin-homology, central acidic (VCA) domain of the Neuronal Wiskott Aldrich Syndrome protein (N-WASP) to CRY2 led to its blue light-induced oligomerization, which could rapidly facilitate the nucleation of actin polymers. The complex process of axon pathfinding in nerve cells involves signal transduction from soluble chemical cues via oligomerization of membrane-located axon guidance receptors such as DCC to mediate intracellular protein phosphorylation resulting in actin polymerization that drives filopodial axon movement. A photo-activatable DCC where DCC was fused to CRY2 could oligomerize in response to blue light and could direct neurite outgrowth in DRG neurons [117]. A similar design could also direct VD motor neurons to the dorsal nerve cord *in vivo* in the nematode worm *C.elegans*. Another approach to modulate actin polymerization is via its regulatory proteins like Rho and their guanine exchange factors (GEFs). In a recent study, Valon and colleagues [118] recruited CRY2-fusions of the catalytic domain of a RhoA GEF ARHGEF11 to a plasma/mitochondrial-membrane-located CIBN to modulate cellular forces and to rapidly activate mechanotransduction pathways such as the YAP pathway. In another study [119], a different blue light-activated optogenetic pair, LOVpep-PDZ, was employed in recruiting the catalytic DH domain of a Rho GEF, LARG, to the plasma membrane thereby inducing local RhoA activation. In this case, light-induced perturbation in the cytoskeleton set the stage for investigating the mechanical properties of the cytoskeleton.

Optogenetics has also been used to inhibit target proteins to arrest cytoskeletal processes. The Heo group [120] developed a light-activated reversible inhibition by assembled trap (LARIAT) which could sequester proteins by clustering under blue light. CRY2 was fused to or targeted by nanobodies to cytoskeletal modifiers such as Vav2, a Rho GEF or PI3 kinase (Pi3K) and co-expressed with CIBN. Blue light-mediated CRY2 clustering trapped these proteins and blocked their activity, which resulted in membrane retraction and protrusion in the illuminated and the non-illuminated sides of the cell, respectively. This approach has been utilized to confirm the activity of new GEF proteins such as PLEKHG3 which is involved in the actin-mediated regulation of cell migration and cell polarity [121]. Another approach to inhibiting actin-dependent cell contractility involves depleting the phosphoinositide PI(4,5)P2 at the plasma membrane with light [122]. A membrane-located CIBN could attract a cytosol-located CRY2 fusion of the catalytic domain of a PI(4,5)P2 phosphatase OCRL under blue light and deplete membrane PI(4,5)P2 and arrest actin dynamics. This system was used to achieve local modulation of cell contractility in the morphogenetic process of ventral furrow formation during Drosophila embryogenesis.

In addition to actin filaments, microtubules mediate cell motility as well as essential functions such as cell division, intracellular transport, and cell polarization. An optochemical approach to depolymerizing microtubules with spatiotemporal control has been developed [123]. A microtubule-destabilizing drug combretastatin was engineered to acquire light sensitive conformational switching between its active and inactive isomeric forms. Violet and green light could switch the drug to inactive and active states, respectively, conferring light control over microtubule depolymerization at the cellular level with potential for subcellular control when appropriate optics can be incorporated. Optochemical approaches have similarly enabled control of other cytoskeletal elements such as myosins involved in cell contraction. For instance, light-induced bursts of calcium emerging from a photolabile Ca^{2+} chelator *o*-nitrophenyl EGTA could stimulate cell contraction and consequently tissue morphogenesis via local non-muscle myosin II activation [124].

Besides photoactivatable proteins, light-responsive nanoparticles have been used in conjunction with light to manipulate cell-level forces in processes such as cell protrusion, migration, and mechanotransduction. These optomechanical actuator (OMA) nanoparticles [125], which are composed of a heat-responsive polymer coated on gold nanorods, can be targeted to specific cell surface receptors. Absorption of near infrared (NIR) light by the gold nanorods enables localized heat generation which collapses the thermoresponsive polymer and generates piconewton forces that can be transduced to the cell via the targeted receptors. These new light-based tools enable the spatiotemporal dissection as well as perturbation of the cytoskeleton and its forces in ways not previously available.

E. Control cargo trafficking along the cytoskeleton

As discussed earlier, the crowded intracellular milieu of the cell renders diffusion inefficient and unreliable. For a complex, polarized cell like the neuron, materials have to be synthesized at one location and transported over long distances without compromising the direction of transportation or the content of the cargo. Such targeted transport is achieved by cargo-carrying motor proteins moving on microtubule tracks with defined directionality. Experimental modulation of cargo trafficking has been a daunting task due to the spatiotemporal requirements in this process. The recent

DOI:10.1063/1674-0068/31/cjcp1806152

evolution of optogenetic and optochemical systems has enabled perturbation at a level suitable for accomplishing these tasks. For a more elaborate discussion on these new experimental strategies for modulating cargo trafficking, we refer the readers to our recent review [126]. Here, we present a succinct discussion of recent optogenetic and optochemical approaches to achieving experimental cargo trafficking.

To control the intracellular movement of cargoes such as organelles, they need to be coupled to molecular motor proteins such as kinesins, dyneins, and myosins or adaptor proteins such as BICD2 (Bicaudal D2). Optochemical systems have been developed to facilitate such targeted association between protein pairs. One such system [127] uses a photocaged dual ligand as a dimerizer to crosslink two fusion proteins—one fused to a Haloenzyme (Halotag) and the other fused to *E. coli* dihydrofolate reductase (eDHFR). The dual ligand is a synthetic, cell-permeable small molecule and is a composite of the ligands for the haloenzyme and eDHFR. The eDHFR ligand, trimethoprim (TPM) is photocaged to prevent its association with eDHFR. UV light application uncages the ligand and promotes dimerization of halotag and eDHFR fusions. This ligand, termed NTH, could facilitate light-inducible mitochondria and peroxisome trafficking in neurons with directional control when the Halotag and eDHFR were fused to organelle-tethered proteins and motors or motor effectors, respectively [128]. This approach was used to evaluate certain mammalian proteins Hook1 and Hook3 for their capacity to act as molecular adaptors and to determine their mechanism of activity [129].

Light-induced dissociation between cargoes and motors was more recently achieved by moving the caging group in NTH to the middle of its two ligands, thereby leading to the constitutive association of the motor and the cargo in the presence of the dimerizer, which could be broken by UV exposure to reverse the dimerization [130]. Using a similar design, but adding a coumarinyl caging group, Chen *et al.* [131] synthesized a light-switchable chemical dimerizer termed 'CONC' for both light-induced association as well as dissociation at orthogonal wavelengths. Using CONC, the authors demonstrated photoswitchable chemically induced dimerization (psCID) on early endosomes. Other small molecules and modules for optochemical control include photocaged rapamycin [132, 133], variant forms of abscisic acid [134, 135] and gibberellic acid [136] and a light-activated crosslinker for SNAPTag and HaloTag [137].

Alternatively, optogenetic approaches use light-induced interactions between photoactivatable proteins and their partner proteins to bring cargoes and motors/motor effectors together. The blue-light inducible optogenetic pairs CRY2-CIB1 and LOVpep-ePDZ (light, oxygen,voltage-peptide epitope-engineered PDZ domain) have been utilized to achieve light-induced cargo transport [138, 139]. Reversible modulation of motor protein directionality was possible when the lever arm of myosin or kinesin motors was engineered with the light, oxygen, and voltage domain LOV [140]. Recently the red light-dependent PhyB-PIF system was employed for organelle transport using similar principles and was used in conjunction with the blue light-inducible TULIP system to orthogonally control the positioning of two different organelles within the cell [141]. A very recent study employed the photoactivatable pair iLID-SspB to recapitulate endoplasmic reticulum(ER)-mitochondria tethering by expressing fusions of this pair with mitochondrial and ER-targeting peptides, respectively [142]. This study highlighted the feasibility of generating organelle interfaces reversibly with high spatiotemporal resolution, simulating a higher level of intracellular organelle positioning associated with cell signaling.

F. Control gene transcription and translation

A particularly important application of non-neuronal optogenetics is to control transcription [102] (FIG. 4). Optogenetic transcription control of up to three sets of genes using orthogonal photoactivatable systems employing UV, blue and red light has been achieved without cross-activation [143]. Moreover, combining optogenetic inputs with chemical inputs enable further tunability and enable tighter control of genetic circuits [144]. For a recent review of the systems used in the optogenetic regulation of transcription, please refer to [145]. Here we discuss various strategies which can be adopted for achieving transcriptional control.

1. Light-mediated hybrid system

By mimicking a yeast-two-hybrid system, a DNA binding domain is fused to a photoactivatable protein, whose binding partner is fused to a transactivation domain such as VP16 or VP64 [146–148]. Light-mediated binding between photoactivatable proteins leads to a recruitment of the transactivation domain to the DNA-binding domain, which in turn activates transcription (FIG. 4(A)). A human P65 activation domain has also been employed in fruit flies using the above strategy to monitor neuronal function and modify behavioral patterns [149]. A more sophisticated scheme has been recently developed based on a CRISPR/Cas9 system, where DNA binding was achieved by RNA-guided, catalytically inactive Cas9 fused to one of the dimerizers. The other dimerizer was fused to an activator, which was recruited to dCas9 upon light illumination [149–153]. Additionally, split-Cas9 systems have been developed for use with both light-induced and chemically-induced dimerization. These systems diminish undesired background activity and offer a new modality for gene-editing [154, 155]. Very recently, a robust single-chain photoswitchable Cas9 was constructed us-

DOI:10.1063/1674-0068/31/cjcp1806152

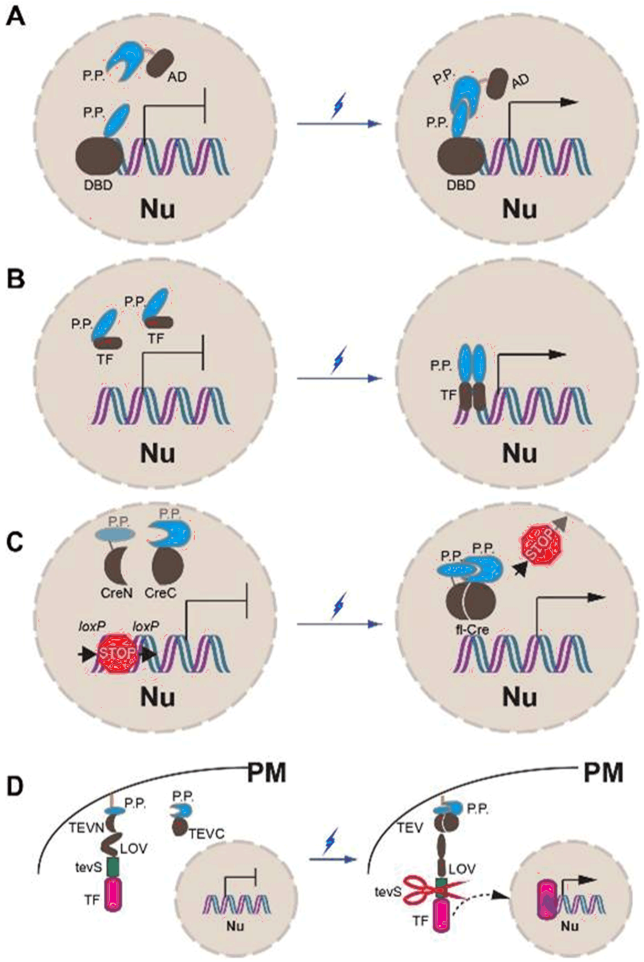

FIG. 4 Current non-neuronal optogenetic approaches to regulating transcription based on a light-mediated two-hybrid system (A), TF dimerization (B), split Cre recombinase (C), and TF release from the plasma membrane (D). TF: transcription factor.

ing pdDronpa [156]. In this system, Dronpa dimerization in the dark occludes the DNA-binding cleft of Cas9 fused to a transactivation domain, whereas cyan light dissociates the dimers, permitting DNA access to the cleft and thereby promoting transcription.

2. Light-induced dimerization of transcription factors

In this modality, transcription factor (TF) is optogenetically dimerized to activate transcription (FIG. 4(B)). The well-characterized DNA-binding domain of Gal4 consists of a DNA-recognition element and a dimerization domain (1-65). Wang *et al.* constructed a LightOn system that contains a fusion protein of Gal4(Δ1-65)-VVD [157]. Gal4(Δ1-65) lacks the dimerization domain essential to DNA binding. VVD dimerizes under blue light illumination, which leads to the homodimerization of Gal4(65) and its subsequent binding to the upstream activating sequence (UAS) of Gal4. This system has been improved by modifications to its UAS [158] and has been incorporated into a bidirectional cassette to control multiple genes [159]. Motta-Mena *et al.* engineered a light-sensitive bacterial transcription factor, EL222, for mammalian transcriptional control [160]. EL222 contains a photosensory LOV domain and a helix-turn-helix (HTH) DNA binding domain. In the dark, LOV binds to HTH, covering its 4α helix essential to dimerization and DNA binding. Blue light illumination triggers the formation of a protein-flavin adduct within the LOV domain, disrupting the LOV-HTH inhibition and allowing EL222 to dimerize and bind to DNA. By adding a VP16 and a nuclear localization signal (NLS) to EL222, light-mediated transcriptional control in mammalian cells was achieved. This system was adapted for transcription control in Zebrafish embryos after replacement of its transactivation domain [161].

3. Split Cre recombinase

A third strategy is based on Cre-lox recombination (FIG. 4(C)). A transcriptional stop sequence is flanked by loxP sites preceding gene of the target. By constructing a split Cre recombinase [162], whose two halves are fused to each component of a dimerizer, one can control gene expression in cell cultures [81, 163], Drosophila [164], and mice [165, 166].

4. The release of a plasma membrane-sequestered transcription factor

Very recently, two independent studies (blue-light-inducible TEV protease or BLITz [167], and fast light- and activity-regulated expression or FLARE [168]) utilized light to induce nuclear translocation of transcription factors to regulate reporter genes driven by Gal4 or tetracycline inducible Tet Repressor-operator systems (tTA). Unlike previous studies, this strategy sequesters the transactivation domains to the plasma membrane in the dark. Upon light illumination, transactivation domains are released from the plasma membrane and translocate into the nucleus (FIG. 4(D)).

Similar to sequestration, another recent strategy exploits aggregation and its light-induced release as a way to control transcription factors. In a technique termed PICCORO (PixD complex-dependent transcription control) [169], a blue-light cyanobacterial protein PixD which forms decameric complexes in the dark is used to aggregate and inactivate a TF fused to PixE, a regulator of PixD. Blue light illumination reduces the PixD decamers to dimers, which forces the release of the PixE-coupled TF. This system was used in Zebrafish embryos by the pioneers of this technique to achieve transcriptional repression of a T-box transcription factor's target genes. A similar approach has been shown

DOI:10.1063/1674-0068/31/cjcp1806152

to work with the TF Bicoid where its fusion with CRY2 repressed its transcriptional activity [170].

An approach for bidirectional control of gene expression has emerged from an intriguing observation of CRY2. It was observed that certain synthetic transcription factors fused to CRY2 exhibited a clustering-dependent slow nuclear clearance and consequently exhibited diminished TF activity upon blue light illumination. Bidirectional control over gene expression was achieved [171] by coupling this light-dependent transcriptional disruption to light-dependent degradation of its transcriptional target protein [172].

Transcription of exogenously expressed genes in eukaryotic cells can also be controlled using an optochemical caging strategy. Binding of the transcription factor TFIID to the TATA box in the promoter region of genes is a prerequisite for virtually all RNA polymerase II-dependent transcription, which makes this interaction an attractive node for transcription control. Hemphill and colleagues installed 1-3 NPOM-caged thymidine residues into the TATA box region of plasmids encoding target genes to block the TFIID-TATA-box interaction in those genes [173]. They then used UV light to uncage the TATA box to specifically express those genes in subsets of HEK cells within a population of transfected cells. This technique also worked in live Zebrafish embryos.

Interestingly an "all-optical" regulation of transcription has been achieved [174]. This method termed laser-induced "Ca^{2+}-comb" (LiCCo) relies on cytosolic Ca^{2+} level-dependent nuclear localization of a universal TF NFAT. It was observed that femtosecond pulses of the laser could release ER Ca^{2+} into the cytosol in a power-dependent fashion. By fusing target TFs to NFAT, their nuclear localization and subsequently transcription can be controlled.

As mentioned earlier, most approaches to transcription control employ transcription activation or repression domains fused to DNA-binding domains. However, these constructs often do not capture the inherent complexity of TFs, which exhibit multiple interactions and regulate several genes. A more tailored approach to controling specific TFs involves developing dominant-negative versions of TFs to inhibit them and use a stimulus to relieve this inhibition to promote transcription. Using computational designs, Ali and colleagues engineered photoactive yellow protein (PYP) to achieve control of CREB-dependent transcription [175]. Large blue-light induced conformational changes in PYP were transduced to a DN-CREB to relieve its dark-state inhibition of CREB. While the design itself is specific, the design principles are generalizable. For instance, Paonessa *et al.* used a similar approach to modulate the gene targets of the transcription factor REST in neuronal cells [176].

Another approach that partially preserves the features of endogenous TF uses light-enabled nuclear import. This strategy resembles the sequestration method discussed earlier, but the target TFs are retained in the cytosol other than at the plasma membrane. This system was modeled by Yumerefendi and colleagues [177] and uses photoaging of a nuclear localization signal (NLS) by the AsLOV2 protein which is fused to target proteins. Additionally, a nuclear export signal (NES) serves to retain the fusion protein in the cytosol in the dark. This NES-AsLOV2-NLS complex termed the light-activated nuclear shuttle (LANS) is engaged by uncaging of the NLS upon blue light illumination, which overpowers the NES and drives a nuclear import. A modified TF where LANS replaced some of its motifs including its NLS was successfully tested by this group. Conversely, a light-induced nuclear export system (LEXY) has also been developed using the same AsLOV2 protein where NES is the caged unit and NLS a supporting unit [178]. Control of both synthetic transcriptional repressors and of a full-length transcription factor p53 has been achieved in that work.

In addition to controlling transcription with light, an approach to controling translation has been developed and improved by Schaffer and Kane [179]. The premise is that translation of an mRNA can be initiated in eukaryotes by targeting the initiation factors eIF4E or eIF4G to sites upstream of the mRNA coding region. A specific RNA-binding domain fused to CIBN could recruit a CRY2-eIF4E fusion under blue light and initiate translation of a reporter mRNA [179]. In this case, the RNA-protein interactions were imported from a naturally strong interaction occurring in the λ-phage and incorporated into this system to target the RNA-binding module to the reporter mRNA. To improve applicability and extend this ability to control endogenous mRNAs would require sequence-specific RNA binding. The authors recruited the Pumilio and FBF (PUF) domains which had the capability of being engineered to target any octameric RNA sequence, with the potential to target longer sequences by combination [180]. This PUF domain could functionally replace the RNA-binding module in the previous design, thereby conferring versatility to the system. In addition, both activation and repression of translation were achievable using PUF.

IV. CONCLUSION

Macromolecules within a cell undergo constant interactions which modulate their chemical and physical states. This modulation is critical for the regulation of metabolism, proliferation, migration, differentiation, cell death, and many more. Probing macromolecular interactions within cells presents significant challenges when the inhomogeneous, compartmentalized, and crowded nature of the intracellular environment is considered. These challenges for life scientists have increasingly invoked the help of other disciplines such as chemistry, physics, material sciences, and engineer-

DOI:10.1063/1674-0068/31/cjcp1806152

ing, thereby promoting a multidisciplinary approach to problem-solving. Furthermore, these challenges have impelled the development of tools and technologies in these related sciences to further our comprehension of the world of the cell. To demonstrate this idea, here, we highlight several recent applications that use light as a tool to probe and control the intracellular processes. We attempted to follow the direction of information flow in signal transduction from the extracellular space to cell interior and highlight the power of light in perturbing cellular processes at every level along that axis. We believe that development in chemical and physical tools will continuously push forward the frontier in biological sciences.

V. ACKNOWLEDGEMENTS

The work was supported by the School of Molecular Cell Biology at the University of Illinois at Urbana-Champaign.

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DOI:10.1063/1674-0068/31/cjcp1806152