Published in final edited form as:

J Phys Chem Lett. 2013 July 3; 4(13): 2138–2146. doi:10.1021/jz400682m.

Labeling Cytosolic Targets in Live Cells with Blinking Probes

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Abstract

With the advent of superresolution imaging methods, fast dynamic imaging of biological processes in live cells remains a challenge. A subset of these methods requires the cellular targets to be labeled with spontaneously blinking probes. The delivery and specific targeting of cytosolic targets and the control of the probes' blinking properties are reviewed for three types of blinking probes: quantum dots, synthetic dyes, and fluorescent proteins.

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Keywords

Single molecule localization microscopy; Superresolution imaging; Optical nanoscopy; Quantum dots; Synthetic dyes; Fluorescent proteins

Optical microscopy and especially fluorescence microscopy have played key roles in studying cellular structures and functions, down to subcellular resolution. The high contrast, high sensitivity and minimal invasiveness attributes of fluorescence have afforded continuous imaging of live cell processes. However, due to the diffraction of light¹, these methods were limited to spatial resolution of around 250–300 nm. In a quest to increase resolving power while maintaining the advantages of fluorescence microscopy, several far field super resolution (SR) techniques have been introduced during the past decade^{2–7}.

A sub-set of these methods relies on single molecule localization, often referred to as “localization microscopy” methods (including PALM⁸, fPALM⁹, STORM¹⁰, dSTORM¹¹, and GSD¹²). Localization microscopy methods are based on the precise localization of individual molecules via temporal separation, achieved either by photoactivation (PALM, fPALM, STORM, dSTORM and GSD), photobleaching (gSHRImP¹³), binding and dissociation (PAINT¹⁴, BALM¹⁵), or stochastic blinking (GSD, STORM and dSTORM).

Recently, we introduced a superresolution method dubbed ‘superresolution optical fluctuation imaging’, or SOFI¹⁶, that is based on stochastic blinking of probes, but does not rely on single molecule localization. Instead, a high-order statistical analysis of the probes’ temporal fluctuation is performed on the data set^{16–22}. The method exhibits an attractive trade-off between achievable resolution and speed: Its resolution enhancement is not as high as in localization microscopies, but it can acquire and analyze the data much faster, and therefore it is suitable for SR imaging in live cells¹⁷.

Quite a few reports of SR imaging in live cells^{23–43} and even in live organisms^{44–47} have been published in recent years. Specific labeling of molecular structures of interest with SR-compatible fluorophores in live cells is still quite challenging. Since probes that are conjugated to antibodies are not suitable for work in live cells, specific targeting has therefore been mostly limited to genetic markers (requiring genetic manipulation). However, the photophysical properties of such probes are not necessarily optimized for SR. Much work is still needed to improve SR probes and to develop better methods for targeting them to structures of interest in live cells.

This perspective focuses on targeting cytosolic structures in live cells with stochastically blinking probes that are suitable for SOFI and some of the other SR methods (STORM/dSTORM, GSD, PALM/fPALM). We will discuss several approaches, including delivery and targeting of blinking quantum dots (QDs), delivery and targeting of permeable blinking dyes to genetically encoded tags, delivery and targeting of permeable fluorogens to genetically encoded activating peptides (FAPs), and genetically encoded blinking fluorescence proteins (FPs). We conclude with a discussion and future outlook.

Quantum dots (QDs)^{48,49} have gained prominence in fluorescence imaging applications due to their unique photophysical properties. Some of their drawbacks (such as size, toxicity, non-specific binding) have, however, hindered their wider dissemination. Another QDs’ attribute that has been considered to be a drawback is their blinking⁵⁰, a property that affects their brightness and undermines their performance in single molecule tracking experiments.

Blinking, though, could also be advantageous in some uses. Lidke et al. were first to take advantage of QDs’ blinking to achieve SR imaging by single molecule localization⁵¹.

However, since QDs' blinking is stochastic and is governed by a power-law statistics⁵⁰ it is not ideal for localization microscopy because it is difficult to control the number of QDs in the 'on' and the 'off' states in each movie frame as is done with photoswitchable probes. In contrast, QDs blinking is ideal for the SOFI method (Fig. 1)¹⁶. Due to QDs' high brightness and extraordinary photostability, long term acquisition can be achieved with low dose excitation light, possibly reducing phototoxicity in live cells. QDs could therefore serve as ideal probes for fast live cell SR imaging.

The big challenge is, of course, to label cytosolic structures in live cells with QDs. QDs conjugated to antibodies, streptavidin, and other small biomolecules or haptens have been successfully applied to fixed cells (immunocytochemistry) and to membrane proteins in live cells^{52–63}. Although many works report on successful targeting of QDs to cytosolic structures⁶⁴, this task is still very challenging. It requires an efficient delivery that escapes the endocytotic pathway, supplying freely diffusing QDs inside the cytosol. In addition, QDs should have minimal steric hindrance (i.e. small size) and minimal nonspecific binding so that they can find and specifically bind to their target(s). Extensive efforts have been vested in surface coating^{57,61,64–69}, labeling strategy^{52–63,70} and developing cytosolic targeting methods^{64,66,71–77}. As for the cytosolic delivery methods, they can be classified into two main categories: (i) Facilitated delivery strategies, using cell penetrating peptides^{76,77}, proton sponge polymer carriers^{74,75}, pinocytosis^{64,73,78}, and transfection reagents⁶⁴. Methods belonging to this category provide high throughput delivery, but suffer from low efficiency release of endosome-free QDs. As a result, most QDs still end up trapped in endosomes and provide background. (ii) Active delivery methods include electroporation^{71,72} and microinjection⁶⁶. Electroporation offers an efficient way of delivery by temporally destabilizing the plasma membrane to create transient pores using high voltage electrical pulses. However, this method causes low cell viability, aggregation of the payload and low uptake of large objects⁶⁴. The other active method, microinjection, is the most efficient and direct way to deliver QDs into the cytoplasm. For example, Lisse et al. delivered fluorescent nanoparticles to target intracellular structures through this technique⁷⁹. The delivery is through a sharp glass microcapillary tip (with a diameter < 0.5µm) that mechanically penetrates the cell membrane^{80,81}. However, the injection of QDs and QDs-protein conjugates is difficult and inconsistent due to QDs aggregation and repeated tip clogging. Recently we successfully delivered tubulin-QD conjugates into the cytoplasm of HeLa cell by the photothermal nanoblade^{82–84}. As shown in figure 2a, a green laser pulse was applied on a titanium coated glass capillary pipette tip that was positioned in a close proximity to the cell membrane. Surface plasmon absorption of the metal coating conducted heat into the local liquid medium, which in turn generated explosive vapor bubbles right next to the cell membrane. Those bubbles produced temporal destabilization of the membrane. Simultaneously, a pressure pulse was applied to the capillary, generating a transient liquid flow through the transient membrane pores into the cytosol. The injected QDs-tubulin conjugates (Figure 2b) were incorporated into growing microtubules in the cell and showed clear filamentous structure as shown in Figure 2c. The photothermal nanoblade delivery technique greatly reduces the mechanical damage to cells (compared to conventional microinjection) since the tip does not puncture the cell membrane. Most importantly, the relatively large size of the tip's orifice (2µm) enables it to deliver aggregation prone QDs-protein conjugate efficiently and consistently. The method suffers, however, from a low throughput (only one cell is injected at a time). Nonetheless, one can envision higher throughput delivery inspired by the photothermal nanoblade method.

The example shown in Fig. 2 gives a hint to future possibilities that could be opened-up by dynamic live cell SR imaging with blinking QDs. For this vision to materialize, smaller, brighter, more inert QDs, and higher throughput delivery and targeting methods, need to be developed.

QDs are not the only fluorescent probes that blink. In fact, all conventional organic dyes blink as well, due to intersystem crossing to a shelved triplet state or other dark states^{85–90}. STORM, dSTORM and GSD are based on these phenomena.

The Sauer group has extensively studied the mechanisms of dyes' blinking using a combination of reducing and oxidizing agents in the buffer (Reducing Oxidizing System or ROXS buffer)^{85,86,88}. These agents can greatly affect the radical anion and radical cation states of the fluorophore and hence modulate its blinking behavior.

Variants of ROXS buffers have been shown to work for Rhodamine, Cyanine and Oxazine dyes, covering the visible spectral range. However, for the localization microscopy methods, a stable off-state is required (e.g. the $\tau_{\text{off}}/\tau_{\text{on}}$ should be high enough for precise localization). In addition, independent tuning of ROXS buffers to achieve stochastic sparse blinking of multiple dyes simultaneously is not possible, making multicolor imaging very difficult. Of particular interest to this perspective, fine-tuning of the ROXS buffer and illumination intensity could generate blinking rates that are too fast or have an unfavorable $\tau_{\text{off}}/\tau_{\text{on}}$ ratio for localization microscopy, but are ideal for SOFI^{20,22}, perhaps even allowing multicolor analysis in spite of the differences in blinking dynamics.

Although ROXS buffers have been successfully applied to in vitro sample imaging, the effect of exogenous ROXS to living cells is yet unknown. Fortunately, the presence of the endogenous molecules glutathione and oxidized glutathione (GSH and GSSG) in the cells serves as an effective, natural, ROXS. Indeed, several recent STORM and dSTORM works report on dyes blinking in live cells^{30,33}. Fast blinking kinetics are essential for fast SR imaging. Blinking rates ($k_{\text{on}} = 1/\tau_{\text{on}}$ and $k_{\text{off}} = 1/\tau_{\text{off}}$) could be easily increased by increasing the lasers' intensities for excitation and photoactivation. This, in turn, however, could increase phototoxicity and cell death, and therefore is suitable only for short observation times. Alternatively, one could take advantage of the fact that concentrations of GSH and GSSG vary in different cell types, and even within organelles in the same cell, in order to tailor the required blinking behavior.

As in the case of QDs, synthetic dyes also need to first be delivered into the cytosol in order to target structures in live cells. However, the size and chemical form of dyes can make them cell permeable, simplifying the cytosolic delivery substantially. In addition, typical dye-adducts are constitutively monovalent (achieving a 1:1 labeling stoichiometry), simplifying analysis of labeling density and reducing the chances of valency-based biological perturbation.

One approach for dye targeting can rely on the large body of work invested in developing various cellular indicators. These are permeable dyes that spontaneously recognize and non-covalently bind cytosolic structures (and / or specific proteins in them). In particular, some commercially available plasma membrane dyes, mitochondrial-tracker, ER tracker, and lysotracker have been shown to blink and to perform well for STORM imaging⁴¹, and DNA-based dyes have been used to visualize nanostructures and chromosome organization in fixed cells¹⁵.

Dyes can also be specifically targeted using genetically encoded tags that are fused to proteins of interest, in conjunction with the expression of post-translation modifying enzymes that catalyze covalent bonding between the dye and the target⁹¹. Some of these approaches, like Halo-tag⁹², SNAP-tag^{93,94} and CLIP-tag⁹⁵ have even been commercialized. As an example, the SNAP-tag is engineered from the human repair protein O6-alkylguanine-DNA alkyltransferase (hAGT) that covalently reacts with benzylguanine derivatives. This tag protein can be genetically fused to any protein of interest. A dye labeled with benzylguanine recognizes the tagged protein and reacts with it to form a

covalent bond (Figure 3a). This and similar technologies were recently used for live cell STORM³³ and dSTORM³⁰ imaging. Similarly, we used the dye Dy505 to label the histone protein H2B that was fused to the CLIP tag to image chromatin in live cells by SOFI (Fig. 3b–3c, and to be published).

One disadvantage of tag-based technologies is the background due to non-specific binding. Sun et al.⁹⁶ elegantly designed a molecule in which both a fluorescent dye and a quencher are conjugated to the benzylguanine moiety. Upon labeling to the SNAP-tag, the quencher with the benzylguanine group is cleaved away, leaving behind the dye which is now covalently conjugated to the tag while liberating its fluorescence. Another disadvantage of tag-based technologies is the irreversible nature of the covalent bond formation. Once the dye is bleached, the tag site is no longer available and therefore the overall occupancy of dyes on the targeted structure is diminished over time due to photobleaching. The FAPs technology that is described next solves this problem.

It is important to note that synthetic dyes are smaller than QDs and FPs, an attribute that can be important in some applications (but the tags themselves have a molecular weight ‘penalty’ similar to that of a fluorescent protein). Dyes have moderate brightnesses and photostabilities in comparison to QDs. Finding bright dyes that blink well under natural physiology conditions in live cell and methods for their specific targeting will greatly assist live cell SR imaging.

Stochastic blinking of probes could be achieved not only by controlling their photophysical properties, but also through binding and dissociation of probes freely diffusing in solution onto/from stationary targets. During the brief immobilization period, the diffused and dim signal of a mobile probe converts into a stable, high signal coming from a spot that can be localized with good accuracy. Furthermore, the quantum yield of the probe could increase during immobilization due to its rigidification when bound to the target. Blinking in this case is achieved by controlling the ‘on’ and ‘off’ rates of the probes to their targets. The localization microscopy method dubbed ‘point accumulation for imaging in nanoscale topography’, or PAINT, is based on this concept¹⁴.

In PAINT, normally quenched, freely diffusing probes brighten-up when they bind to their targets. This brightening enhances the contrast and signal-to-noise ratio. Fluorogen activating peptides (FAPs)^{97–99} are a realization of the PAINT concept that affords specific molecular targeting (in contrast to traditional approaches that are selective for chemical environments such as membranes or DNA)^{14,15}.

FAPs are genetically encoded tags that noncovalently interact with weak (quenched) fluorophores (fluorogens) to activate bright fluorescence upon binding. The equilibrium process of binding and unbinding can be exploited to achieve stochastic blinking at the receptor (FAP) sites, where relative rates of binding and dissociation result in cycles of activation and de-activation at the single molecule level. The duty cycle of such protein-dye complexes can be controlled by the concentration of free dye and the rates of bleaching and/or dissociation of the protein-dye complex. Feasibility of this concept for SOFI imaging is shown in Fig. 4, where blinking frames were generated by labeling a fixed cell that expressed a FAP-actin fusion construct with a low concentration of fluorogen in vitro.

Current FAPs are based on single chain fragment (scFv) antibody molecules. Several cell permeable, normally dim dyes, such as malachite green, have been shown to reversibly bind, brighten-up, and dissociate (therefore ‘blink’) from the corresponding scFv^{99,100}. scFv molecules have shown to be effective labels for the cell surface, and with suitable modifications can also function in the cytosol of living and fixed cells¹⁰¹.

Alternative affinity molecules and paired permeable dyes that function in the cytosol need to be developed and optimized in order for this concept to work well in live cells. The ability to control the equilibrium properties by adjustments of dye concentration makes this approach attractive for multicolor labels. Because kinetic properties control blinking rates, it may be more practical to generate stochastically blinking families of FAP-fluorogen pairs, than it would be to generate these under photoswitching or ROXS mediated blinking control typical of other SR imaging protocols.

Difficulties in delivery and targeting of QDs and synthetic dyes are all eliminated for fluorescent proteins (FPs), which are easily and specifically targeted to cytosolic structures (or proteins) via genetic manipulations. Mutations in the original green fluorescence protein (GFP) have led not only to a large color pallet of FPs, but also to the development of photoactivatable (or photoswitchable) FPs (PA-FPs)^{102–107} which are at the foundation of the localization microscopy techniques^{8,9}.

PA-FPs can be switched from a dark state or a given wavelength fluorescence state to a different wavelength fluorescence state upon irradiation with a specific wavelength ‘switching’ laser which is usually different from the one used for fluorescence imaging. Adjusting the intensities of the two lasers could result in stochastic blinking of the FPs. For some of the PA-FPs, however, stochastic blinking could be achieved with a single wavelength source. Many options in colors and switching behaviors have been developed. Among them, the reversibly switchable FPs (rsFPs) have shown to be of great utility. For example, structured illumination microscopy (SIM)¹⁰⁸ of nuclear pores and actin filaments in live cells labeled with the rsFPs Dronpa exhibited nonlinear response at relatively low intensity excitation, resulting in enhanced resolution at favorable live cell imaging conditions¹⁰⁹.

Dedecker et al. have recently demonstrated dual-color SOFI live cell imaging using the rsFPs Dronpa¹⁰⁴ and rsTagRFP¹¹⁰. We have also used Dronpa labeling and a low dose LED excitation lamp in a simple wide-field fluorescence microscope to image the focal adhesion proteins FAK, zyxin and Hic6 in live HeLa cells (Fig. 5). The combination of a genetic marker (Dronpa in this case), a simple lamp-based microscope, and a simple computational algorithm (SOFI) greatly simplifies SR imaging and makes it more affordable.

rsFPs suffer, however, from poor photostability and low blinking rate, limiting the speed and duration of the data acquisition. With better insight into their protein structure and blinking mechanism, more stable rsFPs have recently been developed^{111,112}. Grotjohann et al. engineered novel rsFPs dubbed ‘reversibly switchable enhanced green fluorescent proteins’ (rsEGFP and rsEGFP2). When compared with Dronpa, rsEGFP displayed an exceptional photostability (> 1000 on-off cycles) and fast blinking¹¹². Using RESOLFT microscopy, they were able to image keratin and dendritic spines labeled with rsEGFP in live cells with a resolution of 40nm and minimal phototoxicity. Since rsEGFP and rsEGFPs can work either in a photoactivation mode or in a stochastic blinking mode, it is likely that they will preform well with other SR methods.

Traditionally, FPs have been optimized for high brightness and good photostability. With the advent of SR methods, several FPs have been evolved to display favorable photophysical properties for SR. Since they offer such an easy way to specifically target and label cytosolic structures of interest, more work is needed to allow specific tailoring of attributes to specific SR methods.

Various SR techniques have their own advantages and limitations. None of them can exhibit at the same time the highest spatial and the highest temporal resolutions and therefore a trade-off has to be tailored for a specific application. Dynamic SR imaging in live cells

could be further improved by: (i) improving the photophysical properties of the probes. Brighter probes will allow longer observations with reduced phototoxicity and will result in better signals. Faster blinking will allow faster SR imaging; (ii) improving the delivery and specific targeting of the probes. Specific labeling reduces the background and enhances the image contrast; (iii) improving the speed and sensitivity of the cameras used for acquiring SR data, allowing faster SR imaging and better signals.

This perspective mainly focused on points (i) and (ii) by discussing several delivery and targeting approaches for dyes, QDs and FPs. Promising live cell dynamic SR imaging results have already been demonstrated with the various probes and delivery methods discussed above. However, long-term live cell SR imaging with both high spatial and temporal resolutions is still quite challenging. Luckily, there is ample of room for improvements. It is likely that better understanding of the probes' structures and photophysical properties will lead to significant enhancements in their performance.

As for QDs, a major challenge is to develop efficient and high throughput endosome-free delivery methods⁶⁴. The thermal nanoblade described here may provide inspiration for developing a transfection reagent that is based on plasmonic nanoparticles together with wide-field pulsed NIR illumination. Better control over QDs surfaces will enhance their photophysical properties and reduce non-specific binding. Thin coating will reduce their steric hindrance and increase targeting efficiency into crowded environments (such as organelles).

As for synthetic organic dyes, development of a larger pallet of bright and stable permeable dyes and the development of smaller molecular weight tags that act together with enzymes to catalyze conversion to covalent bonding (such as Sortase¹¹³, Formylglycine generating enzyme¹¹⁴, Transglutaminase¹¹⁵ and others) are needed. For long-term SR time-lapse microscopy, affinity binding such as PAINT and FAPs is an attractive approach for minimizing ill effects of photobleaching. This approach requires the development of a pallet of permeable fluorogens that are normally quenched, but brighten-up upon binding to their targets. These targets should consist of small molecular weight scaffolds that specifically bind and brighten-up the fluorogens. Molecular evolution techniques coupled to a photophysics-based screening assay could be used to evolve additional and/or more efficient such fluorogen-tag pairs with the desired specificities and association and dissociation rates.

As for FPs, intense effort is currently being conducted to evolve brighter, longer-lived photoswitchable FPs. Molecular evolution with photophysics-based screen will be very useful here too, and in particular for the evolution of spontaneously blinking FPs. Development of hybrid approaches could also provide powerful solutions. For example, Izeddin et al. have recently fused PA-FP to Lifeact, a weak actin binder¹¹⁶. The FP-Lifeact fusion reversibly binds and unbinds actin, allowing for the long-term PALM imaging of dendritic spines' cytoskeleton.

The field of live cell SR imaging is at its infancy. Many more exciting technological advances are anticipated. Some of these advances will be along the lines presented in this perspective. Unexpected advances are also very likely for this fast-moving field. It is going to be a fun ride.

Acknowledgments

We thank Atsushi Miyawaki for the free gift of Dronpa constructs, Pei-Yu Chiou and Michael Teitell for assistance with thermal nanoblade delivery. This work was supported by NIH grant #5R01EB000312 and NIH grant #1R01GM086197 and NIH grant # 1R01GM086237.

Biographies

Jianmin Xu received his Ph.D in Chemistry from University of Miami in 2008. He is currently a postdoc scholar in the Department of Chemistry and Biochemistry at UCLA. His current interests include nanoparticle surface modification, bioconjugation, cellular fluorescent labeling and optical super resolution microscopy.

Jason Lin Chang is an undergraduate Biochemistry major at UCLA, performing research in the Weiss Lab. He studies photophysical properties of fluorescent dyes and fluorescent proteins and optimal conditions for super-resolution imaging. He is a recipient of the Bruce Merrifield Undergraduate Research Award at UCLA.

Qi Yan received her Ph.D in department of Biological Sciences at Carnegie Mellon University under mentoring of Marcel Bruchez in 2012. She is currently a Scientist at Sharp Edge Labs Inc. During her PhD study, she focused on characterizing the Fluorogen Activating Peptide (FAP) and fluorogen system and developed FAP-based fluorescent probes for performing super-resolution imaging and studying GPCR trafficking.

Thomas Dertinger received his PhD in Physics and Physical Chemistry at University of Cologne, Germany. He was a post-doctoral scholar in the Department of Chemistry and Biochemistry at UCLA from 2007–2011. During this time he developed SOFI. He is the founder of SOFast GmbH in Germany and currently puts his focus on intellectual property management.

Marcel Bruchez is an associate professor in Department of Chemistry and the Department of Biological Sciences at Carnegie Mellon University. He received his PhD at the University of California, Berkeley in Physical Chemistry in 1998. His research group focuses on developing new tools for specifically labeling biological targets in living cells and organisms with bright, stable fluorescent molecules, and delivery of active-sensing molecules to specific cellular sites. Prior to joining the faculty at Carnegie Mellon University in 2006, Marcel was the co-founder of Quantum Dot Corporation. His development of quantum dots as biological detection was recognized as one of the Top Ten Scientific Innovations of 2003. He was a TR100 awardee in 2004 and received the Lord Rank Prize for Optoelectronics in 2006 for the realization of quantum dots for biological labeling. He is also the founder and Chief Scientific Officer of Sharp Edge Laboratories.

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Shimon Weiss is professor in the Department of Chemistry and Biochemistry and the Department of Physiology at UCLA. His lab has been working on ultrasensitive single molecule spectroscopy methods and applications. They were the first to introduce the single molecule FRET method and together with the Alivisatos group they were the first to introduce quantum dots to biological imaging. Shimon received his PhD from the Technion in Electrical Engineering in 1989. His postdoctoral training was in AT&T Bell Laboratories. He was a staff scientist at Lawrence Berkeley National Laboratory between 1991–2001. In 2001 he joined UCLA. Shimon is the Dean M. Willard Chair and a full Professor in the Department of Chemistry & Biochemistry. He is a fellow of the Optical Society of America, and received the 2001 Michael and Kate Barany Biophysical Society Award, the 2006 Rank Prize in opto-electronics, and the Humboldt Research Award in 2012.

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Fig. 1.

(A) A wide-field image of the microtubule network of a fixed 3T3 cell immunostained with QD800; (B) A second-order SOFI image of the same cell as in (A); (C) A Fourier reweighted second-order SOFI image of the same cell as in (A); Scale bar: 10 μm . Lower panel: Cross-sections as highlighted in (A) and compared for (A), (B) and (C): (A) in black, (B) in blue and (C) in red. (From ref 21, Reprinted with permission of the Optics Society for Optics Express.)

Fig. 2.
 (A) Photothermal nanoblade delivery of tubulin-QD conjugates into the cytosol; (B) Scheme for tubulin-QDs conjugation; (C) Wide-field image of a live HeLa cell after photothermal nanoblade delivery of tubulin-QD conjugates; (D) Zoom-in of boxed area in (C). Scale bar: 8 μm . (From ref 82, Reprinted with permission of the American Chemistry Society for Nano Letters.)

Figure 3.

(A) Scheme for snap-tag conjugation with a Benzylguanine dye; (B) A wide-field image of dye505 labeled H2B clip tag in a live HeLa cell; (C) Second-order SOFI image of the cell in (B). Scale bar: 5 μm .

Fig. 4. (A) A wide-field image of a malachite green ester analog dye bound to MG-FAPs fused to human β -actin in formaldehyde fixed and permeabilized HeLa cells; (B) Corresponding second-order SOFI image of the network in (A).

Fig. 5. Wide-field images of Dronpa fused to focal adhesion proteins (A) FAK, (C) Hic5 and (E) Zyxin in live HeLa cells; Corresponding second-order SOFI images are shown in (B), (D) and (F). Scale bar: 6 μm .