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Review

Advancing cell biology with nanoscale fluorescence imaging: essential practical considerations

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Recently, biologists have gained access to several far-field fluorescence nanoscopy (FN) technologies that allow the observation of cellular components with ~20 nm resolution. FN is revolutionizing cell biology by enabling the visualization of previously inaccessible subcellular details. While technological advances in microscopy are critical to the field, optimal sample preparation and labeling are equally important and often overlooked in FN experiments. In this review, we provide an overview of the methodological and experimental factors that must be considered when performing FN. We present key concepts related to the selection of affinity-based labels, dyes, multiplexing, live cell imaging approaches, and quantitative microscopy. Consideration of these factors greatly enhances the effectiveness of FN, making it an exquisite tool for numerous biological applications.

Highlights

Here, we provide an overview of the key factors to consider when using fluorescence nanoscopy (FN), including biological questions that can be addressed and aspects that might improve the reliability and effectiveness of FN experiments.

We cover the main aspects related to sample preparation, including the selection of appropriate fixation, affinity-based labels, and fluorescent dyes.

We discuss current limitations and possible future developments in the field that would facilitate a broader application of FN.

We discuss multiplexing possibilities (allowing the simultaneous detection of multiple targets in a single experiment), live cell imaging for the study of cellular and molecular dynamic processes, and quantitative workflows.

Revealing the biological world at the nanometer scale with FN

Conventional fluorescence microscopy, including widefield and confocal microscopy, has been essential for studying the morphology and composition of various cellular organelles and the localization of molecules. The **resolution** (see Glossary) of these techniques, usually above ~200 nm, is a limit for the study of macromolecular arrangements and nanoscale structures. The study of biological processes with nanoscale resolution (<20 nm) is a major step forward for microscopy, because it bridges the world of subcellular biology with that of macromolecules. This effort has been greatly facilitated in recent decades by the use of electron microscopy (EM), which provides exquisite morphological information at the molecular level [1] at the expenses of limited molecular identifications and lack of live cell applicability. These limitations are overcome by fluorescent microscopy, the achieved resolving capabilities of which are progressively reaching those attained by EM.

Several recent approaches based on FN enable researchers to address questions at <20 nm that were difficult to answer with classical fluorescence microscopy. Paradigmatic examples of FN applications include the characterization of the periodicity of actin rings and synaptic sites in neurons, the structure of nuclear pore complexes, the maturation of viral particles, the organization of mitochondrial cristae, the mechanisms of apoptosis, and the functioning of signaling pathways [2]. Recent FN studies have reached extremely high resolutions [3] and even enabled researchers to follow molecular events such as the stepping behavior of kinesin *in vitro* and in cells [4,5] and to render the structure of individual molecules, such as GABA receptors, with a level of detail almost comparable to that of cryo-EM [6].

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In this review, we present a compendium that summarizes practical aspects to keep in mind for harnessing the power of nanoscopic imaging in the field of cell biology. By bridging theory and practice, we provide a roadmap for researchers, equipping them with the essential know-how to successfully navigate the intricacies of implementing, executing, and deriving meaningful data from FN experiments. The design of FN experiments starts from the selection of the most suitable microscopy technique, each with its own specific limitations and strengths (Figure 1A; for recent reviews of the selection of different microscopy approaches, see [7,8]).

Two major far-field fluorescence microscopy strategies are currently able to reliably provide <20-nm resolution in biological samples: camera-based single-molecule localization microscopy (SMLM) [9] and minimal photon fluxes (MINFLUX) [10]. Based on the mechanisms utilized to perform the on–off switching of the **fluorophores** required to obtain a super-resolved image, camera-based SMLM technologies have different names; for example, stochastic optical reconstruction microscopy (STORM), photoactivated localization microscopy (PALM), and DNA-points accumulation for imaging in topography (DNA-PAINT) [11]. In addition to the above two strategies, depending on the exact imaging settings, stimulated emission depletion (STED) [12] can achieve resolutions of ~50 nm and below, although the power of the depletion laser required for FN applications is usually not compatible with conventional biological samples. In addition to these technologies, expansion microscopy is a sample preparation approach aimed at enlarging the sample, which can then be imaged using either conventional diffraction-limited or nanoscopy approaches [13]. With the exception of expansion microcopy, FN technologies are compatible with living specimens, opening the avenue to the understanding of molecular dynamics in native conditions (Box 1). Overall, these four microscopy technologies have the potential to uncover as-yet unexplored biological aspects with exquisite detail. At the same time, their high resolving capability requires the use of sample preparation protocols that limit the introduction of artifacts and the use of labeling tools that have minimal **linkage errors**.

Is nanometer-scale imaging always the preferred option?

The short answer to this question is 'no', because not all biological problems require molecular resolution to be solved. Careful consideration should be given to whether FN is not necessary for some biological questions. For example, FN is not required to determine whether a protein of interest (POI) is localized to lysosomes or mitochondria, but would be required to determine whether a mitochondrial POI is in the outer mitochondrial membrane or the inner mitochondrial matrix, which are ~20 nm apart.

In general, the strength of FN is that it provides increased precision in the localization of individual biomolecules compared with diffraction-limited imaging (Figure 1B,C). A simple rule of thumb that can be used to decide whether FN is needed is to understand whether spatial information in the order of tens of nanometers allows the formulation of fundamentally different biological hypotheses for the process under investigation. At the same time, serendipitous observations were made and, in some cases, FN revealed new structures that were not observable at lower resolutions [14]. For this reason, exploratory experiments should be considered.

Challenges in FN: a brief overview

Very high-resolution imaging is informative, but aspects that could be ignored in conventional fluorescence microscopy become challenges in FN. Live FN would be the ultimate goal in biological studies, but it is still difficult to achieve due to the high **phototoxicity** of several FN technologies (Box 1). The field of FN is in its early stage and it is foreseeable that relevant advances will be made in the near future. At the same time, FN approaches in fixed samples are already changing the biology field; therefore, we concentrate here on fixed specimens (Figure 1D–F).

In an experimental workflow for 'non-live' FN, the sample must first be fixed and the sample preparation must be optimized to avoid artifacts. After fixation, the FN approach requires the use of **affinity-based labels**, such as antibodies, to reveal molecular identities and position the reporter fluorophores in their proximity. To obtain relative or even absolute biologically relevant numbers from FN images (quantitative FN), further measures and considerations must be taken. In Box 2, we review the key issues to be considered when performing quantitative imaging at the nanoscale and explain why **labeling density**, imaging tool stoichiometry, and linkage error are key aspects in this context. Finally, in Box 3, we explain why there is not a single fluorophore that could perform best in all conditions.

Essential aspects to consider to optimally fix a sample and prepare it for labeling

At the beginning of **sample labeling**, the fixation process should preserve the structure of the specimen reflecting as closely as possible the state of the living sample. However, this procedure is prone to the introduction of artifacts. While this is true for both conventional imaging and FN [7], artifacts can be observed when using FN that would be otherwise undetected at lower resolutions [15]. Artifact examples include membrane loss, extraction or blebbing (which might interfere with organelle identification), organelle fusion, relocalization of proteins, and changes in macromolecular complexes (Figure 1D). Fixation can be chemical or thermal. Chemical fixation usually relies on aldehydes (e.g., formaldehyde commonly referred to as PFA, glutaraldehyde, and glyoxal [16]), alcohols (e.g., ethanol and methanol), ketones (e.g., acetone), and acids (e.g., acetic acid). These chemicals crosslink or precipitate biomolecules, thus keeping them 'fixed' in place. Chemical fixation is not instantaneous and enzymatic reactions can still occur for several minutes to hours. Furthermore, remaining reactive groups must be quenched (e.g., with ammonium chloride, glycine, or sodium borohydride when fixing with aldehydes). A universal fixation protocol is not available, because fixatives that are optimal in certain conditions are suboptimal in a different context [16]. For example, 4% PFA is commonly used because it provides sufficient morphology and epitope preservation. However, it requires time to work; although the addition of 10% ethanol can speed up its penetration, it may affect organelle morphology. By contrast, cold methanol fixation is commonly used for some cytoskeletal components but does not preserve cell membranes. Glutaraldehyde and glyoxal often provide better sample preservation, but they might also modify target epitopes, reducing their immunogenicity and decreasing the activity of affinity-based labels. Moreover, each fixation method might lead to different artifacts in different contexts and might be more or less suitable for preserving certain molecular structures [17]. Therefore, for optimal results, it is advisable to test different fixatives for each target structure or model system used.

Low-temperature fixation (cryofixation) using high-pressure freezing, which instantly vitrifies molecules in their native state, is a clear way to minimize artifacts. Cryofixation has been used in the field of EM for decades [18]. The use of cryofixation in FN is a clear current trend [19], and we anticipate that the community will continue to work on implementing simpler and refined fixation methods to increase reproducibility.

Depending on the FN technique applied downstream after fixation, sample preparation might be very different. First, one should keep in mind that samples are noticeably autofluorescent in various wavelengths, and negative controls without fluorophores might be required. Second, membrane permeabilization steps will always extract lipids and cavitate cellular membranes and organelles. The detergents used for this purpose will also extract membrane proteins and compromise the overall nanomorphology of the sample. To avoid this step, smaller or membrane-permeant affinity-based labels can be used (Table 1). Third, due to poor fixation, a fraction of biomolecules might remain mobile, especially in the case of membrane-associated proteins [20]. As a consequence, protein localizations might be different from

Glossary

Affinity-based label: molecule that binds with high specificity to a target molecule (e.g., a protein of interest). Affinity refers to the strength of the binding.
Fluorogenic dye: fluorophore, the fluorescence of which increases upon binding to the target structure.
Fluorophore: molecule, or a part thereof, that can absorb light at a specific wavelength and re-emit it at a longer wavelength. Here, 'fluorophore' is also referred to as 'reporter'.
Labeling density: number of fluorophores in a defined volume. To increase the labeling density, multiple affinity-based labels for different target regions could be used.
Labeling efficiency: ratio between the labeled sites and the total number of available sites.
Linkage error: displacement of the detected fluorophore relative to the actual locations of the target structure; depends on the labeling strategy and the size of the affinity-based label.
Live cell imaging: visualization of the molecules of interest in a living sample, generally aimed at detecting morphological changes in the structure of interest while imaging molecular ensembles.
Localization precision: reflects the uncertainty in estimating the position of a fluorophore and is usually used in single-molecule localization microscopy (SMLM) techniques.
Multiplexing: detection and imaging of multiple targets or molecules within a single sample.
Photostability: ability of a fluorophore to retain its fluorescence properties over prolonged imaging periods, ensuring consistent signal detection.
Phototoxicity: harmful or undesirable effects of absorbed light in living cells or organisms, compromising the viability of the sample.
Quantum yield: ratio of the number of photons emitted to the number of photons absorbed. This measure correlates to the fluorophore brightness.
Resolution: in imaging, shortest distance between neighboring objects that can be resolved in an image. In practice, it is often approximated by the full width at half maximum (FWHM), which is defined as the width of an intensity profile curve perpendicular to the structure at half the maximum value. FWHM

physiological distributions and the use of multivalent probes (i.e., antibodies, streptavidin, and functionalized nanoparticles) might promote the artifactual clustering of their targets that do not form nanoclusters in a native situation [21]. To overcome probe-induced clustering, monovalent affinity-based labels might be used [22], as could more thorough fixation protocols [16,20]. To ensure that sample preparation reflects a quasi-native state, it is advisable to perform controls at intermediate steps to check sample preservation, using live cell conditions as an ideal reference (Figure 1D).

To maximize the **signal-to-noise ratio (SNR)**, especially in the case of low-abundant targets, an appropriate strategy for blocking unspecific signal should be put in place. This step minimizes the unspecific interactions of the desired probe and classically relies on animal sera or proteins derived from them, such as albumin fractions, when performing protein-based labeling.

At the end of the sample preparation workflow, the labeling step of the relevant targets is performed. This is a crucial step since the fluorophores are the molecules that ultimately will be detected under the microscope, and is extensively addressed below.

depends on the linkage error and the resolving power of the microscope.
Sample labeling: procedure involving the use of affinity-based label and fluorophores utilized to reveal (and distinguish) a target molecule/structure. Operationally, this procedure involves the complete sample preparation steps comprised between fixation and imaging.
Signal-to-noise ratio (SNR): ratio of the desired signal (or fluorescence intensity) to the background noise in an imaging experiment. A high SNR leads to improved resolution, image quality, and accuracy.
Stokes shift: difference in wavelength between the maxima of absorption and emission spectra of a fluorophore.

Revealing the targets of interest at the nanoscale: underlying principles

The labeling strategy aims to maximize the **labeling efficiency** while keeping the linkage error to a minimum. Indeed, some techniques can resolve two fluorophores at distances of a few nanometers and have the capacity to visualize each molecule in the sample. The use of bulky affinity-based labels (e.g., antibodies) positions the reporter fluorophores up to ~15 nm away from the target structure and hinders the recognition of neighboring epitopes [23]. Thus, the probe revealing the target of interest should be small enough to increase the labeling density and have only a minimal linkage error between the target and the detected fluorophore (i.e., maximizing the accuracy obtained from FN imaging). Smaller probes also diffuse more easily through the sample and, therefore, tend to find more target molecules, especially in crowded regions or difficult-to-access structures [24]. An exception where the linkage error and epitope masking caused by the probe are neglectable is expansion microscopy, when labeling is performed after the physical expansion of the sample [25].

Besides providing high labeling efficiency and minimal linkage error, the ideal probe (Figure 2) should be easily soluble in aqueous buffers and offer an exquisite specificity and affinity to the target, which in turn minimizes background signal and results in better SNR. Innovative developments for minimizing background are **fluorogenic dyes** [26]. For quantitative imaging, it is also desirable that the readout from a labeling strategy accurately represents the number of target molecules. For this, probes binding a single target molecule (monovalent) and bearing a controlled number of fluorophores are preferred. The ideal stoichiometry for quantitative imaging is to decorate single target molecules with only one fluorophore (Figure 2A). Lastly, strategies to reveal targets of interest requiring genetic manipulation of the sample should impose minimal perturbation of the system physiology. For example, strong overexpression of a protein may affect its localization or the stability of its interaction partners. The development of improved and new labeling strategies for FN is an active field of research and an extensive list of labeling approaches is summarized in Table 1.

Labeling strategies: basic considerations and available tools

Biology relies on diverse biomolecules to function, including proteins, lipids, sugars, and nucleic acids. Developing FN-compatible labeling approaches that enable imaging of all these biomolecular types is a primary goal in the field. However, the fluorescent labeling of lipids and sugars has not been as straightforward as the detection of nucleic acids or proteins.

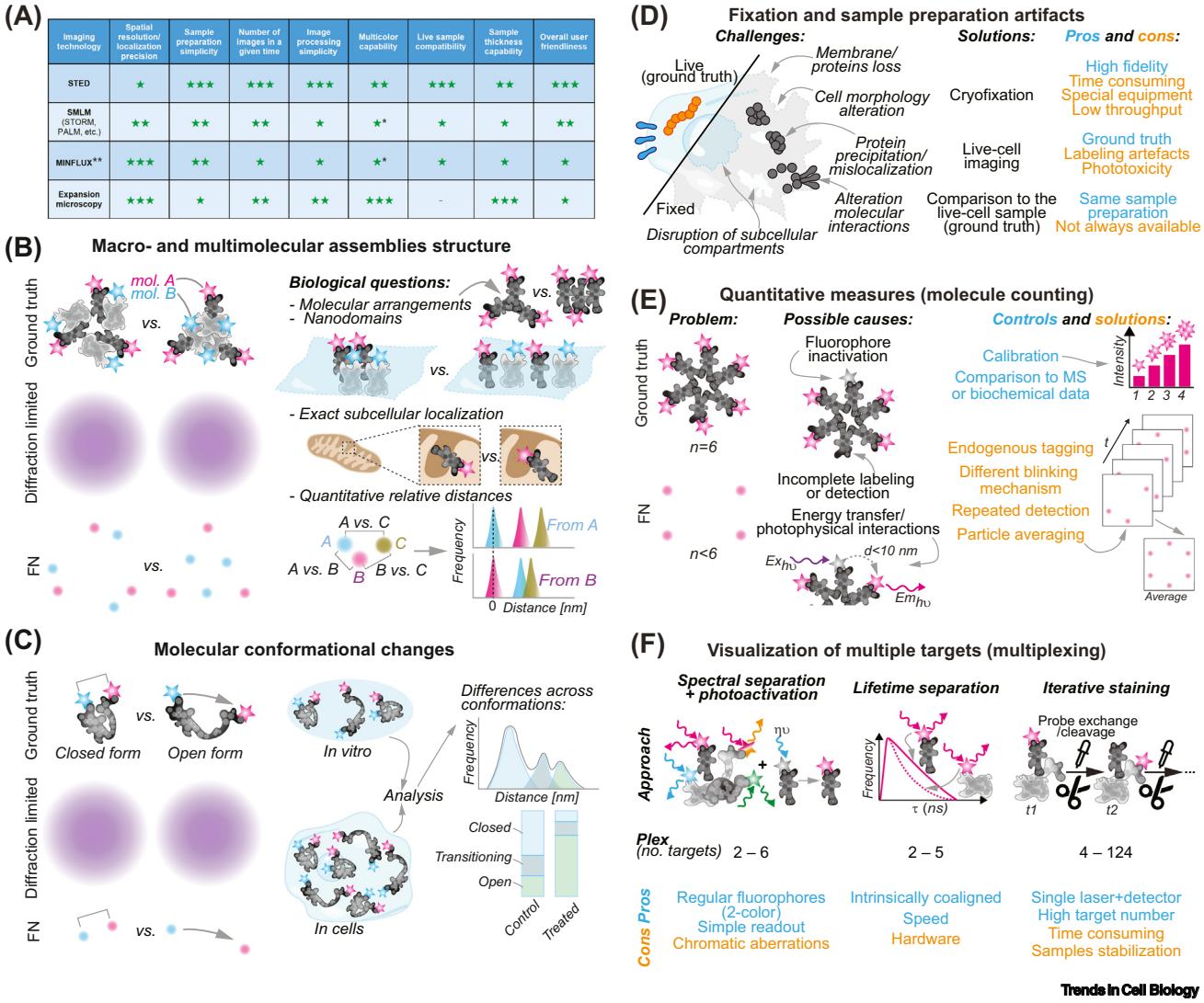

Figure 1. Fluorescence nanoscopy (FN) at a glance: techniques, applications, and challenges. (A) A visual guide to imaging strategies to achieve FN. Stars represent arbitrary rating. Note that in confocal-based methods [stimulated emission depletion (STED) and expansion microscopy], the term 'spatial resolution' is used to define the minimum resolvable distance between neighboring objects, while in single-molecule localization approaches [single-molecule localization microscopy (SMLM) and minimal photon fluxes (MINFLUX)], the term '**localization precision**' is used. *SMLM/MINFLUX microscopy using reversible binders or DNA-points accumulation for imaging in topography (DNA-PAINT) can provide substantial benefits in terms of multiplexing. **For the purpose of this review, only MINFLUX imaging (not single-molecule tracking) is considered. (B,C) Possible applications of FN. (B) Study of the structure of macromolecular or multimolecular assemblies and (C) quantitative analysis of molecular conformers. From left, the schemes represent: (i) the ground truth (the actual molecular arrangement); (ii) the imaging output with a light diffraction-limited approach; and (iii) how the fluorophores would be separated by FN. Panels on the right describe the applications in more detail. (D–F) Challenges of, and opportunities for, FN. (D) Schematic of fixation and sample preparation challenges, with artifacts and possible solutions. (E) Schematic of challenges in quantitative measures. Left: expected versus observed signal; middle: reasons for the reporter loss; right: controls and alternative approaches. (F) Schematic of approaches that can be used for the visualization of multiple targets of interest in a sample. Abbreviations: MS, mass spectrometry; PALM, photoactivated localization microscopy; STORM, stochastic optical reconstruction microscopy.

The main approach used to reveal proteins in chemically fixed samples is indirect immunofluorescence (IF) using primary antibodies binding the POI followed by signal amplification using fluorescently conjugated secondary antibodies. Antibodies are preferred because of their specificity, large target diversity, and commercial availability. One caveat for FN is that antibodies are large

Box 1. Nanometer-scale fluorescent imaging in living cells: are we there yet?

FN **live cell imaging** visualizes entire cellular structures over time as a 'whole', allowing detection of morphological changes, and is recommended for organelles such as the endoplasmic reticulum, in which conventional chemical fixation often introduces artifacts that are visible in FN [19]. In our opinion, at least four connected aspects limit the implementation of live FN applications as detailed herein.

Phototoxicity

Phototoxicity arises when the absorbed light generates free radicals and reactive oxygen species, ultimately causing genomic damage, mitochondrial stress, and protein degradation (see Figure 1D in main text). Light might be absorbed by both endogenous molecules or the fluorophores and can additionally increase the local temperature in the sample. To limit some of these effects, combinations of image reconstruction strategies, adaptive illumination approaches [75], and event-triggered approaches are emerging [76]. Furthermore, novel fluorophore classes of self-blinking dyes or red photoswitchable proteins exist, which do not require blue light and can be excited with lower light doses [77].

Low image acquisition frequency

Low image acquisition frequency is often required to increase resolution, but compromises the interpretation of fast biological processes. In SMLM, a limiting factor is the localization of a sufficient number of molecules, while, in scanning-based approaches, the limiting steps are the brightness of the fluorophore and size of the field of view. Reducing the field of view or the time spent on each pixel (dwell time) can speed up the imaging, although at the expenses of decreased contextual information and SNR. FN will strongly benefit from parallelization and the use of deep learning to improve temporal performances.

Availability and impact of labeling on living cells

The availability and impact of labeling on living cells cannot be ignored. Some probes are drugs that bind to their target with high affinity, often interfering with the physiology of the targeted molecule (e.g., phalloidin). An alternative is to use genetically encoded tags or, even better, minimal tags combined with genome-editing approaches [78] (see Table 1 in main text).

Imaging depth and large field of view

Imaging depth and a large field of view are important because many biological samples are not single cell monolayers. Although its feasibility specialized has been demonstrated [79], imaging deeper than 10–50 μm in both SMLM and STED methods is challenging. The field would benefit from the use of engineered illumination, adaptive optics, image restoration algorithms, and multiphoton excitation.

Ultimately, the best FN technology for live cell imaging must be selected based on the experimental setup and the precise biological question being addressed, and it is essential to include controls for possible phototoxic effects, such as conditions that are present that might cause damage to biological processes but are not considered. This should be done by using settings that cause less perturbation of biological conditions and can be achieved by troubleshooting the selection of appropriate labeling strategies and imaging conditions. For example, far-red light commonly used in the STED depletion laser is less phototoxic compared with the 405-laser used for SMLM at the same irradiance.

molecules and, in indirect IF, the primary–secondary antibody sandwich increases the distance between the target and the fluorophores up to ~30 nm. Due to their smaller sizes, antibody fragments, such as Fab fragments or camelid single-domain antibodies (nanobodies), provide an advantage in FN (Figure 2B). Nanobodies can be designed to carry a defined number of fluorophores, an advantage for quantitative imaging. Unfortunately, the discovery and validation workflow that leads to a well-performing nanobody is not yet as straightforward and, thus, the catalog of primary nanobodies available remains limited. A practical alternative to this issue is using an IF approach where primary antibodies are revealed by secondary nanobodies. This results in a smaller linkage error than when using polyclonal secondary antibodies. Moreover, due to the monovalency of nanobodies, this strategy allows one-step IF by premixing primary antibodies with secondary nanobodies, eliminating the need to use primaries of different species for **multiplexing** [22].

Another strategy to image proteins is to introduce a gene to express the POI carrying a modification that can be revealed with fluorescence. This can be achieved by adding a tag to the POI,

Box 2. Quantitative FN: absolute versus observed number of molecules

Quantitative FN requires that the number of detected fluorophores matches (or, more precisely, correlates) with the real number of target molecules in the sample. Therefore, prerequisites for quantitative FN are a high and controlled labeling efficiency and the ability to detect all fluorophores.

A high and controlled labeling efficiency is best achieved with monovalent affinity probes carrying a single (or at least a fixed number of) fluorophore reporting it (see Figure 2A in main text). Moreover, probes that utilize covalently linked labeling strategies that ensure that the targets are stably labeled with a single reporter should be preferred. The use of multivalent polyclonal reagents stochastically labeled with fluorophores (e.g., secondary antibodies carrying approximately one to six fluorophores) should be avoided since the correlation between the number of reporters and the target molecules might be inconsistent.

Even in the ideal case in which all target molecules are decorated with an affinity-based label, the possibility exists that only some of the fluorophores decorating the affinity-based label are functional. Indeed, fluorophores might be inactivated, damaged, or not detected during the imaging procedure (see Figure 1E in the text). Detecting all single fluorophores in densely labeled samples is challenging, a problem known as fluorophore crowding. When the distances between fluorophores are on a single-digit nanometer scale, photophysical interactions occur, resulting in undesired alterations of fluorescence properties. For example, this happens between two fluorophores on the same structure, either by Förster resonance energy transfer (FRET) or even H-dimer formation if the two fluorophores are separated by molecular-scale distances [80]. Notably, interactions between fluorophores have been reported for fluorophores decorating an antibody [81], with one fluorophore serving as a 'super emitter' while others remain in the dark state. Some technologies, such as expansion microscopy, allow fluorophore crowding to be reversed, by physically creating a distance between molecules. Other technologies, such as DNA-PAINT, deal with the problem differently by modulating the concentration of the imaging probe or using light-controllable fluorophores [82] (see Figure 2D in main text).

While relative quantification is more easily achieved, absolute molecule-counting approaches have been proposed for several approaches and a comprehensive review has recently been published on the quantification challenge [83]. Importantly, all these methods need calibration to be benchmarked against known markers or biochemically (e.g., by quantitative western blot, liquid chromatography, or mass spectrometry).

which is fluorescent *per se* or can interact with an exogenous fluorophore or affinity probe reversibly or covalently. Fluorescent proteins (FPs), with GFP as a pioneer, are the most frequently used genetically encoded fluorescent molecules for tagging a POI. However, the **photostability**, brightness, and spectral properties of FPs are limited, encouraging scientists to develop other tagging methods (Table 1), such as the fusion of an engineered enzyme to the POI that can form a covalent (e.g., SNAP-, Halo-, CLIP-, and TMP-tag, which derives from dihydrofolate reductase) or reversible (e.g., reHaloTag, TMP-tag, FAP, and FAST) bond with a fluorescent substrate [27]. While covalent binders are better established, with SNAP- and Halo-Tag being commonly used (with Halo having the fastest kinetics), reversible binders are an emerging field because bleached fluorophores can be exchanged with fresh ones, enabling long-term imaging. Furthermore, the transitory binding of the fluorescent substrate to the tag can be used as a switching mechanism to obtain a single-molecule regime. DNA-PAINT is the most prominent implementation of this refreshable concept [28].

FPs fused to POIs can interfere with the physiological function and localization of the POI. Protein engineering that avoids disruption of important domains and incorporates a flexible linker between tag and POI can help ensure that the tag does not interfere with protein function and localization. Moreover, to avoid overexpression, CRISPR/Cas9 knock-in strategies can be used and, although the efficiency of these manipulations remains limited, even a few positive cells may be sufficient for microscopy experiments [29]. Smaller tagging systems requiring only small peptides (<3 nm) fused to the POI and a corresponding tagging enzyme include biotin ligase, lipoic acid ligase, and Tub-tag (Table 1). However, these tagging methods need specialized enzymes; thus, labeling is limited to surface proteins or fixed cells. Alternatively, FlAsH-tag and His-tag require small-molecule ligands (Table 1), but at the same time show high background and their implementation is not straightforward. Other approaches require tagging proteins with a small

Box 3. Fluorophore selection and why there are no one-size-fits-all solutions

A variety of fluorophores have been developed to fulfill the specific requirement of each FN technique (Table I). Factors that need to be considered when selecting a fluorophore include its chemical structure and charge, fluorescence **quantum yield**, and photostability (see Figure 2C in main text). While STED and expansion microscopy requires photostable fluorophores, SMLM and MINFLUX rely on molecules that reversibly switch between non-emitting and emitting states. For the possible switching mechanisms, we refer the reader to Figure 2D in main text.

The most used fluorophore scaffolds in FN are cyanines and rhodamines. Among cyanines, Alexa Fluor 647 is considered the gold standard in SMLM and blinks in the presence of reducing agents and UV light [84]. Rhodamines can relatively easily be modified to tune their spectral properties [85], membrane permeability [86], and equilibrium between the open fluorescent and the closed non-fluorescent forms. The regulation of this latter equilibrium induces live cell-compatible spontaneous blinking [86].

Other frequently used fluorophores are based on coumarin, oxazine, or BODIPY scaffolds (see Figure 2E in main text). Coumarins are among the smallest fluorescent dyes and can be modified to generate variants with a large **Stokes shift**, which is advantageous for low background and multiplexing FN imaging [87]. Oxazines can be live cell compatible [88], have a red-shifted absorbance, high extinction coefficient, and the ability to 'blink' in buffers containing reducing oxidizing agents. Finally, BODIPY dyes are valued for their sharp absorption and fluorescence spectra combined with very high quantum yield and extinction coefficient [89]. Although their application to FN is limited due to their highly hydrophobic nature and poor off-switching properties, recent low light-dose photoactivatable variants make them attractive for the field [90].

In practice, fluorophores should be selected only after the most appropriate FN technique has been identified, always considering the specifications of the available instrument (e.g., lasers and detectors). The choice should also be driven by specific experimental needs, such as multicolor or live FN, the presence of autofluorescence in the sample, the need to reduce background, or counting molecules. An important consideration is that naked fluorophores may themselves have specific affinities for some cellular structures (e.g., lipophilic fluorophores may stain membranes). This should be evaluated when designing experiments, for example, by using fluorophores (reporters) without their targeting moiety as controls whenever necessary (see Figure 2B in main text). In general, we recommend that inexperienced FN users consult with their local expert to select the best dye for their application.

Table I. Properties of commonly used fluorescent dyes

Fluorophore class	Coumarins	Rhodamines	Cyanines	BODIPYs	Oxazines
Commercial examples	Alexa Fluor 350, Pacific Blue	Alexa Fluor 488, silicon-rhodamine, TMR	Alexa Fluor 647, Alexa Fluor 555, Cy5	BODIPY FL, BODIPY TMR	Atto 655, Atto 680
Spectral range (nm)	360–700	500–750	500–1000	500–700	600–750
Extinction coefficient ($cm^{-1}M^{-1}$)	15 000–60 000	80 000–150 000	130 000–250 000	60 000–100 000	110 000–130 000
Quantum yield	0.4–0.9	0.1–0.9	0.1–0.6	0.8–0.9	0.1–0.6
Photostability	++	+++	+	+++	++
Compatibility with FN methods	STED, SMLM	STED, SMLM, MINFLUX, expansion microscopy	STED, SMLM, MINFLUX, expansion microscopy	STED, SMLM	SMLM

epitope tag recognized by an intrabody [30,31] or nanobody/antibody in fixed samples. An example is the rationally designed ALFA tag (13 amino acids), recognized by a nanobody/intrabody carrying various reporters [32]. The smallest possible tag for a protein is a single fluorescent amino acid (unnatural amino acid, UAA), which is incorporated into the protein sequence at a specific position. This method, which is still laborious and has some limitations, requires a dedicated tRNA synthetase that adds the UAA at an amber STOP codon in the mRNA of the POI.

Table 1. Comparison of different labeling strategies[a]

Tag or method name	Maximum reaction rate (k_{react}) or affinity (Kd)	Labeling mode	Approximate size of tag[b]	Detection of tag and size	Refs
Protein-labeling methods					
Ligand-directed covalent modification	k_{react} up to 10^4 $M^{-1}s^{-1}$	Covalent	Small molecule (1–2 nm)	N.A.	[35]
Unnatural amino acid	k_{react} up to 10^5 $M^{-1}s^{-1}$; TCO/tetrazine	Covalent	One amino acid (~0.5 nm)	Clickable dye (~0.5 nm)	[36]
Antibodies and nanobodies	Kd range pM–nM	Reversible	Range of sizes	Antibody (10–15 nm), nanobody (~3 nm)	Various
His-tag	Kd 270 nM; up to 10^3 $M^{-1}s^{-1}$	Reversible or covalent	Six amino acids (~2 nm)	Specialized fluorophore (~0.5 nm)	[37]
Tetracysteine (FlAsH/ReAsH)	Kd 2.4 nM	Reversible	Six amino acids (~2 nm)	Specialized fluorophore (~0.5 nm)	[38]
Tetraserine (RhoBo)	Kd ~400 nM	Reversible	Six amino acids (~2 nm)	Specialized fluorophore (~0.5 nm)	[39]
Epitope tags for nanobodies (e.g., ALFA, SPOT, C Tags)	Kd: ALFA tag ~20 pM; SPOT tag ~1 nM; C tag ~10 nM	Reversible	Four to 13 amino acids (1–3 nm)	Nanobody (~3 nm)	[32]
Epitope tags for antibodies (e.g., FLAG-tag, HA-Tag)	Kd range pM–nM	Reversible	Eight to 13 amino acids (2–3 nm)	Antibody (10–15 nm)	[40]
Biotin ligase (AviTag)	Kd_{avidin} 0.001 pM	Covalent	15 amino acids (3 nm)	Avidin/streptavidin (tetrameric, ~5 nm)	[41]
LAP-tag	k_{react} 10^2 $M^{-1}s^{-1}$	Covalent	13 amino acids (~3 nm)	Clickable moiety (~0.5 nm)	[42]
FKBP	Kd 0.4 nM	Reversible	108 amino acids (~3 nm)	FK506-fluorophore (~2 nm)	[43]
PYP-tag	k_{react} 4×10^3 $M^{-1}s^{-1}$	Covalent	125 amino acids (~3 nm)	Binder fluorophore (~1 nm)	[44]
TMP/DHFR-tag	k_{react} 53 $M^{-1}s^{-1}$	Covalent or reversible	159 amino acids (~4 nm)	Fluorescent inhibitor (~2 nm)	[45]
SNAP-tag	k_{react} up to 10^5 $M^{-1}s^{-1}$	Covalent	182 amino acids (~5 nm)	Fluorescent substrate (~2 nm)	[46]
Halo-tag	k_{react} up to 10^8 $M^{-1}s^{-1}$	Covalent or reversible	290 amino acids (~5 nm)	Fluorescent substrate (~2 nm)	[47]
GFP-tag	N.A. (covalent)	Covalent (no exogenous chromophore)	238 amino acids (~4 nm)	N.A.	[48]
DNA-labeling methods					
Metabolic labeling	Up to 10^2 $M^{-1}s^{-1}$; alkyne/azide	Covalent via 'click' chemistry	Single nucleotide	Clickable moiety (~0.5 nm)	[49]
Small-molecule dyes	Kd range nM–μM	Reversible or covalent	Four to 30 base pairs	Specialized fluorophore (~0.5 nm)	[50]
Triplex-forming oligonucleotides	Highly sequence dependent	Reversible	13–30 base pairs	Fluorophore (~0.36 nm per base pair)	[51]
Polyamides	Kd range 1–100 nM	Reversible	Four to 24 amide units	Fluorophore (~0.34 nm per base pair)	[52]
Bacterial repressor (LacI or TetR)	Kd range 0.1–1 nM	Reversible	19–24 base pairs	Protein size (~5 nm)	[53]
dCas9 protein	Kd nM	Reversible	20–30 base pairs	Large DNA–RNA–protein complex (~10 nm)	[54]
TALEN proteins	Kd range 1–100 nM	Reversible	13–20 base pairs	Programmable protein (~5–10 nm)	[55]
DNA-FISH	Highly sequence dependent	Reversible	~22 base pairs–few kilobase pairs	Fluorophore (~0.34 nm per base pairs)	[56]

(continued on next page)

Table 1. (continued)

Tag or method name	Maximum reaction rate (k_{react}) or affinity (Kd)	Labeling mode	Approximate size of tag[b]	Detection of tag and size	Refs
RNA-labeling methods					
Metabolic labeling	Up to 10^2 $M^{-1}s^{-1}$ (alkyne/azide)	Covalent via click chemistry	Single nucleotide	Functionalized nucleotides with 'clickable' moiety (~0.5 nm)	[57]
Small-molecule dyes (aptamers)	Kd range 1–100 μM	Reversible	Small molecule (1–2 nm)	Specialized fluorophores (~0.5 nm)	[58]
MS2 coat protein	Kd range 1–10 nM	Reversible	Protein size (~5 nm)	GFP-MS2 fusion (~5 nm)	[59]
Pumilio homology domain (PHD) proteins	Kd range 1 nM–1 μM	Reversible	Programmable protein (~5–10 nm)	GFP-PHD fusion (~5–10 nm)	[60]
dCas13a protein	Kd 100 nM	Reversible	Large RNA–protein complex (~10 nm)	Large RNA–RNA–protein complex (~10 nm)	[61]
RNA-FISH	Highly sequence dependent	Reversible	~22 base pairs–few kilobase pairs	Fluorophore (~0.3 nm per base pairs)	[56]
Lipid (membrane)-labeling methods					
Cholesterol derivatives	N.A.	Reversible	Small molecule (1–2 nm)	Covalently attached fluorophore (~1 nm)	[62]
Lipophilic dyes	N.A.	Reversible	Small molecule (1–2 nm)	Fluorescent dye	[63]
Functionalized lipids	Up to 10^5 $M^{-1}s^{-1}$ (TCO/tetrazine)	Reversible	Small molecule (1–2 nm)	Functionalized lipids with clickable moiety (~0.5 nm)	[64]
Fluorescent lipids	N.A.	Reversible	Small molecule (1–2 nm)	Fluorophore-attached lipids (~1 nm)	[65]
Phosphoinositides	Kd 10–1000 nM	Reversible	Protein size (~4 nm)	GFP-pleckstrin homology or FYVE domains fusions (~5 nm)	[66]
Mitochondrial lipophilic dyes	N.A.	Reversible	Small molecule (1–2 nm)	Fluorescent dye	[67]
Carbohydrate-labeling methods					
Metabolic labeling	Up to 10^2 $M^{-1}s^{-1}$; alkyne/azide	Covalent	Functionalized sugars with clickable moiety (~0.5 nm)	Functionalized sugars with clickable moiety (~0.5 nm)	[68]
Wheat germ agglutinin	Kd range 1 nM–1 μM	Reversible	Protein size (~5 nm)	Covalently attached fluorophore (~1 nm)	[69]

[a]Note that, due to space limitations, only a subset of the available strategies are summarized here.
[b]The approximate size of the tag affects the linkage error, which is the distance between the biomolecule of interest and the fluorescent reporter. This size is not simply linearly scaled and has been approximated from crystal structures, known distances of elemental components, or reported in base pairs when referring to polymers, such as DNA or RNA. Please use this as an initial guide; also note that the linkage error is further increased by the detection system used.

Subsequently, the modified protein will contain the UAA, which can be used for specific conjugation of a fluorophore (e.g., via click chemistry) [33]. Genetic manipulation of the system may result in several artifacts and should be considered on a case-by-case basis. Therefore, an ideal labeling system should avoid genetic manipulations and use small fluorogenic dyes that interact with the target specifically with minimal perturbations.

A widely used example of such a small primary probe is phalloidin, a natural ligand that binds to, and enables imaging of, actin filaments. Leveraging on natural ligands, drugs, or charged groups, multiple FN-compatible probes have been developed over the past decade, highlighting their usefulness [34].

Various strategies for labeling DNA, RNA, lipids, and carbohydrates have been used in FN, although their application to this type of super-resolution imaging remains limited (see Table 1 for examples). While there are many strategies for fluorescently labeling different molecules of interest,

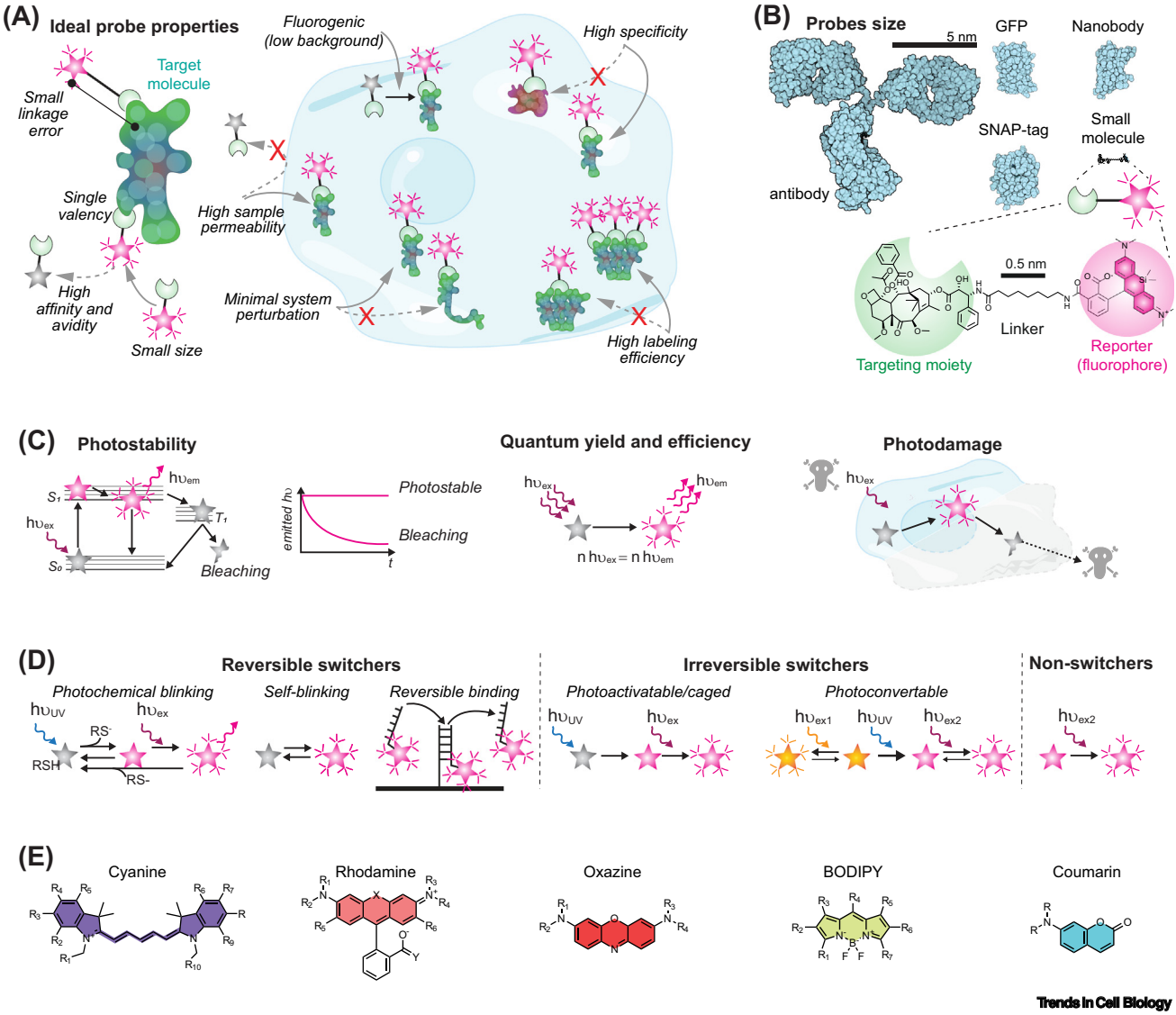

Figure 2. Probes and reporters. (A) Representation of the structure and size of common probes and their desired properties (B). (C) Representation of relevant aspects to consider when choosing a fluorophore. Note that photodamage can be caused by both the direct irradiation or the fluorophore itself and its photobleaching byproducts. (D) Types of switching mechanism and (E) classes of reporters that can be used in fluorescence nanoscopy (FN).

and the list is growing rapidly, there is no single solution that can be applied to all imaging modalities (Box 3). We refer the reader to Table 1 for examples with potential FN applications.

Multiplexing: detecting multiple targets in the same sample

Being able to simultaneously target multiple structures within the same specimen is crucial to understand the regulation of complex biological processes. However, the sample preparation and imaging procedures constitute a significant hurdle in FN.

The separation of different probes within the same sample can occur by distinguishing them based on their spectral or photophysical properties (Figure 2D). Even though progress has

been made in the development of new FN-compatible fluorophores, chromatic separation approaches are prone to bleed-through between the different channels and rely on the usage of separate laser lines, which could introduce chromatic aberrations. To overcome this limitation, lifetime imaging or spectral separation based on crosstalk analysis can provide valuable solutions. In lifetime imaging, photons are assigned to different fluorophores species based on their arrival time to a single detector after a pulsed excitation [70]. In spectral separation based on crosstalk analysis, photons of spectrally similar dyes are excited with the same wavelength light and separation is achieved by mathematical analysis of the signal collected by detectors in different spectral windows [71]. Typically, commercial FN setups based on chromatic and lifetime separations achieve up to three to four colors/targets, but a combination of the above-mentioned strategies is also possible.

The limit to the number of targets imaged by FN can be also circumvented by sequential labeling and imaging cycles. In this way, the targets are uniquely identified because they are present in the sample and are imaged at different time points. An example of this approach is Exchange PAINT [72]. Sequential imaging and inactivation of the fluorophores [73], the use of chemicals for the scission of click-labeled substrates [74], or removal of the probes with harsh washing have also been proposed and could be applied to FN. While these strategies require simpler microscopes and are not prone to chromatic effects, they are not live cell friendly, with notable exceptions [74], and are time-consuming since the repetitive labeling and imaging procedure has to be performed on the microscope stage by using microfluidic approaches.

In summary, while simultaneous imaging of three to four targets can relatively easily be achieved, imaging of several dozens of targets in FN at the sub-20-nm scale is not only a challenge, but also a trend toward which the field is moving with the support of advanced data analysis approaches [28].

Concluding remarks

In this review, we provide a curated collection of aspects that scientists should consider when establishing experiments based on FN technologies. FN is a field that is growing at a remarkable pace, driven by advances in engineering, chemical synthesis, and the development of labeling strategies, automation, machine learning, and neural networks for image analysis. Thanks to this rapidly evolving field, cell biologists are finding new ways to answer previously inaccessible biological questions. At the same time, essential aspects that will remain central for cell biologists are the ability to optimally prepare samples, avoid artifacts, use optimized affinity-based labels, improve their quality, and ensure that experiments based on imaging are informative and reproducible, bridging the gap between the world of molecular processes and that of cellular events.

Future developments in FN promise to further push the limits of biological imaging (see Outstanding questions), even in live settings (the unique prerogative of FN over EM), to obtain absolute molecular numbers for complexes, using optimized calibration tools, and to uncover previously unexplored cellular dynamics. To achieve this, direct collaboration between biologists, microscopists, chemists, affinity-based label developers, and data analysists will be critical.

Outstanding questions

Which new fixation strategies (e.g., cryofixation) can be utilized to preserve native morphologies, halt enzymatic activity, and reduce fluorescent background in FN while still being simple and having a relatively high throughput and limited costs?

How can we develop novel fluorophores or labeling techniques that minimize phototoxicity while still providing excellent photostability and brightness, enabling prolonged and repeated imaging of live cells and tissues? Can fluorophores be generated *in vivo* to avoid external addition and circumvent cell permeability issues in living cells?

Can the process of developing affinity probes based on small molecules be streamlined, ultimately achieving a large choice of affinity-based labels comparable to what is currently available for antibodies?

What are the most promising approaches for developing imaging probes that can selectively target specific cell types or subcellular structures *in vivo*, allowing researchers to study complex biological processes at high resolution within their native environment?

How can we achieve effective multiplexing *in vivo* using FN, enabling simultaneous imaging of multiple cellular targets or dynamic processes in living organisms, without crosstalk or spectral overlap issues?

What strategies can be used to improve the speed of FN imaging, enabling the visualization of fast cellular dynamics and processes in real time while maintaining high spatial resolution?

How can we enhance the user-friendliness of FN imaging techniques and data interpretation allowing researchers with limited expertise to perform high-resolution imaging experiments efficiently and accurately?

How many images are needed to extract biologically meaningful data? Will the community agree on a standard for required dataset sizes and analysis procedures?

Author contributions

All authors wrote, commented, and finalized the manuscript in a collegial manner.

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What are new biological questions that one can now address with FN?

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Declaration of interests

G.L. has filed a patent application (PCT/EP2011/064750, applicant EPFL, status: granted) on SiR derivatives. R.L. is co-inventor of a patent application (WO2023284968A1) covering PaX dyes, filed by the Max Planck Society. F.O. is a shareholder of NanoTag Biotechnologies GmbH. All other authors declare no competing interests.

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