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Fluorescent probes for nanoscopy: four categories and multiple possibilities

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Abstract

Nanoscopy enables breaking down the light diffraction limit and reveals the nanostructures of objects being studied using light. In 2014, three scientists pioneered the development of nanoscopy and won the Nobel Prize in Chemistry. This recognized the achievement of the past twenty years in the field of nanoscopy. However, fluorescent probes used in the field of nanoscopy are still numbered. Here, we review the currently available four categories of probes and existing methods to improve the performance of probes.

Graphical Abstract

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Keywords

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1. Introduction

Revealed nanostructures empower us to understand our world better, providing enormous information on a studying object at the molecular level. Nanostructural information is extremely useful in fields such as biology, biomedical engineering, material science, and nanotechnology. In the 1930s, Ruska and Knoll pioneered the construction of an electron microscope (EM) [1]. Afterwards, two types of EMs were developed: a scanning EM and a transmission EM (SEM and TEM). They are still widely used to visualize the “nanoworld.” In 1986, Ruska was awarded with the Nobel Prize in Physics. An EM requires tedious sample preparation procedures; and sample fixation, dehydration, ultra-thin sectioning, and heavy metal staining may cause artifacts [2]. In addition, samples are viewed in high vacuum in an EM (except an environmental SEM), which is not physiological relevant for biological samples. Besides EM, other techniques, such as scanning tunneling microscope (STM) [3], atomic force microscope (AFM) [4], X-ray computed-tomography [5], Magnetic resonance imaging (MRI) [6], ultrasound [7], and light microscopy (LM) are used to extract nanostructural information. Among them, LM stands out since it uses light, a non-ionizing radiation, other than electron or X-ray. Therefore, it is desirable to reveal nanostructures by light, especially for biological samples such as cells, tissues, and organisms.

A light microscope, since its invention in the early 1600s, has evolved tremendously especially over the past 3 decades: confocal laser scanning microscopes (CLSM) enable researchers to acquire three-dimensional (3D) images; nonlinear optical microscopes (NLOM) provide deeper images with less photo-damage [8–10]. The main advantages of LMs over EMs are as follows: lower invasiveness, better dynamics, and specificity. These

advantages are extremely important for bio-imaging. For instance, via antigen-antibody binding, proteins of interest can be easily identified by a fluorescence microscope. Nonetheless, a traditional LM cannot suppress the Abbe's diffraction limit [11]. Images obtained by visible light blur below ~250 nm [12]. To overcome the diffraction limit and achieve the sub-diffraction resolution, scientists have developed several approaches. This barrier was first overcome by applying near-field optical scanning microscopy in 1972 [13]. For far-field microscopy, the first approach was proposed in 1994, known as stimulated emission depletion (STED) microscopy [14]. In the early 2000s, to reveal biomolecular complexes and intracellular structures (resolution ~20 nm), two other types of microscopy were developed: stochastic optical reconstruction microscopy (STORM) and (fluorescence) photo-activated localization microscopy ((F)PALM) [15–17]. In addition, several new types of microscopy were developed such as reversible saturable optical fluorescence transition (RESOLFT), super-resolution optical fluctuation imaging (SOFI), structured illumination microscopy (SIM), and ground state depletion microscopy followed by individual molecule return (GSDIM) [12, 18–22]. All these types of microscopy are also known as super-resolution microscopy or nanoscopy. In 2014, three scientists shared the Nobel Prize in Chemistry to recognize the achievement of the past two decades in this field. Although the instrumentation and working principles of the nanoscopes differ, they mostly rely on fluorescent probes to visualize nanostructures. Not every fluorescent probe is suitable for every nanoscope. For example, STED requires bright and photo-stable probes whereas STORM requires photoswitchable probes.

In this review, we first briefly discuss the principles of the nanoscopy. Next, we focus on the recent development of fluorescent probe technology, especially for nanoscopes. Fluorescent proteins (FPs), organic small molecules, (OSMs) and quantum dots (QDs) are three major types of fluorescent probes that are extensively used for nanoscopes [23–30]. Recent emergence of nanoparticles (NPs), such as carbon dots (CDs), graphene quantum dots (GQDs), gold nanorods (AuNRs), polymer-based nanoparticles (P-dots), and aggregation-induced emission dots (AIE dots), broadens beyond these three types [31–35]. These four categories of probes constitute the “fluorescence toolbox” for nanoscopes. Using this toolbox, we can apply the right probe to each nanoscope to visualize biological and chemical processes obtaining 3D multicolored pictures in real time.

2. Working principles

In this section, we briefly discuss the working principles of nanoscopy, focusing on three approaches: 1) SIM, 2) STED and 3) STORM and (F) PALM.

SIM uses Moiré effect to overcome the Abbe's diffraction limit [36–38]. First, a bar code-like, excitation pattern of light is illuminated on the testing sample, generating a Moiré interference. Then the orientation of the excitation pattern is tilted to generate another set of images. After a series of such images are collected, they are further processed by a computer algorithm to achieve a super resolution image of the sample (Figure 1, SIM column).

STED uses two laser beams, one for excitation and the other for depletion, to illuminate samples. Fluorescent probes were first excited by the excitation beam and then returned back

to the ground state by the depletion beam. When these two laser beams are superimposed, only the fluorescent probes that reside in the center of the depletion beam can emit fluorescence, generating a “focal spot” (Figure 1, STED column). A super-resolution image is recorded by scanning this type of “focal spot” across the sample.

STORM and (F)PALM use practically the same approach. This approach relies on photo-switchable fluorescent probes, which can be switched between an “on” (bright) and an “off” (dark) state. At each given time, a sparse set of the fluorescent probes are activated to produce single-molecule images (represented by yellow circles in Figure 1 STORM/PALM/FPALM column) that do not overlap. A super-resolution image is constructed by taking a sequence of such images.

3. Fluorescent probes

Despite the recent advances in the development of super-resolution microscopy or nanoscopy, the quest to fluorescent probes with high brightness, high photostability and low phototoxicity remains an un-met need. Based on their working principles, we summarize the fluorescent probes for each approach of nanoscopy in Table 1. They rely on commercially available fluorescent probes, such as Alexa Fluor®, EosFP and Si-R [15, 39, 40]. These probes perform fairly well. However, they are not perfect. For example, EosFP, a fluorescent protein named after the goddess of dawn in Greek mythology, is not as bright as Dronpa [23]. Advances in designing new fluorescent probes can significantly enhance the performance of the nanoscopes. Recently, Skylan-NS was used as a new probe for SIM [41]. By using this probe, researchers can not only reduce the SIM’s resolution from 110 nm to 60 nm, but also improve the speed of SIM imaging, pushing SIM imaging technology to a new level [41, 42].

In order to find out how frequent the probe of each category was used, we performed the literature search mainly by Google Scholar with the keywords listed in Figure 2. Total hits for each category were recorded. As can be seen in Figure 2, FPs are the most commonly used probes for nanoscopy followed by OSMs, QDs, and NPs.

Here, we review the four categories of fluorescent probes and the major subtypes in each category. We examine these probes in terms of their brightness, photostability, and phototoxicity. With the widespread use of nanoscopes, this review will serve as a guide for researchers to choose the right probe for their bio-imaging applications.

4. Category one: Fluorescent Proteins (FPs)

In Figure 2, FPs are shown to be the most frequently used as fluorescent probes at the current stage. They have a relatively short history and are used for bio-imaging at the current stage only. Green fluorescent protein (GFP), the first and most famous FP, was discovered and extracted from jellyfish, *Aequorea victoria*, in the 1960s [43]. Since the 1990s, the GFP has been genetically engineered to produce a vast number of mutants. In addition, other FPs were discovered in other species and extracted from them. The results are demonstrated in a full color palette (Figure 2). Based on colors (the emission wavelength), the FPs can be classified as UV (e.g., Sirius), blue (BFP, e.g., EBFP2), cyan (CFP, e.g., Cerulean), green

(GFP, e.g., EGFP), yellow (YFP, e.g., EYFP), orange (e.g., mOrange), red (RFP, e.g., mCherry), far-red (e.g., mPlum), and near infra-red (NIR, e.g., iRFP) proteins [44]. Unlike other fluorescent probes, FPs can be genetically coded and label cellular structures with minimum invasion [44]. Therefore, an FP is an important tool for bio-imaging. In 2008, three scientists were awarded the Nobel Prize in Chemistry for their contribution to the discovery and development of the GFP. The total number of the FPs available now is in thousands [27]. However, the FPs suitable for STORM/PALM/FPALM nanoscopy are numbered. They are a special subgroup of FPs, known as the photo-activatable FPs (PA-FPs) or the smart labels [23, 25–27, 45–50]. PA-FPs generally can be divided into three types:

1. Photo-convertible proteins/irreversible photo-converters, e.g., EosFP [51]
2. Photoactivatable proteins/dark-to-bright photo-activators, e.g., PamCherry [52]
3. Reversible photochromism/reversible highlighters, e.g., Dronpa [53] and Dreiklang [54]

Figure 4 shows their photophysical characteristics. Some PA-FPs can irreversibly photoswitch their peak emission from green to red (type 1) upon light irradiation; Other PA-FPs can irreversibly convert from a dark to a bright state (type 2) or reversibly (type 3) in response to light irradiation at specific wavelengths. These probes are extremely useful for nanoscopy to visualize the molecular details of sub-cellular organelles, proteins, and signal pathways. Other FPs, though bright and photostable, are rarely used by nanoscope researchers.

The history of the PA-FPs is relatively short in comparison with FPs. In 2004, EosFP was found to be able to change from green to red upon near-UV irradiation [51]. Afterwards, other FPs with similar photo-physical properties were found, including asFP595 [55], Dronpa [53], mMaple [56], mGeo [57], PAmCherry1 [52], Dreiklang [54], and more. New PA-FPs are mutants modified from the existing FPs. So far, there is no clear strategy to design a superior PA-FP. It is still based on trial-and-errors. This is mainly because of the unique structure of an FP. It is hard to predict the maturation rate of a new FP unless someone tries it out [44, 46]. In Figure 5, we list all PA-FPs that are reviewed in this paper in chronological order. By carefully choosing the right PA-FPs, researchers now push the limit of nanoscopy to a new level.

PA-FPs are usually thought to be not as bright as OSMs. The brightness, by the definition, “was calculated as extinction coefficient $\epsilon \times$ quantum yield $\Phi/1000$ at the on-state of the proteins, corresponding to the peak intensity” [58]. If we compared the literature brightness values between Dronpa and ATTO532, we found they are quite similar. The literature brightness values of Dronpa are 72 based on Ref. [59], 81 based on Ref. [23], and 85 based on Ref. [58]. ATTO532, an OSM, has a brightness of 104 based on Ref. [23]. Another OSM, ATTO565 has a brightness 108 based on Ref. [25]. Although the literature values of the probe brightness vary depending on the measurement conditions, their magnitude is roughly within the same order.

Next, we examine the photostability of PA-FPs. There are several ways for evaluating the photo-stability. The most common way to measure it is to perform a photobleaching kinetic

study [46]. However, the use of different laser intensities and buffer for such kinetic studies or the evaluation of FPs by using different colors may result in different readouts. Reports showed that Kohinoor is more photo-stable than Dronpa and Dreiklang [60]. Skylan-S also is superior to Dronpa in terms of photostability [58].

Other photo-physical parameters of PA-FPs, such as the number of switching cycles and photons per switching event, are much lower than that of OSMs (see Tables 1 and 2 in Ref. [25], for example). Therefore, currently, the research efforts are made to enhance these parameters. For example, Skylan-S shows almost the same fluorescent intensity whereas Dronpa shows only one third of its original intensity after 21 switching cycles [58]. The contrast ratio of on/off-states is another important photo-physical parameter. “On” is a fluorescent state whereas “off” is a dark state. The contrast ratio of PAmcherry1 is approximately 4000, which is one of the highest among all PA-FPs [25, 27].

The phototoxicity of FPs are well studied by the researchers. With irradiation, FPs can produce toxic reactive oxygen species (ROS) [61]. However, their toxicity effect is quite low and can be ignored in many cases [27, 61]. Dronpa did not show any severe toxic effect under light irradiation [62].

Besides the mild phototoxicity issue, FPs tend to form oligomers, which could lead to cytotoxicity [27, 46]. As for PA-FPs, some form dimers (e.g., mMaple [63]) or tetramers (e.g., EosFP [26]). Oligomerization of tdEos and dEos cause the compromised performance as a probe for STORM/PALM/FPALM nanoscopy since more than one chromophore are present in each localized probe molecule [64]. On the other hand, the performance of mEos2 (a variant to EosFP) was not hampered by oligomerization [64].

Because FPs are bright, photostable and with low phototoxicity, they are widely used as probes for nanoscopes. Dual-color nanoscopy was realized in 2007 by using PA-FP pairs (Dronpa/EosFP and PS-CFP2/EosFP) [65]. The major problem of dual-color or multicolor imaging is the overlap of emission between the PA-FP pairs. Recently, a new class of FPs, oxFP, was developed, which provided a full color palette (ranging from blue to red) [66].

FPs offer researchers a useful tool for bio-imaging, especially for studying subjects like cellular organelles, cells, and tissues. The major advantages of FPs over other probes are that they can be genetically coded and labeled by one step. However, FPs have disadvantages as well: 1) the number of available probes is still limited (Figure 5); 2) the size of the probes is usually larger than that of OSMs; 3) and oligomerization may cause cytotoxicity. Progress is underway to generate new types of FPs, which are brighter, more photo-stable, and can be switched on/off multiple times.

5. Category two: Organic small molecules (OSMs)

Many OSMs are commercially available with trademarks such as Abberior, Alexa Fluor, ATTO, and DyLight Fluor [67, 68]. The current collection of OSMs spans from the visible to near-infrared (NIR) region of the spectrum. Examples include coumarins (blue to green), xanthenes (yellow to red), and cyanines (red to NIR). Besides them, oxazines (leuco dyes)

are another type of OSMs. Here, we examine these four families of OSMs: coumarins, xanthenes, cyanines, and oxazines. Their chemical structures are shown in Figure 6.

5.1 Coumarins

Coumarin 102 is a commonly used OSM in STED microscopy [35]. However, it emits green fluorescence upon excitation, far below the “therapeutic window” (near-infrared window is also known as optical window or therapeutic window, in the range of 650–1350 nm [69]). Nonetheless, it is an ideal dye for bio-imaging. One derivative with a large Stokes shift, 7-dialkyl-amino-4-trifluoromethyl coumarin, was designed. This molecule emits red fluorescence [70]. The technique of combinatorial chemistry is successfully applied for building a coumarin library. By screening this library in terms of optical properties, a new probe with high-fluorescence quantum yields was identified [71]. One of the disadvantages of coumarins is their poor water solubility. This limits their application in bio-imaging.

5.2 Xanthenes

Rhodamines are the most famous members in the xanthene family. When Hell and Wichmann first illustrated the concept of STED in 1994, a rhodamine B dye was used [14]. It is bright and photostable, offering many advantages over other OSMs and FPs. Moreover, rhodamine B is permeable to cellular membranes, which makes it more suitable for intracellular labeling [23]. Furthermore, it is photoswitchable. The photoswitch mechanism of this rhodamine molecule is simply light-induced isomerization without adding other molecules. In addition, a derivative of this molecule can be switched on with two-photon absorption (2PA) [72]. 2PA is superior to one-photon absorption (1PA) for two reasons. 1PA utilizes UV light whereas 2PA utilizes red or NIR light. UV light may lead to the damage of biological samples and autofluorescence. On the contrary, red or NIR light is preferred for biological samples, known as “therapeutic window.” Rhodamine 6G is hydrophobic. When it is dissolved in aqueous solutions, it forms dimers or aggregates, which hampers its application in bio-imaging. To circumvent this problem, sulfonation was used to modify rhodamine 6G and rendered it good water solubility. Hence, the new derivative of rhodamine 6G can be used for bio-imaging [73]. Amino or thiol reactive groups were attached to rhodamines via a carboxyl reactive site. This type of conjugation makes the new derivatives more suitable for bio-imaging [74]. Rhodamines were also modified using different polar groups to render even high-fluorescence quantum yields [67]. Another method to enhance the fluorescent properties of rhodamine is the replacement xanthene oxygen with quaternary carbon. This type of dyes is known as carborhodamines, a promising probe for nanoscopy [75]. Rhodamine NN, a caged OSM, was also synthesized as a new class of probes for nanoscopy. A caged compound refers to a fluorescent dye chemically linked with a photosensitive masking group. This compound is nonfluorescent until the mask is cleaved-off by UV light, and then the compound becomes fluorescent [76]. Besides bio-imaging, rhodamines are also commonly used as chemosensors. The mechanism for a nonfluorescent rhodamine derivative to become fluorescent is through a ring-opening reaction. A comprehensive review can be found elsewhere [77]. Caged Q-rhodamine is a promising PALM and STED probe [78]. The methods applied by researchers to modify rhodamine are reviewed in Ref. [79] and Ref. [30]. Silicon-rhodamine (SiR) is structurally related to the well-known family of rhodamine fluorophores and was recently developed as a promising

probe [80–82]. It is bright, photostable, non-cytotoxic and permeable to cell membranes. So far, SiR is one of the best candidates as a fluorescent probe for nanoscopes in the xanthene family.

5.3 Cyanines

STORM utilized a molecular switch made of a pair of cyanine dyes (Cy3–Cy5) when it was first introduced in 2006 [16]. This switch can be turned “on” and “off” for hundreds of cycles. And each cycle can yield thousands photons, sufficient for STORM imaging [16, 83, 84]. The photoswitching mechanism is determined as thiol concentration dependent [84]. Cyanines can react with primary thiols and change from a fluorescent to dark state. Secondary thiols do not facilitate photoswitching [84]. Not only Cy3–Cy5 can be a switch, other combinations, such as Cy3–Cy5.5, Cy3–Cy7, and Cy2–Cy5, are investigated as molecular switches. These combinations make multi-color super-resolution imaging possible [85]. Dempsey et al. systematically investigated 26 commercially available OSMs and found that the best-performing dye is Alexa Fluor 647 (a cyanine dye, similar to Cy5) [68]. Cy5 was conjugated with cyclooctatetraene, 4-nitrobenzyl alcohol or Trolox, to enhance its photo-stability [86, 87]. One of the disadvantages of Cy3, Cy5, and Cy7 as probes for STORM is their low-fluorescence quantum yield. To enhance the quantum yield of these cyanine dyes, heavy water (D₂O) instead of normal water (H₂O) was used, and their performance was substantially increased [29]. Cyanines have also been conjugated with ligands to target specific tumors [88, 89]. A detailed review regarding cyanines can be found elsewhere [90]. Recent researches were focused on enhancing the photo-stability and brightness of cyanines [29, 87, 91, 92].

5.4 Oxazines

Oxazines (ATTO655, ATTO680, or ATTO700) have been investigated as molecular switches for years. These OSMs can be switched between “on” and “off” by adding or removing reductant or oxidant [93]. Similar to cyanine dyes, oxazine dyes gain a higher-fluorescent quantum yield and perform better in D₂O compared to that in H₂O [94]. Oxazine and rhodamine probes have a similar chromophore structure and exhibit similar redox properties. Therefore, by adding or reducing agents, such as β -mercaptoethylamine, dithiothreitol, or glutathione (GSH), these probes can be turned “off.” In other words, these probes can be reversible photoswitching by varying redox environment. This is important for live cell imaging as the intracellular GSH level is relatively high (concentration in millimolar level) [95].

In summary, scientists are searching for brighter and more photostable OSM probes by modifying chemical structures of the existing molecules (e.g. rhodamines). Si–R is a perfect example for such effort. As indicated by both oxazine and cyanine dyes, switching solvents from normal water to heavy water can significantly improve these dyes’ performance. This hints that the solubility of these probes is one of the most important factors we should consider when we modify the molecular structures. Screening a library of molecules with systemic changes in molecular structures, such as side chains and functional groups, can help us find the better performing fluorescent probes. Recent advances in computational chemistry can also expedite this searching process.

6. Category three: Quantum Dots (QDs)

QD is another class of probes for nanoscopy. They are commercially available semi-conductive nanocrystals. Since 1998, QDs are widely used as fluorescent probes [96, 97]. It offers many advantages over OSMs and FPs: outstanding brightness, long fluorescent lifetime, superior photo-stability, and versatile bio-conjugation capability. Its emission is tunable by varying the particle size. It also spans from the visible to near-infrared region of the spectrum. Compared with OSMs, it shows a broad absorption band and a symmetric narrow emission band. Detailed reviews can be found in Refs. [24, 28]. For STORM, the control of QD blinking is of crucial importance. A blueing technique was developed to make a QD suitable for STORM (see Figure 7) [98]. For STORM, unlike those cyanine dyes, which require two laser beams for bleaching and reactivation and a thiol-containing agent to enhance photoswitching, QD requires one laser beam and no thiol or any other external chemicals. In comparison with those cyanine dyes, a QD show higher photon counts. QD blinking initially limits its application in STED [99]. This problem was recently resolved. QD surface chemistry was well studied and reported in several reviews [100, 101]. Zrazhevskiy et al. developed a technology called multicolor multicycle molecular profiling (M3P) [101]. This technology is based on linking antibodies to QDs and provides a useful tool for basic biology, disease diagnostics, and drug discovery. Other technologies include using nickel-histidine tags [102], Halotag [103], and high-affinity protein binding (e.g., biotin-avidin) [104]. By changing the surface chemistry, researchers can explore different biological applications. A QD has a core-shell structure. By varying the shell thickness, the optical properties of QD probes can also be improved [105]. Multicolor nanoscopy can be realized with different pairs of QDs, for example QD585 and QD655 [106]. 3D nanoscopy was also demonstrated by a couple of groups [107, 108]. One of the concerns for using QDs is their toxicity as they are made of heavy metals. Several reviews regarding this topic can be found elsewhere [9, 109].

7. Category four: Nanoparticles (NPs)

With the advancement of nanotechnology, several nanoparticles have been developed in recent years. Among them, carbon dots (CDs), graphene quantum dots (GQDs), gold nanorods (AuNRs), polymer-based nanoparticles (P-dots), and aggregation-induced emission dots (AIE dots) are developed as fluorescent probes. Some of them are also applied to nanoscopy. Since these probes are relatively new, the number of research papers is lower compared to FPs, OSMs, and QDs (Figure 1).

CDs are nanometer-sized carbon-based photo-luminescent materials, which are first discovered by Xu et al. in 2004 [9, 110]. Khan et al. recently demonstrated that CDs are photoswitchable fluorescent probes, which make them suitable for nanoscopy [31]. However, the photoswitching mechanism of these probes is still unclear. One possible mechanism of CDs in nanoscopy is illustrated in Figure 8. Unlike QDs, CDs are relatively new to the field of nanoscopy, possibly because they are not commercially available and not well studied. However, CDs can be viewed as alternatives to QDs with lower toxicity, lower cost, and better biocompatibility. Graphene is another type of carbon-based photo-luminescent materials [9]. Stöhr et al. directly visualized graphene through STED in

combination with fluorescence resonance energy transfer (FRET) [111]. Zhang et al. constructed ssDNA-graphene oxide (GO) assemblies and used them as fluorescent probes/biosensors to detect DNAs, proteins, metal ions, and other small molecules [112]. Graphene and GOs have low quantum yields [9]. Therefore, only a few studies for nanoscopy are done. We expect more studies of these carbon-based materials in the field of nanoscopy in the near future.

Gold nanospheres, nanoshells, nanocages, and nanorods (AuNRs) are two-photon luminescence (TPL) materials [9]. Wang et al. first reported both *in vitro* and *in vivo* TPL imaging using AuNRs in 2005 [113]. Blythe et al. visualized fluorescently labeled double-stranded DNA bound to the surface of AuNRs by using a GSDIM nanoscope [34]. The same group directly visualized AuNRs by using different microscopes to measure the point spread functions, which were the critical parameters that limit the resolution of microscopes [114]. Not limited to gold, metal NPs were able to improve the performance of STED-based nanoscopes. This new type of nanoscope was called as NP-STED [115]. In another recent study, metal NPs (gold in particular) were used as probes for nanoscopes [116]. Pellegrotti et al. showed that they can reduce photobleaching by changing the size of AuNPs [117]. Briefly, metal NPs, especially AuNPs, have the potential as nanoscope probes. However, photoswitching of metal NPs was realized by surface coating with molecular photo-switches, such as spiropyran [118].

AIE pheromone was first discovered by Luo et al. [119]. It is opposite to an aggregation-caused quenching (ACQ) feature [120]. Yu et al. recently used AIE dots as fluorescent probes of STED [35]. These AIE dots are brighter and more photo-stable than coumarin 102 (an OSM probe). Organic dots with the AIE properties are superior probes for cell tracking [121]. A detailed review of AIE dots can be found elsewhere [122, 123].

Conjugated polymer nanoparticles (CP-dots) are another type of probes for nanoscopy [124]. CP-dots are nanosized (~4 nm) and bright. Spiropyran, a molecular photoswitch, is commonly used to decorate P-dots [125–127]. Besides surface modification, another method is to encapsulate OSMs (e.g., NIR dyes) into P-dots [128]. These dots can be excited by one-photon or two-photon. Therefore, they have the potential as probes for future nanoscopy (i.e., multi-photon nanoscopy).

8. Conclusion and outlook

In this review, we reviewed all existing probes for nanoscopy, including fluorescent proteins, organic small molecules, quantum dots, and other types of nanoparticles. These probes enable us to visualize nanosized features, especially for bio-imaging.

These probes have pros and cons. FPs can be genetically coded and possess unique features compared to other fluorescent probes. Currently, new FPs are emerging. These proteins are mutants of the existing FPs but with superior photo-physical properties to their parent proteins. OSMs are also improving. For example, the photo-physical properties of SiR, with a single atom change from carbon to silicon, are enhanced. Various modifications of the existing OSMs make them promising as the future nanoscopy probes. Moreover, OSMs are

indispensable as they can be conjugated to antibodies and are widely used for confocal fluorescent microscopy. QDs are superior in terms of their photo-physical properties. Their unique core-shell structure makes them easy to be modified and suitable as probes for nanoscopy. However, they are composed of heavy metal elements, which make them less compatible to the biosystems.

Enormous work was done to improve the performance of these probes. New probes, such as nanoparticle-based probes, have been developed in recent years, increasing the number of existing probes. We expect brighter, more photostable, and less phototoxic probes to be discovered. Live, multicolor, three-dimensional images with an even higher resolution will be realized in the near future.

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Figure 1.
Three approaches of nanoscopy: SIM (column 1); STED (column 2); STORM/PALM/FPALM (column 3) (adapted from Stender et al. [37]).

Figure 2.

Four categories of fluorescent probes used for nanoscopy. Total hits (4398); Fluorescent proteins (FPs, 2390, 48%); organic small molecules (OSMs, 1807, 36%); quantum dots (QDs, 719, 14%); nanoparticles (NPs, 96, 2%). Data were extracted from Google Scholar on April 4th, 2016.

Figure 3.
Spectral diversity of available monomeric FPs (adapted from Chudakov D. M. et al. with permission [46]).

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Figure 4.
Photo-physics of PA-FPs. (adapted from Dedecker P. et al. [48]).

Figure 5.
Timetable of the PA-FPs that were used over the past decade.

Figure 6. Chemical structures of OSMs. (a) Xanthene, (b) coumarin, (c) cyanines: three types of cyanines are shown. I: Streptocyanines; II: Hemicyanines; III: Closed chain cyanines; (d) oxazines: they are cyclic compounds containing one oxygen and one nitrogen. All possible isomers are shown.

Figure 7. Quantum dots blueing mechanism and its application in nanoscopy (adapted from Hoyer et al. [98]).

Figure 8.
A possible photoswitching mechanism of carbon dots (adapted from Khan et al. [31]).

Table 1

Currently available fluorescent probes for each approach of nanoscopes.

Approach	Fluorescent Probes
SIM	Standard fluorescent probes (e.g. rhodamine and GFP)
STED	Bright and photo-stable fluorescent probes (e.g. Si-R)
STORM/PALM/FPALM	Photoswitchable fluorescent probes (e.g. Alexa Fluor® and EosFP)

SIM: structured illumination microscopy; STED: stimulated emission depletion; STORM stochastic optical reconstruction microscopy; (F) PALM: (fluorescence) photo-activated localization microscopy.