Fluorescent Probes for Super-Resolution Microscopy of Lysosomes
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ABSTRACT: Lysosomes are membrane-enclosed small spherical cytoplasmic organelles. Malfunctioning and abnormalities in lysosomes can cause a plethora of neurodegenerative diseases. Consequently, understanding the structural information on lysosomes down to a subnanometer level is essential. Recently, super-resolution imaging techniques enable us to visualize dynamical processes occurring in suborganelle structures inside living cells down to subnanometer accuracy by breaking the diffraction limit. A brighter and highly photostable fluorescent probe is essential for super-resolution microscopy. In this regard, this mini-review deals with the various types of super-resolution techniques and the probes that are used to specifically stain and resolve the structure of the lysosomes.

INTRODUCTION
Lysosomes are small membrane-enclosed cytoplasmic organelles ranging in size from 100 to 500 nm that are responsible for the intracellular protein degradation.1 The biomolecules including carbohydrates, peptides, nucleic acids, and lipids are engulfed within the cell, wherein they are broken down due to the presence of various enzymes in lysosomes. Hence, abnormalities and malfunctioning of these organelles might induce numerous pathologies.2 Therefore, it becomes highly imperative to develop a technique that enables visualization and tracking of lysosomes and eventually would aid in understanding the lysosomal working principle. Additionally, recording the dynamics of lysosomal movement in real time would facilitate the in-depth understanding of the working principle of lysosomes and the kind of organelles it could interact with.

Light microscopy (LM) is a prevailing tool for monitoring cellular biological processes like protein folding and unfolding, cell division, signaling, etc., as it enables a noninvasive real-time signal with high spatial and temporal resolution and sensitivity. However, owing to the diffraction limit, a resolution of only ~300 nm can be achieved by conventional light microscopy.3 To overcome this limitation, super-resolution microscopy (SRM) is proving to be a promising tool for monitoring cellular biological processes. It has drastically evolved over the past few years with new concepts such as stimulated emission depletion (STED4), structured illumination microscopy (SIM5), reversible saturable optical fluorescence transitions (RESOLFTs6), and subdiffraction resolution imaging done by subsequent localization of single molecules, e.g., stochastic optical reconstruction microscopy (STORM7), direct stochastic optical reconstruction microscopy (dSTORM8), photoactivated localization microscopy (PALM9), and fPALM.10 Such techniques which could resolve structures beyond the diffraction limit have been swiftly emerging. Therefore, these approaches, which make breaking the impenetrable fence possible, do so by resolving complex mechanisms that happen inside biological structures smaller than the diffraction-limited spot and are in high demand.

The principal criterion for fluorescent probes that could efficiently be used for SRM (localization-based) is their ability to be photoswitched by the light of a particular wavelength as a means to modify their spectral properties for the detection of selected subpopulations. On the other hand, a highly photostable probe is needed for microscopies like STED and SIM. Hence, the development of photostable nanoprobes for the specific labeling and imaging of lysosomes for real-time monitoring is of vital importance. In this regard, various groups throughout the globe are working to provide meaningful information and efficient probes. There are numerous reviews which deal with the super-resolution imaging of various organelles and the development of the probes for SRM.11−13 In this mini-review, we aim to discuss the emergence of the fluorescent probes that are used in various SRM techniques to specifically and selectively image the lysosomes. We will discuss the advancement of probes for each localization-based and other types of SRM techniques.
Figure 1. (a) Confocal microscopy. (b) STED microscopy images of lysosome structure using carbon dots in fixed MCF7 cells. (c and d) Two representative line profiles obtained from carbon dot signals inside the cell. Gaussian fits (thicker line) and raw data (light color) are shown in STED (green color) and confocal (red color) mode. The fwhm values are (i) 140 nm and (ii) 66 nm in (c) and (i) 98 nm, (ii) 54 nm, and (iii) 30 nm in (d). Reprinted with permission from ref 14. Copyright 2014. The Royal Society of Chemistry.

Figure 2. (a) Confocal and (b) STED microscopy images of lysosomes of living HeLa cells stained with LysoPB Yellow. The right panel of (b) shows the zoomed images from the ROI 1–4. (c) Intensity profiles across the lysosomes (ROI 1 in panel b). (d) STED images of large lysosomes. Reprinted with permission from ref 16. Copyright 2020. American Chemical Society.
PROBES USED FOR PATTERNED ILLUMINATION-BASED MICROSCOPY

In patterned illumination-based microscopic techniques, fluorophores are excited with a pattern illumination in combination with saturation effects to generate a fluorescence signal from a small subset of emitters within a diffraction-limited region. Examples of these methods are STED, RESOLFT, SIM, and their variants.3−5 With these techniques, one can achieve very high-resolution imaging down to a subnanometer level. A small disadvantage in these techniques is the use of complicated instrumentation and optical configuration to pattern the illumination light beam. Lemenager et al. used biocompatible carbon dots for the SRM imaging of lysosomes.14 They used STED microscopy for detection of carbon dot localization in both living and fixed cells and were able to achieve a spatial resolution of lysosome structure down to 30 nm in a fixed cell, while in live cells the resolution of 67 nm was attained. Figure 1 shows the conventional (confocal) and super-resolved images of lysosomes inside living HeLa cells. Grimm et al. designed green-emitting Rhodamine dyes for labeling of various cellular organelles, and with the help of STED microscopy, they were able to resolve the structure of desired organelles.15 In addition to staining the inner matrix of lysosomes, researchers have been successful in staining the membrane of lysosomes as well. Wang and co-workers were the first to design LysoPB Yellow as a new fluorescent probe that could selectively stain the lysosomal membrane.16 Using STED microscopy, the membrane of lysosomes stained with LysoPB Yellow was efficaciously visualized in living HeLa cells, which showed a full width at half-maximum (fwhm) of 70 nm. Figure 2a and 2b displays confocal microscopy and STED microscopic images, showing that lysosomes inside living HeLa cells stained with LysoPB

Figure 3. (a) Picture showing SIM imaging of lysosomes inside living He La cells (scale bar 10 μm). The insets show the zoomed images of the red and blue regions (scale bar 5 μm). (b) 3D surface plot sketching of the SIM picture of lysosomes with pseudocolor demonstrating relative fluorescence intensity. Reprinted with permission from ref 17. Copyright 2018. Springer.

Figure 4. Super-resolution imaging of lysosomes in U2OS cells by SIM using three different probes: (a) Lysosome-488, (b) Lysosome-565, and (c) Lysosome-647, respectively. Reprinted with permission from ref 18. Copyright 2017. Nature Publishing Group.

Figure 5. (a) Compositions of a Cy5-AuNP. (b) SIM images of HeLa cells stained with nanoparticles for different treatment periods. (c) SIM images of HeLa cells stained with nanoparticles in different concentrations for 24 h. Reprinted with permission from ref 22. Copyright 2020. Ivyspring International Publisher.
Two major strategies to visualize the lysosomes have been witnessed in the literature. The first one involves compounds which show weak fluorescence in neutral and basic media and enhanced fluorescence in an acidic environment (e.g., commercially available LysoSensor pH indicators) and other fluorescent compounds whose fluorescent properties are pH-independent (e.g., the commercially available LysoTracker). Zhu et al. developed a naphthalimide-based fluorescent compound, i.e., (E)-6-(4-((tert-butylphenylsilyl)oxy)styryl)-2-(2-(dimethylamino) ethyl)-1H-benzo [de]isoquinoline-1,3(2H)-dione (NIMS). The signaling mechanism toward lysosomes involved an accumulation process due to the protonation of the dimethylamino group in acidic environment and a fluorescence enhancement owing to its interaction with protein. Figure 3a shows the SIM-based SRM image of lysosomes using NIMS. Figure 3b represented the 3D surface plot profiling of the SIM picture of lysosomes with pseudocolor representing relative fluorescence intensity.

Han et al. established a series of cell-permeable organic fluorescent probes that exhibited excellent photostability and high specificity to image lysosomes. The probes are composed of three basic units, i.e., (rR),R2, a peptide capable of cell penetration, a recognition unit epoxysuccinyl scaffold to target the lysosomes, and a fluorescent dye (Atto 488, Atto 565, and Alexa Fluor 647). Using these constructs, they were not only able to visualize the lysosomes’ super-resolved structure but also observed the lysosome—mitochondrion interaction in living U2OS cells at a resolution of ~90 nm over a long time course of ~13 min using SIM. Additionally, they also observed the successive dynamics of lysosomal fission and fusion using lysosome-565 as a fluorescent probe. Figure 4 represents the super-resolved images of lysosomes stained with three different probes (Lysosome-488, Lysosome-565, and Lysosome-647).

Very recently, Chen et al. introduced an M-value to quantitatively examine the mitochondria and lysosome contact (MLC) and mitophagy by employing SIM. They showed that the M-value for mitophagy varies from 0.5 to 1.0, whereas in MLC it is less than 0.4. Using these parameters, they were able to successfully quantify the interaction of lysosomes to mitochondria and autophagosomes. On the other hand, Zhang et al. also developed the cell-permeable dyes by using PV-1 as a cell-permeable peptide. The incubation of the impermeable fluorescent compound, HD-Br, led to staining of the lysosomes of U2OS cells for the live cell SIM imaging. Fang et al. designed a near-infrared lysosome-targeted probe, HD-Br. In this work, 4-bromoresorcinol was treated with the commercially available heptamethine dye IR-780 under appropriate conditions, yielding the hemicyanine product, HD-Br. Based on passive targeting of lysosome via the endocytosis mechanism, the dye formed a nanoaggregate in the aqueous solution that could specifically target the lysosomes. Using this probe, a SIM-based imaging of mitochondria—lysosome contact (MLC) and mitochondria—lysosome interactions in mitophagy was performed. The probe was also used to image the lysosome in Caenorhabditis elegans.

In addition to the various organic dyes, gold nanoparticles were also used as a fluorescent probe for super-resolution microscopy; for example, Qiu et al. used gold nanoparticles (AuNPs) as a fluorescent probe for SIM. Cy5 dye was employed to make a bioinert gold core as a fluorescent material and polyethylene glycol spacer to render its biocompatibility. Figure 5a shows a schematic of the conjugation of Cy5 to AuNP (Cy5-AuNP). Figure 5b shows a time-dependent SIM image of lysosomes in HeLa cells stained with Cy5-AuNP, and Figure 5c shows the SIM images of lysosomes stained with different concentrations of CY5-AuNP.
PROBES USED FOR LOCALIZATION-BASED MICROSCOPY

The basic principle of the localization-based microscopy method lies in the sequential localization of a temporally separated stochastically switching individual fluorescent emitter for building a high-resolution image. These microscopic techniques are relatively more simple than patterned illumination-based microscopy, as they do not require complicated instrumentation and can be done by using any conventional wide-field or total internal reflection fluorescence microscope. The examples of these single-molecule localization-based methods (SMLMs) are photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and their variants. The essential criteria of localization-based SRM techniques are that the fluorophore should display adequate fluorescence blinking between on and off states, a large number of photon counts, and a number of switching cycles. Several studies have also been carried out for the development of various probes that could be used to image the lysosomes and study their dynamics using localization-based super-resolution microscopy techniques.

Zhang et al. designed a far-red photoactivatable borndipyrromethane (BODIPY) to be used as a PALM probe that selectively stained the lysosomes of COS-7 cells.23 One of the major advantages of using far-red probes was to minimize the autofluorescence that poses many obstructions in imaging. The BODIPY fluorophores were coupled to the styryl appendage and a photoswitchable auxochrome to efficiently utilize it as a PALM probe. The high mean photon counts ∼2000 assisted in resolving the lysosome structure up to a subnanometer level. Such high photon counts allow the unprecedented visualization of labeled lysosomes in living cells with localization precision of ∼15 nm. The intensity profiles of the blue and green highlighted regions in Figure 6 specify the size of individual lysosomes to be ∼80 nm.

Figure 7. (a) Scheme showing the construction of probes. (b) Diffraction-limited TIRF image and (c) super-resolution images of stained lysosomes. (d) Zoomed TIRF image of the boxed region in b. (e) Zoomed super-resolution image of the boxed region in c. (f) Cross-sectional profiles of lysosome shown in d, e. Scale bars: (c) 2 μm and (e) 200 nm. Reprinted with permission from ref 24. Copyright 2014. Nature Publishing Group.

Figure 8. (a) Wide-field and (b) PALM image of the lysosomes. (c and d) Zoomed images for the boxed areas from images a and b, respectively. (e) Intensity profile of a single lysosome along the lines in c and d. (f) Histograms of the number of photons per event. (g) Histograms of the localization precision. Scale bars: 2 mm (a and b); 300 nm (c and d). Reprinted with permission from ref 25. Copyright 2018. The Royal Society of Chemistry.
A photoswitchable organic fluorescent probe was developed by Pan et al. The probe consisted of an (rR)₃R₂ cell-permeable peptide, an epoxysuccinyl scaffold serving as the recognition unit to specifically label the lysosomes and Caged-Rh110 as a dye, as it is stable in acidic environment. The nonfluorescent Caged-Rh110 can be uncaged using a 405 nm laser light, and on exciting at 473 nm, it emits at 520 nm. The complete schematic of the formation of the probe has been illustrated in Figure 7. HeLa cells were stained with the above-specified construct, and the images were attained under continuous illumination at 473 and 405 nm (UV light intermittent activation). Finally, the images

Figure 9. (a) Bright-field and (b) conventional fluorescence images displayed the distribution of lysohighlighter. (c) Zoomed image of the conventional fluorescence image shown by the box in part b. (d) SRM image of the same box marked in part b. Images e and f are zoomed views of the boxes marked in parts c and d, respectively (g) Intensity profiles of a pair of neighboring fluorescent points along the dashed lines in part f. Reprinted with permission from ref 26. Copyright 2014. American Chemical Society.

Figure 10. (a) Protonation of Lyso-R produces Lyso-RH in situ in acidic lysosomes. (b) Super-resolved and (c) conventional microscopic images of lysosomes stained with Lyso-RH. (d) Time colored super-resolution images of lysosomes. (e) and (f) are zoomed images of the highlighted region in (b) and (c). (g) Intensity profile from (e) and (f). (h) Histogram of brightness and (i) localization uncertainties of a single molecule. (j) FRC analysis of (b). (k) The survival fraction of molecules. Scale bars: for b and c, 1 μm; for d, 4 μm; for d-1 to 5, 200 nm; for e and f, 300 nm. Reprinted with permission from ref 27. Copyright 2019. American Chemical Society.
were reconstructed using the PALMER algorithm, and 64 nm fwhm was achieved.

In the same direction, He et al. developed a nitroso-caged Rhodamine probe (NOR535) which was efficient for lysosomal PALM imaging. Their findings revealed that the non-fluorescent N-nitrosonaphthalimides exhibited fluorescence when excited with UV light. Although Rhodamine has been well explored for SRM due to its high brightness and photostability, NOR535 was also found to be brighter upon protonation in acidic medium, thus allowing its use to SRM image the lysosome. The PALM imaging of HeLa cells was done using a TIRF microscope. A 405 nm laser was used as an activation laser, and a 532 nm laser was used for its localization. The number of mean photon counts was found to be \( \sim 577 \), and a localization precision of 14.3 nm was obtained. A line profile of a single lysosome in reconstructed super-resolved (Figure 8) structure revealed the fwhm of \( \sim 86.9 \) nm.

Li et al. reported a lysohighlighter poly[NIPAM-co-(DTE-NI)], which was constituted of three units: (a) dithienylethene (DTE), (b) a photoswitching quencher, and (c) naphthalimide (NI). It is a pH-sensitive water-soluble polymer. The covalent conjugation of NI and DTE generated a photoswitchable fluorophore quencher pair, wherein Forster resonance energy transfer (FRET) led to turn on and turn off of the ability to modulate the emission of NI. HeLa cells were incubated with this construct for SRM imaging (Figure 9). Two fluorescent points with a resolution of 40 nm were obtained (Figure 9g).

Ye et al. designed a quaternary piperazine-substituted Rhodamine probe (Lyso-R) for SRM imaging of lysosomes. The N,N-dimethylpiperazine-substituted Rhodamine doubled the brightness while maintaining the biocompatibility. The presence of an electron-withdrawing group; i.e., the quaternary piperazine moiety withdraws the electron density from the amino group, resulting in destabilization of the twisted intramolecular charge transfer (TICT) effect and therefore leading to high brightness. At neutral pH, the Lyso-R probe exhibited weak fluorescence in deprotonated form that strengthens at acidic pH on protonation. This protonation in acidic medium leads to the creation of highly fluorescent Lyso-RH. This protonation induced a bright−dark transformation. SRM of lysosomes in live HeLa cells can be seen in Figure 10.


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Figure 11. (a) and (b) STORM images of HeLa cells stained with ROXSA and LysoTracker Red, respectively. (c) Recognized molecules per lysosome of HeLa cells loaded with LysoTracker Red or ROXSA. (d) Distribution of number of photons perceived for individual ROXSA or Lyso Tracker Red. Reprinted with permission from ref 28. Copyright 2018. American Chemical Society.

| S.N. | probe                           | emission maximum (nm) | techniques used for super-resolution | resolution/fwhm | ref no. |
|------|--------------------------------|------------------------|--------------------------------------|-----------------|--------|
| 1.   | carbon dots                    | 490                    | STED                                 | 30 nm in fixed cell, 67 nm in live cell | 14     |
| 2.   | green emitting Rhodamine dyes  | 522−543 (different dyes) | STED                                 | -               | 15     |
| 3.   | LysoPB yellow                  | 573−642 (with different solvents) | STED                                 | 70 nm           | 16     |
| 4.   | naphthalimide-based fluorescent compound (NIMS) | - | SIM | - | 17     |
| 5.   | cell-permeable organic fluorescent probes using (Alexa Fluor 647, Atto 565, and Atto 488) | as of Alexa Fluor 647, Atto 565, and Atto 488 | SIM | 90 nm | 18     |
| 6.   | Morph-Alexa 488, Morph-Alexa 647, Morph-Cy3B, Morph-Atto 514, and Morph-Atto 488 | as of Alexa 488, Alexa 647, Cy3B, Atto 514, and Atto 488 | SIM | - | 20     |
| 7.   | HD-Br                          | 712                    | SIM                                  | -               | 21     |
| 8.   | CYS®AuNPs                      | as of CYS              | SIM                                  | 550 nm          | 22     |
| 9.   | BODIPY                         | 670                    | PALM                                 | 80 nm           | 23     |
| 10.  | Rh110-based probe             | 520                    | PALM                                 | 64              | 24     |
| 11.  | NOR535                         | 550                    | PALM                                 | 86.9            | 25     |
| 12.  | NIPAM-co-(DTE-NI)              | 524                    | localization based                   | two fluorescent points separated by 40 nm apart can be well differentiated | 26     |
| 13.  | Lyso-R                         | 557                    | SMLM                                 | 70.3            | 27     |
| 14.  | ROXSA                          | 557                    | STORM                                | -               | 28     |
| 15.  | gold nanoclusters              | 625                    | SRRF                                 | 59              | 30     |

The corresponding emission maxima and the resolution obtained in each case are also presented.
The average photon count of $\sim 821$ leads to the localization precision of 13.9 nm. The width of the lysosome was found to be 70.3 nm.

Since Rhodamine is known for its photoswitching behavior between on and off states, Xue et al. reported lysosomal STORM imaging using Rhodamine-X-integrated sialic acid (ROXSA) and compared the results with commercially available lysosome staining dye Lyso Tracker Red. Owing to the higher photon yield of ROXSA over Lyso Tracker Red, the localization accuracy was enhanced in the case of ROXSA. Quantitative analysis revealed that the number of ROXSA molecules entered into the lysosomes was much higher in comparison to that of LysoTracker Red ($399.2 \pm 36.6$ vs $142.6 \pm 14.1$). Figure 11 shows the STROM image of lysosomes stained with ROXSA in HeLa cells.

SRM imaging is one of the prevailing techniques to resolve the ultrastructural dynamics of these structures. In this regard, Shim et al. identified numerous photoswitchable, small-molecule membrane probes and showed their efficacy in STORM imaging of mitochondria, plasma membrane, endoplasmic reticulum (ER), and lysosomes in living cells. The study reveals nanometer-scale morphological dynamics of neuronal processes, ER, and mitochondria, as well as nonuniform membrane diffusivity in neurons.

A recent publication by Yadav et al. has also efficaciously contributed in the field of SRM imaging of lysosomes (Table 1 and Table 2). They developed bovine serum albumin (BSA) coated gold nanoclusters for specific labeling of lysosomes. Gold nanoclusters are well-known for their interesting photoluminescent properties and easy renal clearance from the body. Additionally, the emission in the red region is also

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### Table 2. Chemical Structure of the Fluorescent Probes Used in Various Types of SRM Techniques

| S.N. | Probe | Chemical structure | Reprinted with permission from ref 23. Copyright (2018) The Royal Society of Chemistry. |
|------|-------|--------------------|----------------------------------------------------------------------------------|
| 1.   | Green emitting Rhodamine dye | ![Green emitting Rhodamine dye](image) | |
| 2.   | Lysotracker yellow | ![Lysotracker yellow](image) | |
| 3.   | Naphthalamide-based fluorescent compound (NIMB) | ![Naphthalamide-based fluorescent compound (NIMB)](image) | |
| 4.   | Cell permeable organic fluorescent probes using Alexa Fluor 647, Atto 365 and Atto 488 | ![Cell permeable organic fluorescent probes using Alexa Fluor 647, Atto 365 and Atto 488](image) | |
| 5.   | Morph-Alexa 647, Morph-Alexa 647, Morph-Cy3B, Morph-Atto 544 and Morph-Atto 488 | ![Morph-Alexa 647, Morph-Alexa 647, Morph-Cy3B, Morph-Atto 544 and Morph-Atto 488](image) | |
| 6.   | HD-Rh | ![HD-Rh](image) | |
| 7.   | Cy3-AuNP | ![Cy3-AuNP](image) | |
| 8.   | BODIPY | ![BODIPY](image) | |
| 9.   | Rh103 based probe | ![Rh103 based probe](image) | |

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**Table 2. continued**

| S.N. | Probe | Chemical structure | Reprinted with permission from ref 26. Copyright (2014) American Chemical Society. |
|------|-------|--------------------|----------------------------------------------------------------------------------|
| 10.  | NBD535 | ![NBD535](image) | |
| 11.  | NIPAM-co- (DTE-N) | ![NIPAM-co- (DTE-N)](image) | |
| 12.  | Lyso-R | ![Lyso-R](image) | |
| 13.  | ROXSA | ![ROXSA](image) | |

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beneficial in reducing the autofluorescence from the biological samples. They have used super-resolution radial fluctuations (SRRFs) as the SRM technique, which is a threshold-free algorithm based on analysis of the image sequence. The lysosomes stained with gold nanoclusters were excited using a 532 nm laser, and the images were collected with the help of an EMCCD camera. Finally, the image sequence was analyzed with the help of SRRF, and they were able to find the diameter of the single lysosome ∼59 nm (Figure 12), which is close to the actual smallest size of the lysosome. The average photon counts were found to be ∼7237, and the localization of a single gold nanocluster was found to be ∼24 nm, due to which the lysosomal diameter on such a small range could be resolved.

■ CONCLUSION
In this mini-review, we outlined a variety of nanoprobes that exhibited promising properties for the SRM imaging of lysosomes in living or fixed cells. Numerous reports for the development of the probes for light microscopy have been noted. We hope that this mini-review will be helpful to readers in understanding the current state-of-the-art of the lysosome imaging and finally will allow us to design new fluorescent probes for the lysosomes dynamics in the cell.

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Notes
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Chethana Rao pursued her Master’s degree in physics from NITK, Surathkal, India. In 2017, she started her Ph.D. under the supervision of Dr. Chayan Kanti Nandi, at IIT Mandi, India. Her research is based on exploring methods of exploiting the protein corona for advancing the rational design of drug delivery systems with better clinical success.

Chayan Kanti Nandi received his Ph.D. in 2006 from the Indian Institute of Technology Kanpur, India. He completed two consecutive postdocs from Germany in 2009 and at Princeton University in 2010. Currently, he is an associate professor at the Indian Institute of Technology Mandi, India. He has been awarded as an Alexander von Humboldt fellow in 2007. In 2019, he received the CSIR bronze medal award for his outstanding research in chemistry. His current research is to design new fluorescent nanoprobes for localization-based super-resolution microscopy.

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