Supporting Information

Dual Responsive Specifically Labelled Carbogenic Fluorescent Nanodot for Super Resolution and Electron Microscopy

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Material and Methods:

Materials. All glasswares were washed with aqua regia (3 HCl/1 HNO_3), followed by rinsing several times with double distilled water. Citric acid, urea, EDC and sulpho-NHS were purchased from Sigma-Aldrich. DMF, Methanol was purchased from Fisher Scientific. phalloidin was purchased from aminta phalloides. All the dyes were purchased from Sigma-Aldrich. NaOH was purchased from Merck chemicals. Double-distilled (18.3 MΩ) deionized water (ELGA PURELAB Ultra) was used throughout the entire process. .

(a) Synthesis and Purification of quasi FND:

Supplementary discussion 1:

The citric acid (1 g) and urea (2 g) were reacted at 200° C for 6 h under solvothermal condition in 10ml DMF and then cooled to room temperature. The obtained dark brown solution was mixed with 20 ml NaOH (50 mg mL^{-1}), stirred for 1 min, and then centrifuged at 16000 rpm for 10 min (Sorvall LYNX 6000, Thermo Scientific). The precipitate was collected, dissolved in water and centrifuged (16000 rpm, 10 min) twice to wash off residual salts and alkali. The synthesis condition produces quasi molecular carbogenic nanodots, quasi FND (fluorophore attached to carbgenic core) and also unbound molecular fluorophore. To get the desired quasi FND, it was dialyzed against Milli-Q water for 8 days using 1 kDa dialysis membrane to remove the unbound molecular fluorophore. The water was changed every 24 hours. The removal of the free fluorophore was verified by measuring the absorption and emission spectra of the dialysate in every 24 hours. Thus, after 8 days the dialysate didn't show any absorption or emission spectra confirming the complete removal of the free fluorophore. The retentate was collected for further dialysis using a 3.5 kDa membrane. The dialysate was collected as the desired material and finally it was column purified to get the ultrapure highly homogeneous quasi FND. Interestingly, the perfect matching of the fluorescence spectrum of the dialysate coming out

from 1 kDa membrane (free molecular fluorophore) and the quasi FND suggested that the same molecular fluorophore attached to the carbogenic core.

Supplementary discussion 2: Synthesis of FND-Phalloidin conjugates

To get the covalent conjugation of FND with amino-phalloidin the FND were activated through EDC coupling reaction, which is a popular and versatile method for labelling or crosslinking of carboxylic acids (-COOH) to the amine group. In brief, 0.2 mL of FND (20 mg/mL) aqueous solution and 0.8 mL of amino-phalloidin (0.1 mg/mL, methanol) were mixed and diluted to a total volume of 4 mL in borate buffer (0.2 M, pH 8.5). EDC and Sulfo-NHS were then added into the solution at a stoichiometric molar ratio of FND/EDC/Sulfo-NHS = 1/5/5. The reaction was allowed to carry on for 3h at room temperature with gentle mixing. The obtained product was dialyzed using a 1kDa molecular weight cut off dialysis membrane against Milli-Q water for overnight to eliminate the excess EDC and Sulfo-NHS.

(b) Characterization of quasi FND

UV-Vis Absorption and steady state fluorescence spectroscopy:

The UV-Vis absorption spectra were recorded using Shimadzu UV-Vis 2450 spectrophotometer. The spectra were collected using a quartz cuvette having 10 mm path length and 1 ml volume. All the measurements were repeated at least three times. Steady state fluorescence was measured using Horiba Fluorolog-3 spectrofluorometer. The fluorescence was measured in 1 ml quartz cuvette.

Transmission Electron Microscopy (TEM):

The particle size and dispersity of the synthesized nanoparticles were performed using a TECNAI G2 200 kV TEM (FEI, Electron Optics) electron microscope with 200 kV input voltage. TEM grids were prepared by placing 5 µL diluted and well sonicated sample solution on a carbon coated copper grid and evaporated the solution at room temperature completely. Precautions were taken to avoid contamination from various sources like dust particles and glassware.

X-ray Diffraction (XRD):

Powder X-ray experiment was carried out using powder X-ray diffractometer which is built on 9 KW rotating anode x-ray generator with NaI Scintillation counter detection system. Copper anode were used as the target material with fine focus filament as the cathode. The samples were freeze dried to prepare dry powder before XRD experiment.

Raman Spectroscopy:

The Raman spectrum was measured by the confocal Microscope Raman spectrometer (Horiba Scientific, XploRA ONE) with 633 nm laser. Sample preparation for analysis was done by putting the drop of FND on the quartz slide, followed by drying inside a 37˚ C incubator.

Fourier Transform Infrared Spectroscopy (FTIR):

FTIR spectra of FND were measured using a Perkin-Elmer FTIR spectrophotometer equipped with a horizontal attenuated total reflectance (ATR) accessory containing a zinc selenide crystal and operating at 4 cm^{-1} resolution. The use of the spectral subtraction provided reliable and reproducible results.

X-ray photoelectron spectroscopy (XPS):

X-ray Photo-Electron Spectroscopy (XPS) with Auger Electron Spectroscopy (AES) module PHI 5000 Versa Prob II, FEI Inc. and C60 sputter gun have been used for characterization and scanning the spectra from C1s, N1s, and O1s region. Al Kα X-ray radiation was used as the 4 source for excitation (1482.5 eV, 500 mm). Samples were loaded on copper strips, and surface adherence done by double sided adhesive tape. The sample was freeze dried before loading.

Cytotoxicity test of FND:

HeLa cell line was procured from National Centre for Cell Science, Pune, India. The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco™) and incubated at 37° C in a humid atmosphere containing 5% CO_2. For cytotoxicity experiments, the cells were seeded at a density of 5000 cells/well in a 96 well plate and allowed to adhere for 24h. The adhered cells were treated with 100 µL of different concentrations (1, 10, 25, 50, 100µg/mL) of FND along with positive and negative controls. After 24 h of incubation, 10 µL of 5 mg ml^{-1} 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and was further incubated for 3h. The cells were then lysed with 100 µL Dimethyl sulfoxide and the absorbance was read at 570 nm (reference 650 nm) using Tecan Infinite M200 PRO plate reader.

Confocal Imaging of HeLa cells labelled with FND

Coverslip preparation: The glass slides and coverslips were cleaned by the sonication in ethanol, followed by incubation in HNO_3 and KOH solution for 20 min each and slides were washed by MiliQ water after every step and finally dried by Nitrogen.

Cell Culture, fixation and staining: HeLa cells were grown in Dulbec co's Modified Eagle Medium (DMEM) with 10% fetal bovine serum. The cells were grown in 6-well plate on the

coverslips. Each well was filled with 2 ml of cell suspension in growth medium and the cells were allowed to grow overnight for the proper confluency. The growth and the attachment of the cells to the coverslips were examined by an optical microscope. Once the cells reached proper confluency, they were fixed by incubating with 4% paraformaldyhyde solution in 1x PBS buffer for 5min. After fixation the cells were permeabilized by 10min incubation in 0.1% tritonX . The fixed and permeabilized cells were washed 4-6 times by PBS buffer to remove extra agents. These cells were incubated with the 1 μg/ml FND solution for 2hrs at room temperature to achieve enough labelling density for confocal microscopy or SRRF. The Atto 647N-phalloidin labelled cells were prepared in a similar manner. The DAPI was used to stain the nucleus for the two color confocal imaging. The samples were washed rigorously with PBS to remove any nonspecific attachment of the dye or FND on the coverslip to reduce the background. The coverslips were fixed on a glass slide before imaging.

Confocal microscopy: Nikon Eclipse Ti inverted microscope was used for the confocal microscopy and images were acquired using Nikon Nis-Element software. The cell samples were excited by the two lasers 401 nm for DAPI and 561 nm for FND to achieve two color imaging, whereas the Atto 647N labelled cells were excited with 639 nm laser and the images were collected by choosing a proper filter set.

Single molecule time trace and photon counts

FND were spin coated on a clean glass slide to collect single molecule time traces. The diffraction limited spots produced due to single molecule blinking were observed using 100x Nikon TIRF objective. A 532 nm diode laser with 50 mW maximum power was used. An oil immersion Nikon TIRF objective (100x magnification and 1.49 NA) was mounted on a custom build inverted optical microscope. A 532 nm high pass Dichoric (AHF Analysentechnik) was used to separate the excitation and emission light. Another 635 nm excitation laser and corresponding high pass Dichoric (AHF Analysentechnik) were used with 680/20 nm band pass filter (AHF Analysentechnik) for excitation. Andor EMCCD iXon Ultra was used to record the single molecule photon events at the frame rate of 17 MHz and exposure time of 50 ms. The time trajectories were recorded and analyzed using the Andor Solis Software. The incident photons were converted to electrons and subsequent to digital counts by the EMCCD. The Andor Solis and Matlab ware used to extract the total counts along with its maximum, mean and standard deviation values from a group of pixels. For the movies, recorded under the kinetic mode of EMCCD, time/frame trajectories of the counts/intensity at a given pixel, were obtained for the provided exposure time. These time trajectories were saved for further

analysis. The image area of only 64x64 pixels was taken from the total 512x512 pixels of the whole sensor of EMCCD. A small area of the sensor was used for faster image acquisition using the crop-sensor mode of iXon EMCCD. Thus, we used the total image area of 64x64 pixels (10.24µm x 10.24µm) with each pixel size of 106.7 nm using 100x objective and 1.5x extra magnification.

Single Molecule Localization:

For the localization experiments, the above mentioned dichroics and filter combination were used. The image is projected on the sensor of EMCCD using a focussing lens in the emission path. The number of photons per pixel was determined using the photon counting mode of EMCCD and the background was calculated from the image area in the absence of the molecules during the measurement. The mean value of this background was subtracted from the original trajectory to obtain a background free signal. For further correction, background signal (after complete photobleaching) was also set to zero level. GDSC ImageJ Plugins was used for optical reconstruction.(open source code available on http://www.sussex.ac.uk/gdsc/intranet/ microscopy/imagej/smlm plugins). For the localization of single FND, all photons emitted by the FND were used and the localization was performed by SMLM-GDSC plugin which calculate the theoretical limit (precision) for fitting the signal (number of photons) and the XY coordinates (localisation). The background noise is estimated by the PEAK FIT plugin during the fitting process using a global noise estimate per frame. A camera with 16 µm pixel size and a 100x objective with 1.5 magnification (pitch of 16000/150= 106.7 nm) was used. Exposure time of 50 ms, wavelength of 612 nm and 1.40 numerical aperture of the objective was chosen. The standard deviation of the Gaussian approximation to the PSF was calculated by the following formula.

$SD=p\times1.323\times\lambda/2\pi NA$

Where λ is the wavelength (in nm), NA is the Numerical Aperture and p is the proportionality factor. A value of p=1 gives the theoretical lower bounds on the peak width. However, the limitations of the microscope optics and stage drift leads to a fitted width, which is often wider than this limit. The p value was taken as 1.52. Data processing involves the method of fitting Gaussian functions to individual molecule fluorescence blinking events. The EMCCD used here worked with a 17MHz readout rate with 5-50ms exposure times which gives around 200-20 s^{-1} frame rate for the camera. The on time of the FND comes out to be around 0.01-10.00s with 20 mw laser power, which can be detected at these camera frame rates. One of the causes for the artifacts in localization based microscopy is the nonrobust switching which results in the detection of a lower number of fluorophores than the actual and hence the resultant image

contains fewer details. On the other hand, in the absence of proper blinking the background signal or multiple emitters can be detected as a single fluorophore.

Data acquisition for SRRF:

For the cell imaging experiments, same microscope, dichroic and filter combination were used. An image projection lens was used to project images at the sensor of EMCCD. The number of photons per pixel was determined using EMCCD photon counting mode. The Andor iXon Ultra EMCCD camera has a pixel size of 16 µm x 16 µm , so on a system with a 100X objective and 1.5x magnification lens, the pixel size on the image would be 106.7 nm (16 µm/150). The background was calculated from the image area where no molecule was present during the measurement time. The mean value of this background was subtracted from the original trajectory to obtain a background free signal. Data was processed by the open source NanoJ SRRF plugin of ImageJ.

SRRF

The SRRF is a threshold free algorithm based on the analysis of the image sequence. It considers that the image is convoled with a point spread function (PSF). Each of the PSF created by the single molecules contained a higher degree of local geometrical symmetry than the background. It is better than the single molecule detection because it calculates the local gradient convergence or termed as radiality in the whole frame by dividing each pixel to sub pixels, which preserve the information in the gradient field which would be discarded by any other localization technique. The radiality distribution is independent of the PSF intensity and FWHM of this distribution can be adjusted by gradient convergence radius. The full image created by the radiality distribution can acquire the image noise corresponding to non fluorophore associated radiality peaks, but it can further be de-noised by the time series analysis, the increase in FWHM of the radiality distribution or the radiality map waiting with the flourophore intensity. In the time series analysis, the higher order temporal cumulants can be calculated and the noise reduction happens because of the uncorrelated noise peaks in the time series and high order correlated peaks at the center of the actual fluorophore. TIRF imaging of fixed actin filaments was performed using a custom build inverted optical microscope in TIRF mode. A 100X TIRF objective (1.49 Oil immersion, Nikon) was used with additional 1.5 magnification to collect fluorescence onto an EMCCD camera (Andor iXon Ultra), yielding a pixel size of 107nm (16 µm /1.5x100), and excitation was provided by a 532nm laser. To perform single-molecule localization, we recorded a time series with 0.05 s acquisition time

per image. The 8min movie contained 10,000 stacked images, which were analyzed with an open source version of the NanoJ-SRRF on a high performance GPU having 1076 cores. The ring radius chosen for the SRRF analysis was optimized and the best images were obtained at the ring radius 0.5. The chosen radially magnification was 5 times of the original pixel size. Before the SRRF analysis drift correction was done using the correlation method and the final image was displayed as the Temporal Radiality Pairwise Product Mean (TRPPM) method where the final image is the raw second moment of the radiality integrated over the time series and giving a proper noise reduction.

Error analysis

The quality of Superresolution image was further tested using an open source ImageJ plugin called NanoJ-SQUIRREL (Super-resolution Quantitative Image Rating and Reporting of Error Locations). It is a software package designed for assessing and mapping errors and artifacts within any generated super-resolution images. This analysis is done through quantitative comparison of the superresolution image with a reference image of the same structure (typically a widefield, TIRF or confocal image). SQUIRREL produces quantitative maps of image quality and resolution as well as global image quality metrics. First quality parameter is Resolution scaled error and resolution scaled Pearson's coefficient calculation combined with error map generation. Error map is generated by calculating a Resolution Scaled Function (RSF) or superresolution image in comparison to widefield or TIRF image. The superresolution image was divided in time to generate two frames of the reconstructed structure from the data recorded in a different time. SQUARIAL plugin generates the RSE and RSP values for each superresolution frame and an error map shown in yellow-purple colorrmap in which the yellow regions show poor agreement with superrresolution image i.e high error regions and purple regions have low error.

FRC

The FRC analysis was done with SQUARIAL software. FRC measures the similarity of two images as a function of spatial frequency, giving a quantitative estimate of the resolution. Again, FRC map needed two statistically independent superresolution images that were generated by dividing the total image sequence in two parts, separate sequence of even numbered frames and odd numbered frames. This will generate statistically independent superresolution image of the same structure. The whole SRM image, generated by SRRF, is divided into 10x10 equal spatial subblocks(total 100 subblock) and the FRC analysis was performed on each individual subblock. The pixel size of the super-resolution image was chosen 21 nm for the calibration of the FRC results.

TEM imaging of the cells

The cells were grown as described in the cell culture section of methods. The cells were trypsinized and the cell suspension was washed with PBS buffer. Then the cells were fixed in the solution with 4% paraformaldehyde and permeabilized with 0.5% tritonX. The permeabilized cells were washed and centrifuged before staining. The cells were incubated for 4 hours with 2 μg/ml FND solution for staining. After staining the cells were recovered from the FND solution carefully through centrifugation and further by washing. Finally the washed cells were dropcasted on the carbon coated copper TEM grids and frozen at -80^0C for 12 hours. The frozen cells were dried using a lyophilizer for 18 hours before the TEM imaging.

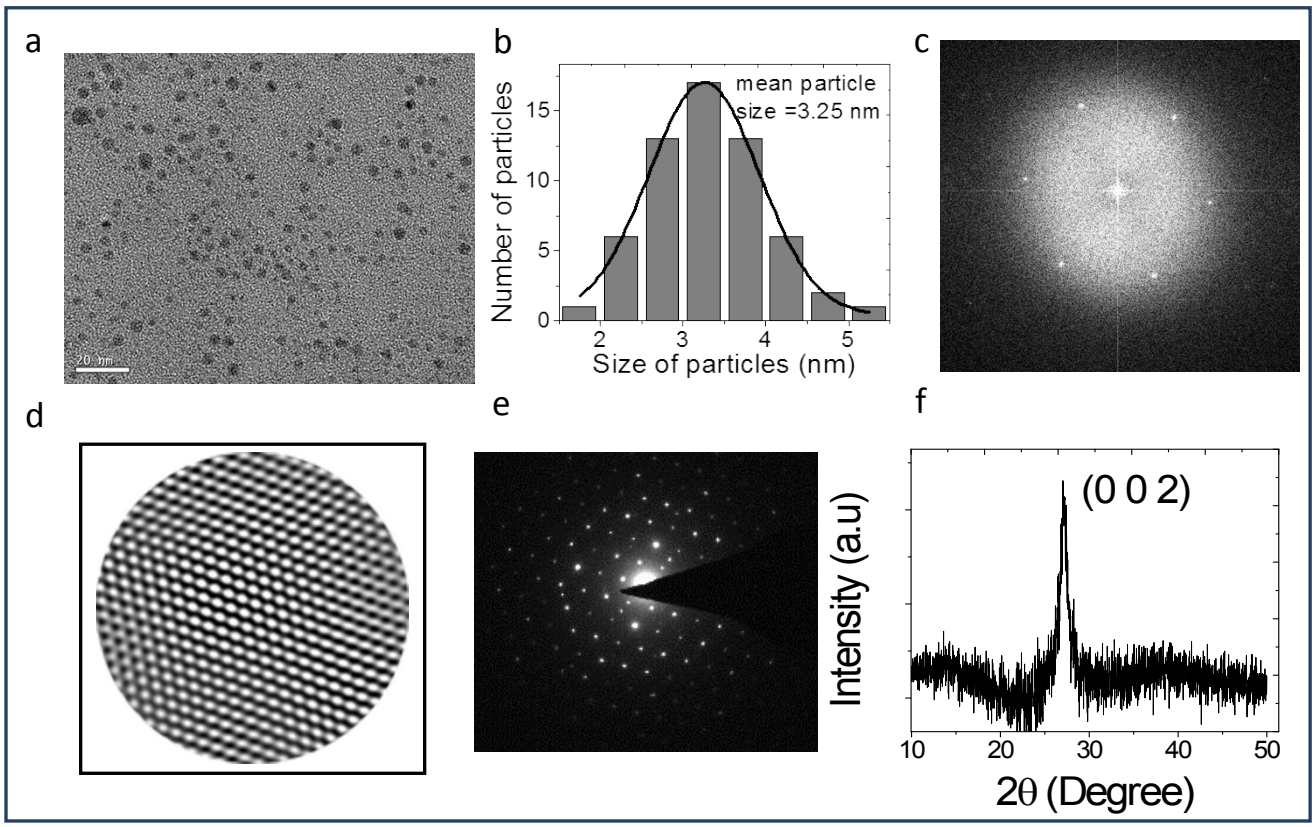

Figure. S1: (**a)** TEM shows the homogeneous particle distribution with average size of 4 nm.**(b)** particle size distribution histogram shows average particle size 3.2 nm (**c-d)**, FFT, inverse FFT and SAED confirms the single crystalline pattern of FND with hexagon al symmetry (**f**) X-ray diffraction pattern shows 002 carbogenic graphitic plane.

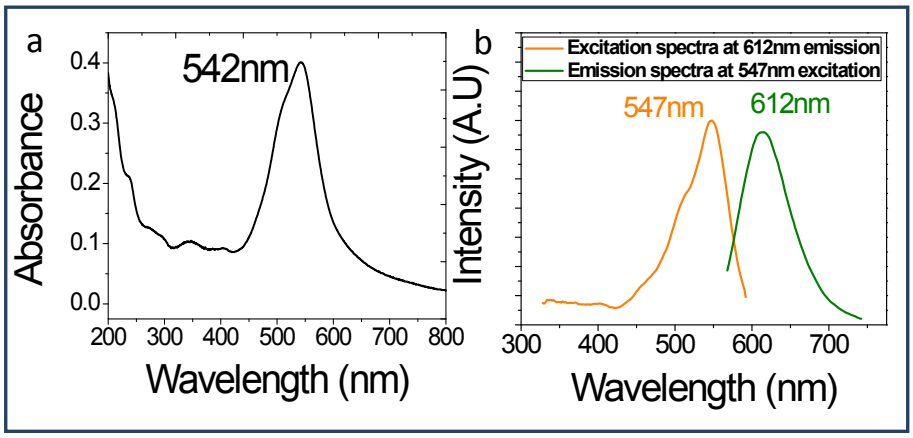

Figure. S2: (a) UV-Visible absorbance of FND shows an absorption peak at 542 nm (**b**) Fluorescence excitation and emission spectra of FND. The excitation peak is at 547 nm and the emission peak is at 612 nm.

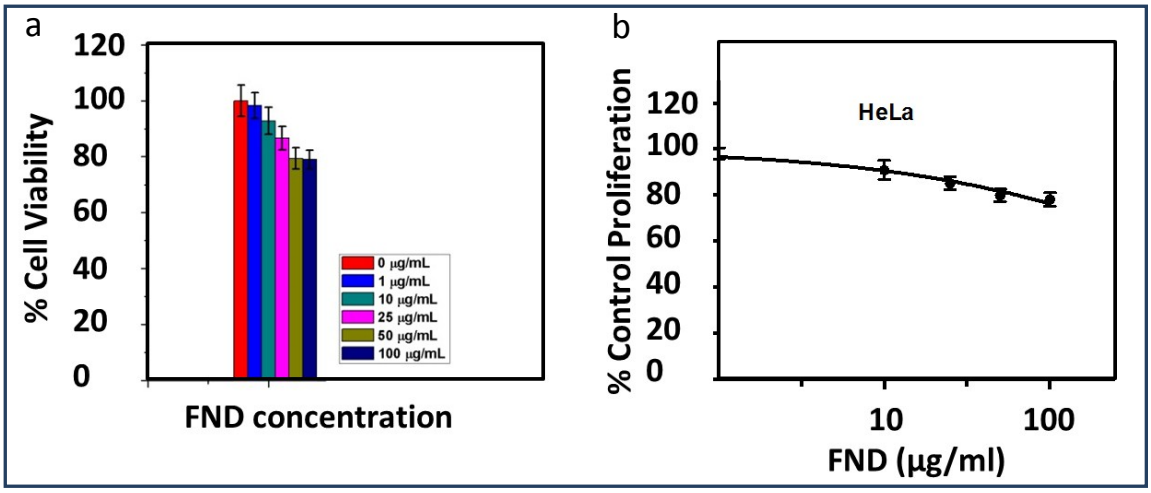

Figure. S3: (a) FND are almost non-toxic as shown by the cytoxicity measurement using MTT assay on HeLa cells with increasing concentration from 0-100 µg/ml. (b) The measured IC50 values: the best fit IC50 that came out upon extrapolation of the graph, was 1169 µg/ml. In our studies, the concentration of FNDs used was 1µg/ml and that was enough for labelling density for confocal microscopy or SRRF.

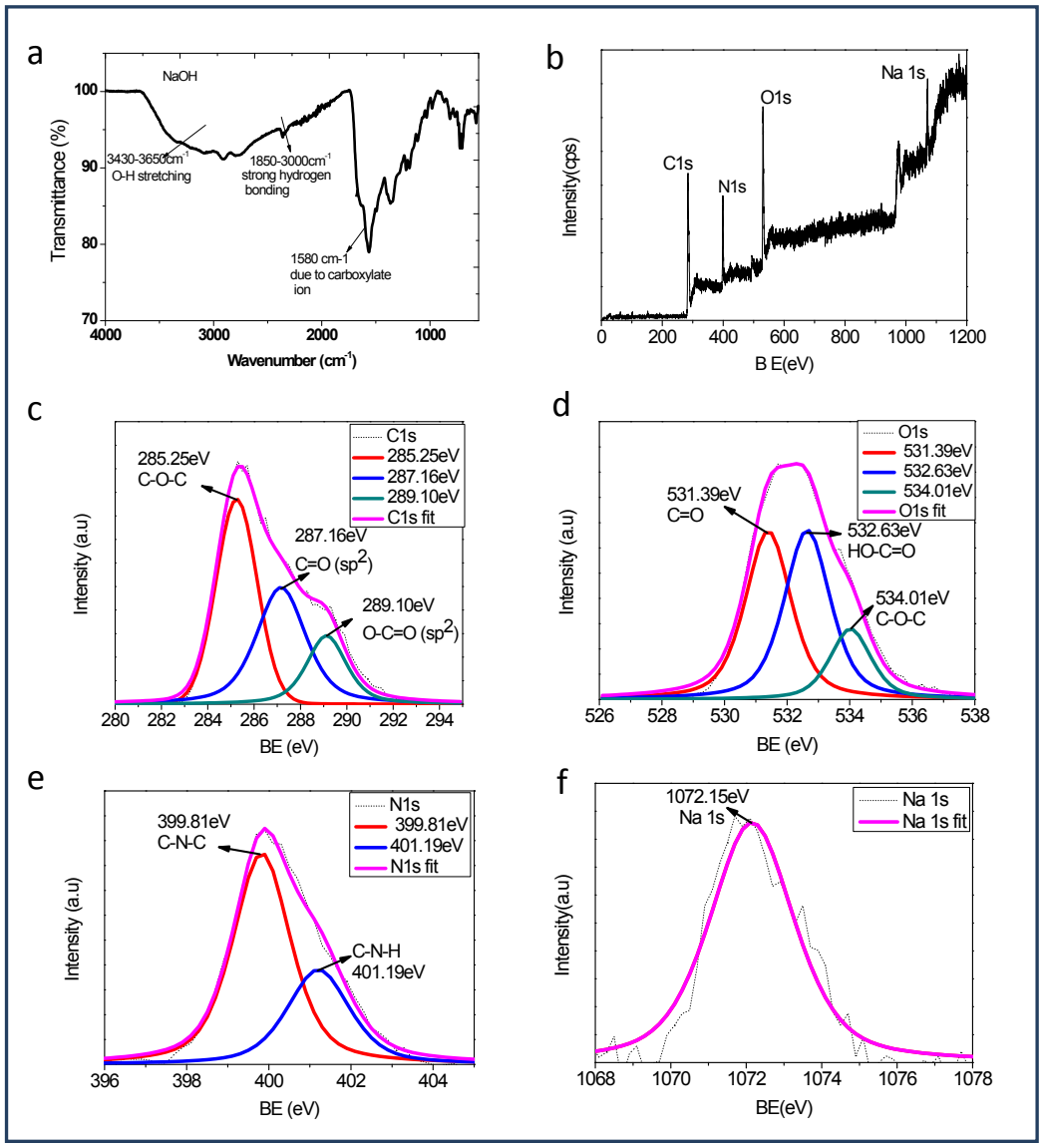

Figure. S4: (a) The Fourier transform Infrared Spectroscopy (FTIR) and (**b-f)** The XPS of the FND suggests the presence of carboxylic acid group that is used for coupling with phalloidin.

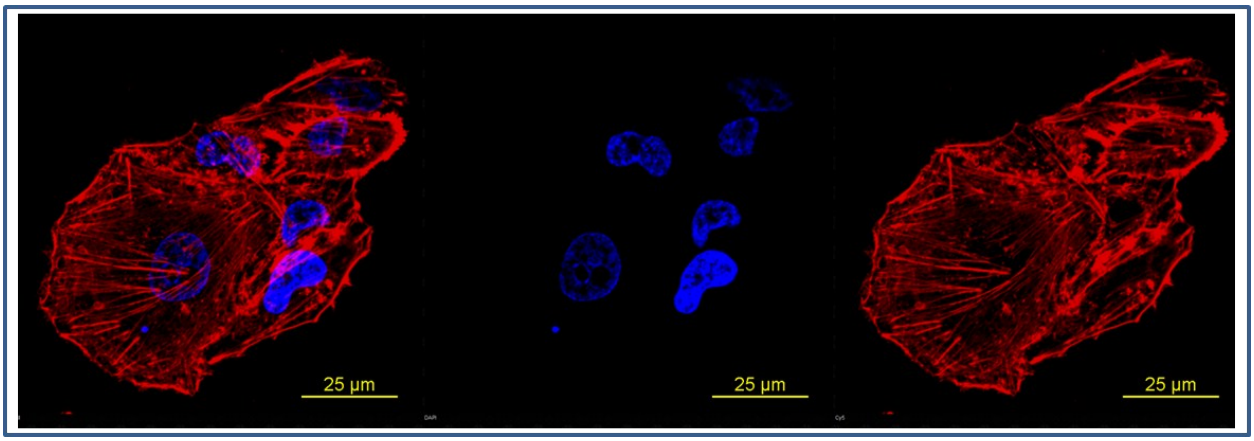

Figure. S5: Two color conofocal imaging of HeLa cells in which actin filaments were stained with Phalloidin-Atto647N shown in red color. Nucleus of the cells is stained with DAPI shown in blue color.

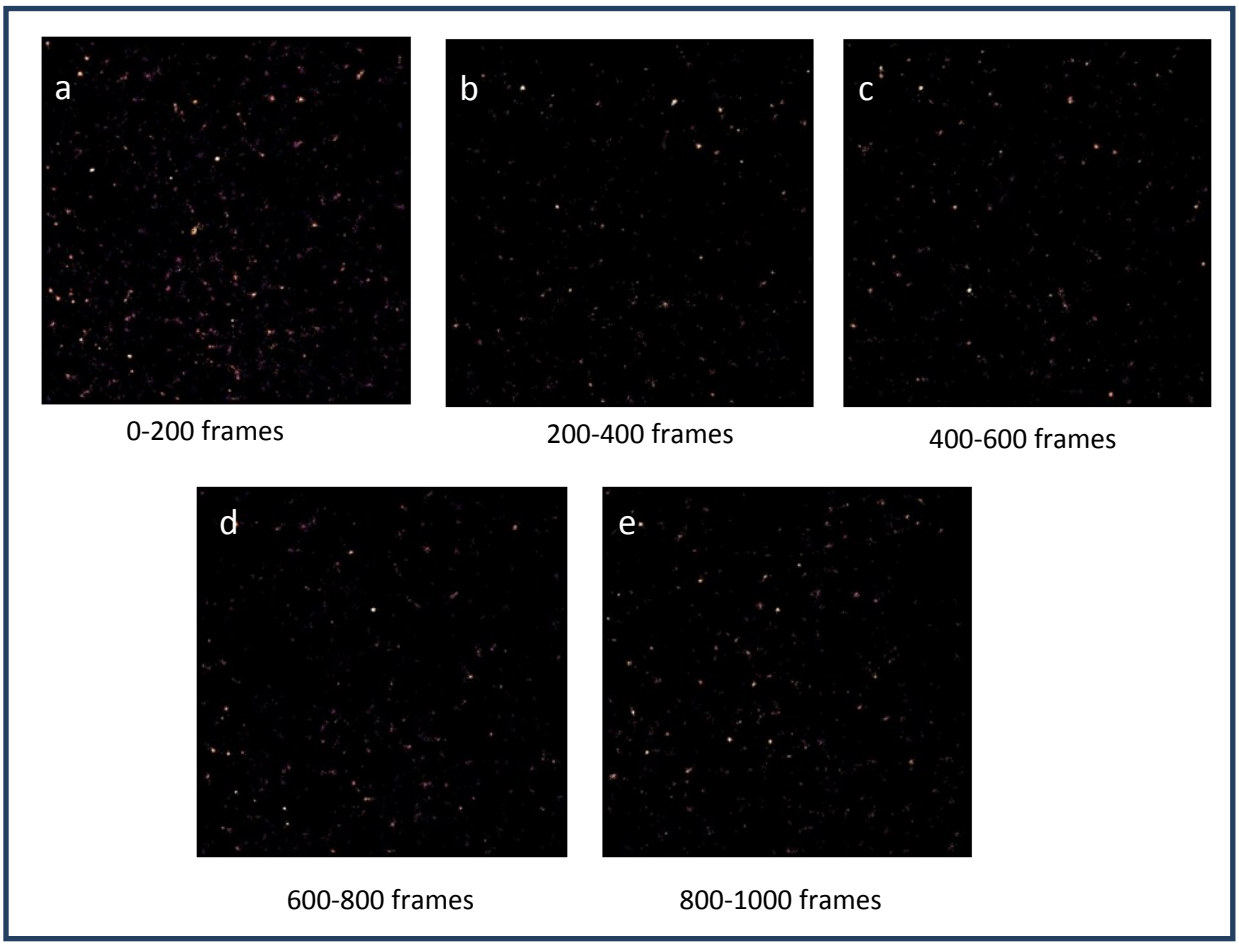

Figure. S6: Single particle localization of FND obtained from spin coated sample and with the excitation using 532 nm laser under TIRF illumination. (**a-e**) The number of localization decreased with increasing frame numbers due to photobleaching with 20% of the molecule survived even upto 1000frame (1 minute at 50 ms/frame)

Table S1. Comparison of the photon counts, on-off duty cycle and number of switching cycles of FND with the reported dyes and fluorescent proteins.

S.N.	Fluorescent probe	Excitation Wavelength (nm)	Emission Wavelength (nm)	Photons counts	Number of switching cycles (mean)
1	**FND (This work)**	542	612	6,879	6.2
	Other dyes[3]				
2	Cy3	550	570	8,158	1.6
3	Cy2	489	506	3,208	0.7
4	Cy3B	559	570	2,057	5
5	Day Light 750	752	778	749	6
6	Cy7	747	776	997	2.6
7	Alexa Fluor 750	749	775	703	6
8	Alexa Flour 790	785	810	740	2.7
	Fluorescent proteins[4]				
9	PAmcherry1	564	595	725	1
10	Dronpa	503	518	120	60
11	Dreiklang	511	529	700	-----
12	MEos2	569	581	1000	2.8
13	MEos3.2	572	580	1000	2.4
14	mMaple	566	583	1000	3.4
15	mMaple2	566	583	800	-----

References

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2. J. V. Staros, R. W. Wright, and D. M. Swingle, Analytical biochemistry 1986, **156**, 220-222.
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