**Supporting Information**

# Multicolor Fluorescent Peptide-Nanoparticle Scaffold: Real Time Uptake and Distribution in Neuronal Cells

Kallol Bera[a], Shounak Baksi[b], Moupriya Nag[a], Subhas Chandra Bera[a], Debashis Mukhopadhyay[b] and Soumen Basak[a,*]

[a]Chemical Sciences Division & [b]Biophysics and Structural Genomics Division, Saha Institute of Nuclear Physics, Kolkata 700064, India

**S1. Materials:**

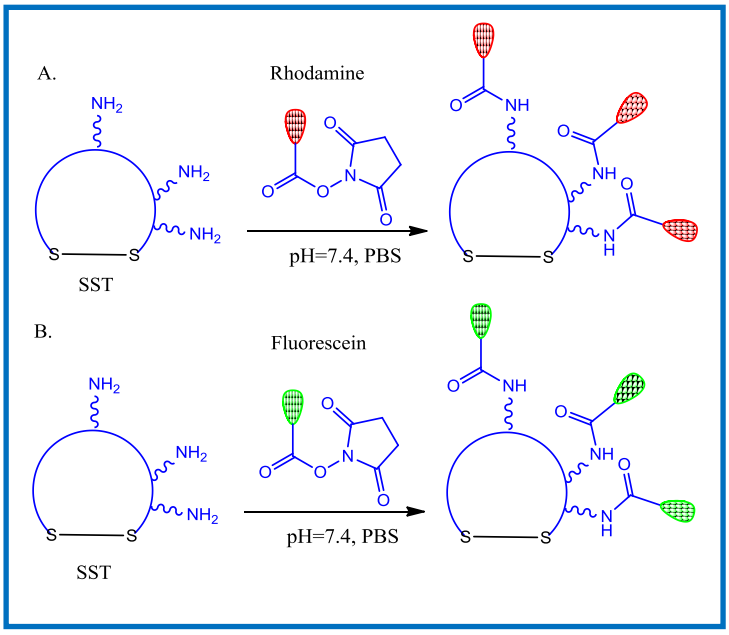
All solvents were of analytical grade and used without further purification. Disodium hydrogen orthophosphate dehydrate ($Na_2HPO_4$) and Potassium dihydrogen orthophosphate ($KH_2PO_4$) were purchased from S.D Fine Chem Ltd (Mumbai, India).Sodium Chloride (NaCl) is obtained from Fisher Scientific (India). Potassium Chloride (KCl) and calcium dihydrate ($CaCl_2.2H_2O$) were obtained from SRL (Mumbai, India. Magnesium Sulphate was purchased from Anala R, Glaxo laboratories (Mumbai India). Somatostatin, $AuCl_4$, $NaBH_4$, Propidium Iodide, poly-L-Lysine and Dulbecco's Modified Eagle's Medium (DMEM), 5-Carboxy-tetramethylrhodamine N-succinimidyl ester, 5-Carboxyfluorescein N-succinimidyl ester were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). Penstrep, Fetal Bovine Serum (FBS), DMEM-F12 media and Trypsin were purchased from Gibco (Grand Island, NY, USA). L-glutamine, B-27 supplement, Hepes, Neurobasal medium were purchased from Invitrogen. 96 well plates and petridish was purchased from Tarsons (Kolkata, India). Fluor 546 F(ab')2 fragment of goat anti-mouse IgG (H+L) (A11018) was purchased from Invitrogen, USA. Early endosomal marker antibody mouse anti-EEA1 (ab15846) was purchased from abcam, USA.

**S2. Synthesis:**

**(a) Synthesis of rhodamine-B and fluorescein labeled somatostatin:**

Rhodamine-B labeled somatostatin [RhB:SST] and fluorescene labeled somatostatin [Fl:SST] were synthesized according to reported procedure[1].

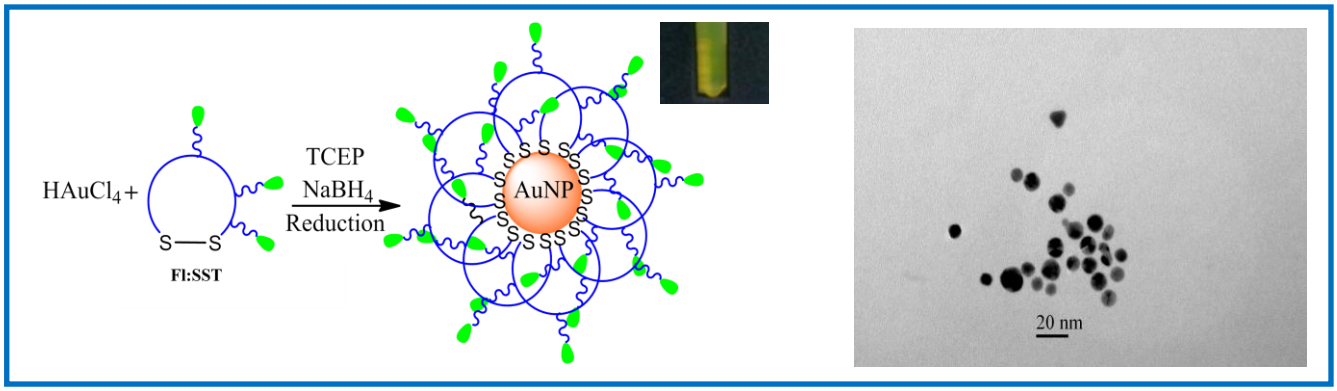
**Scheme S1:** Tagging of Different fluorophores with SST.



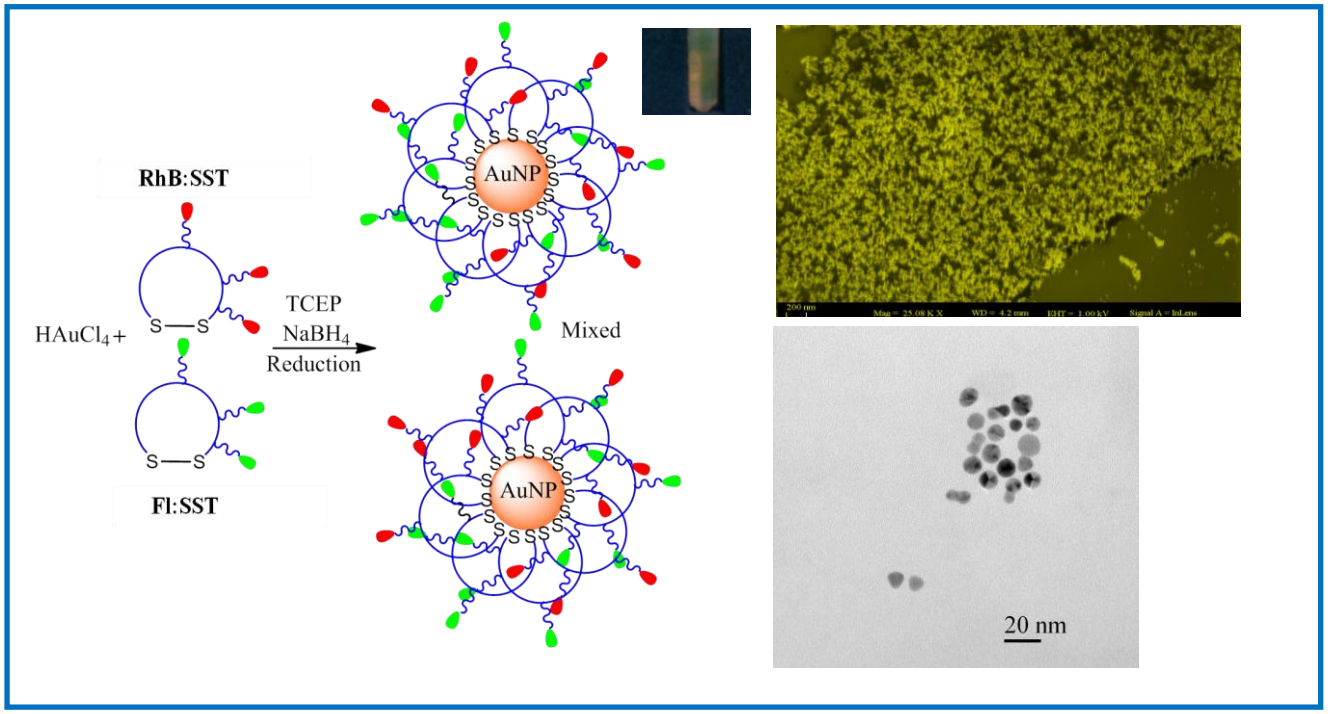

**(b) Synthesis of Fl:SST-AuNPs [2]:**

$HAuCl_4$ solution (50 mM, 20 µL) was incubated with lyophilized Fl:SST (100 µL, 50 mg/mL) and TCEP (200µL,10 mM) for 1 h in an ice-bath to which $NaBH_4$ (100 mM, 20 µL) was added drop wise with constant stirring and the mixture was incubated overnight at $4^0$C. Fl:SST coated AuNP were obtained by further purification via centrifugation (three times) followed by dialysis (three times) to remove free dye and other impurities. Then the solution was further purified by size exclusion chromatography and AuNPs solution was lyophilized and stored at $-20^0$C. The NPs systems were characterized by UV-visible, steady state and time resolved fluorescence, fluorescence correlation spectroscopy (FCS), FT-IR, CD, TEM, SEM and LCSM measurements.

**Scheme S2:** Synthesis of fluoroscein-somatostatin capped AuNPs [**Fl:SST-AuNPs**] and corresponding TEM /SEM image.




**Scheme S3:** Synthesis of RhB-SST and Fl:SST capped AuNP (**RhB/Fl:SST-AuNPs**) and corresponding TEM/SEM image.




**(c) Synthesis of Multicolor RhB/Fl:SST-AuNPs [3]:**

$HAuCl_4$ solution (50 mM, 20 µL) was incubated with lyophilized RhB:SST (50µL, 30mg/ml), Fl:SST (50 µL, 20 mg/mL) and TCEP (200µL, 10 mM) for 1 h in an ice-bath to which $NaBH_4$(100 mM, 20 µL) was added drop wise with constant stirring and the mixture was incubated overnight at $4^0$C. RhB/Fl:SST coated AuNP were obtained by further purification via centrifugation (three times) followed by dialysis (three times) to remove free dye and other impurities. Then the solution was further purified by size exclusion chromatography and AuNPs solution was lyophilized and stored at $-20^0$C. The NPs systems were characterized by UV-visible, steady state and time resolved fluorescence, fluorescence correlation spectroscopy (FCS), FT-IR, CD, TEM, SEM and LCSM measurements.

**S3. Visual Color:**

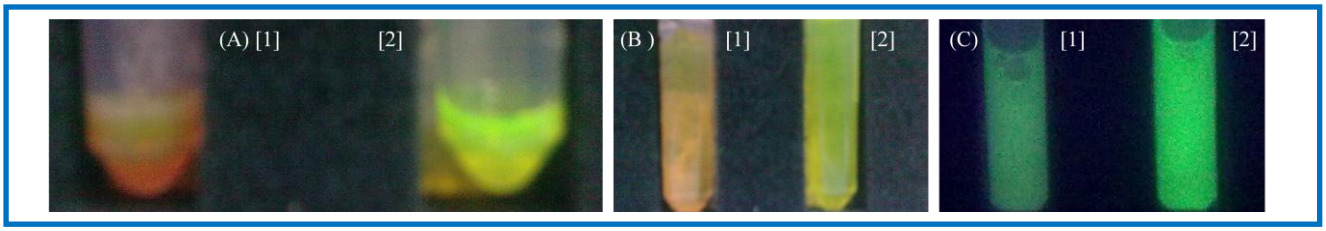



**Figure S1:** (a) Mixture of somatostatin labeled with rhodamine (RhB:SST) and fluorescein (Fl:SST) [1] and only fluorescein labeled somatostatin (Fl:SST) [2]. (b) Multicolor fluorophore (rhodamine and fluorescene) labeled somatostatin capped AuNP (**RhB/Fl:SST-AuNPs**) [1] fluorescein labeled somatostatin capped AuNP under day light (2). (c) Multicolor fluorophore (rhodamine and fluorescein) labeled somatostatin capped AuNP (**RhB/Fl:SST-AuNPs**) [1] fluorescein labeled somatostatin capped AuNP under long wavelength UV-light in PBS at pH 7.2.

**Note: [1]** The proportion of dyes is tunable during the synthesis.

[2] **RhB:SST-AuNPs** were prepared according to same protocol.

**S4. Spectroscopic characterization of RhB:SST-AuNPs:**

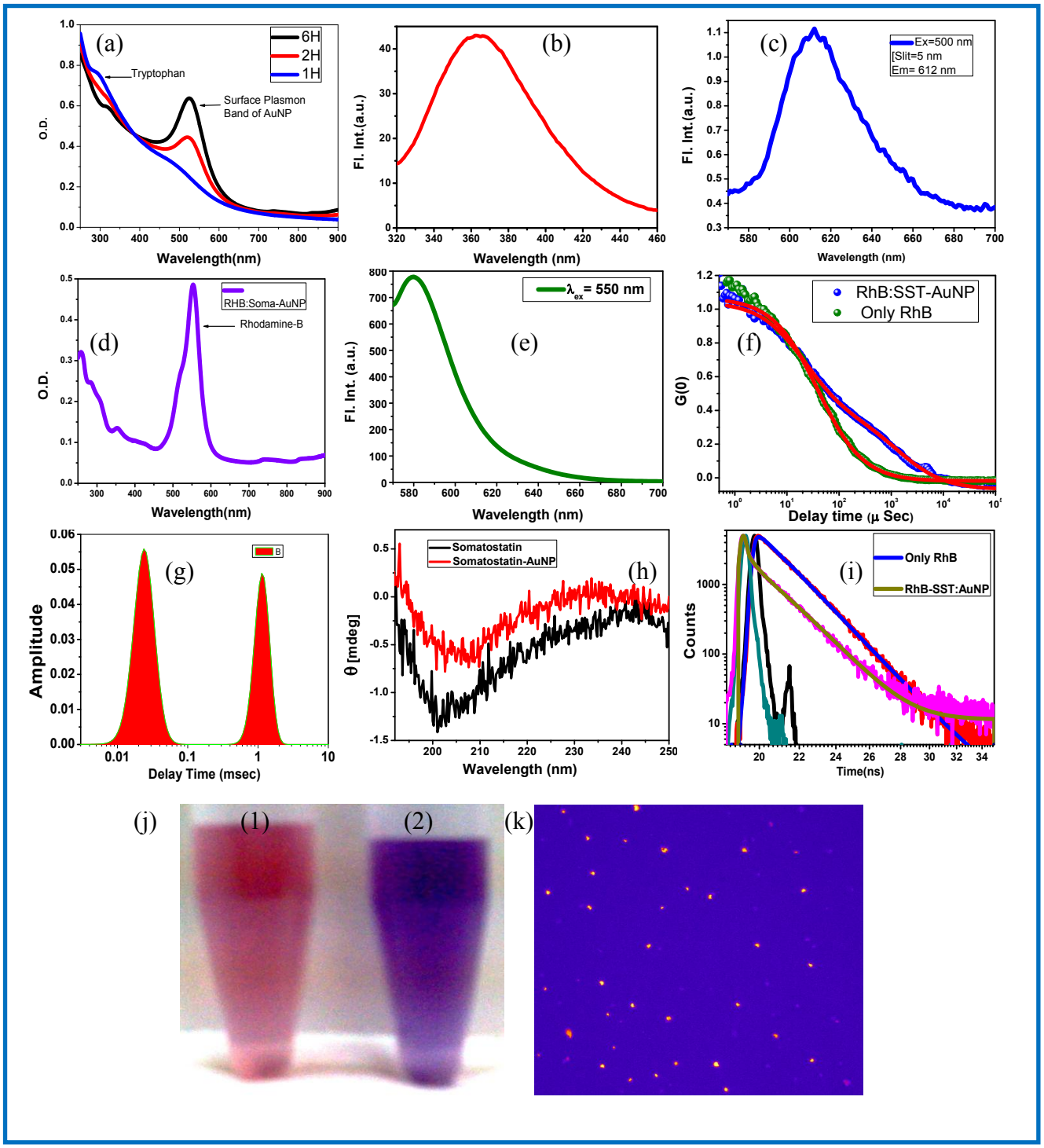



**Figure S2:** (a) The time evolution of AuNP formation was studied by the growth of the characteristic SPR peak at 525 nm (b) Trp fluorescence spectra ($\lambda_{ex}$=280 nm) (c) Luminescence spectra of AuNP ($\lambda_{ex}$=500 nm). (d) Absorption spectra of **RhB:SST-AuNP**s. (e) Luminescence spectra of **RhB:SST-AuNP**s ($\lambda_{ex}$=550 nm) (f) FCS and (g) MEM-FCS of **RhB:SST-AuNPs** (h) CD spectra of somatostatin when free (black) and attached to AuNP (red). (i) Fluorescence total intensity decay trace of **RhB:SST-AuNPs** ($\lambda_{ex}/\lambda_{em}$ = 471 nm/560 nm) (j) Visual color of (1) **SST-AuNP**s (2) **RhB:SST-AuNPs** (k) Fluorescence microscopic images of 10 nm RHB: SST-AuNP immobilized on BSA surface [scheme S4].

**S5. FT-IR measurement:**

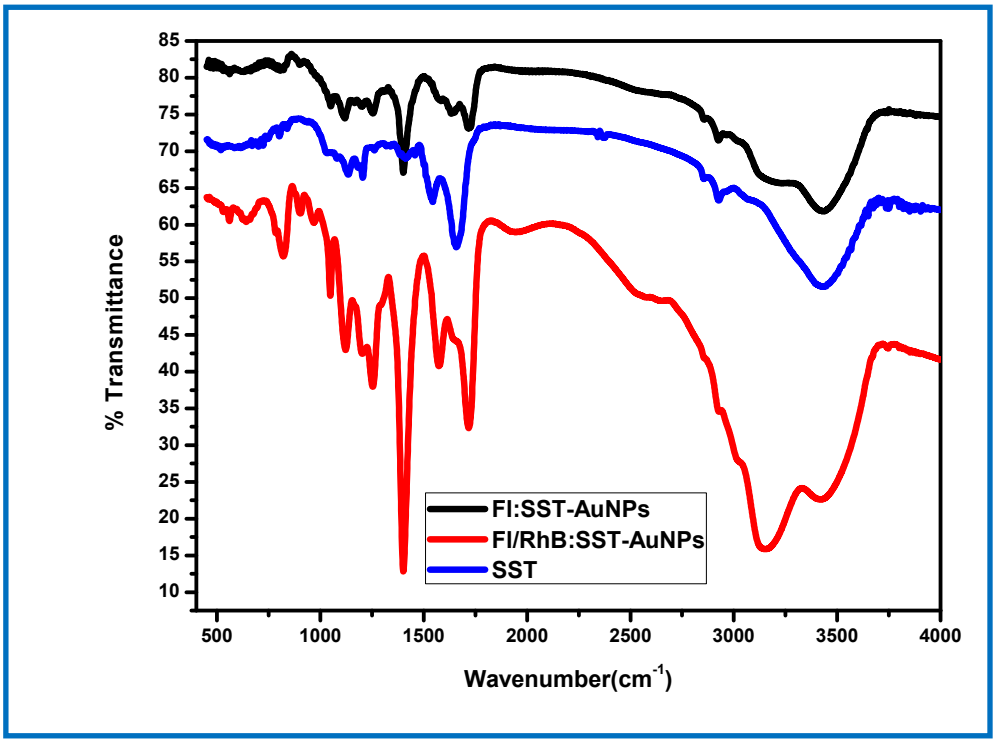
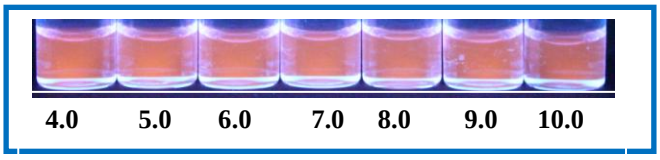



**Figure S3:** FT-IR spectra of (a) the **Fl:SST-AuN**Ps (black) and (b) **RhB/Fl:SST-AuNPs** (red), (c) SST (blue).

**S6. RhB: SST-AuNPs at different pH:** [ pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 ] under UV-Light [100 µg/mL] in PBS.



**S7. FCS of RhB:SST-AuNPs at different pH:**

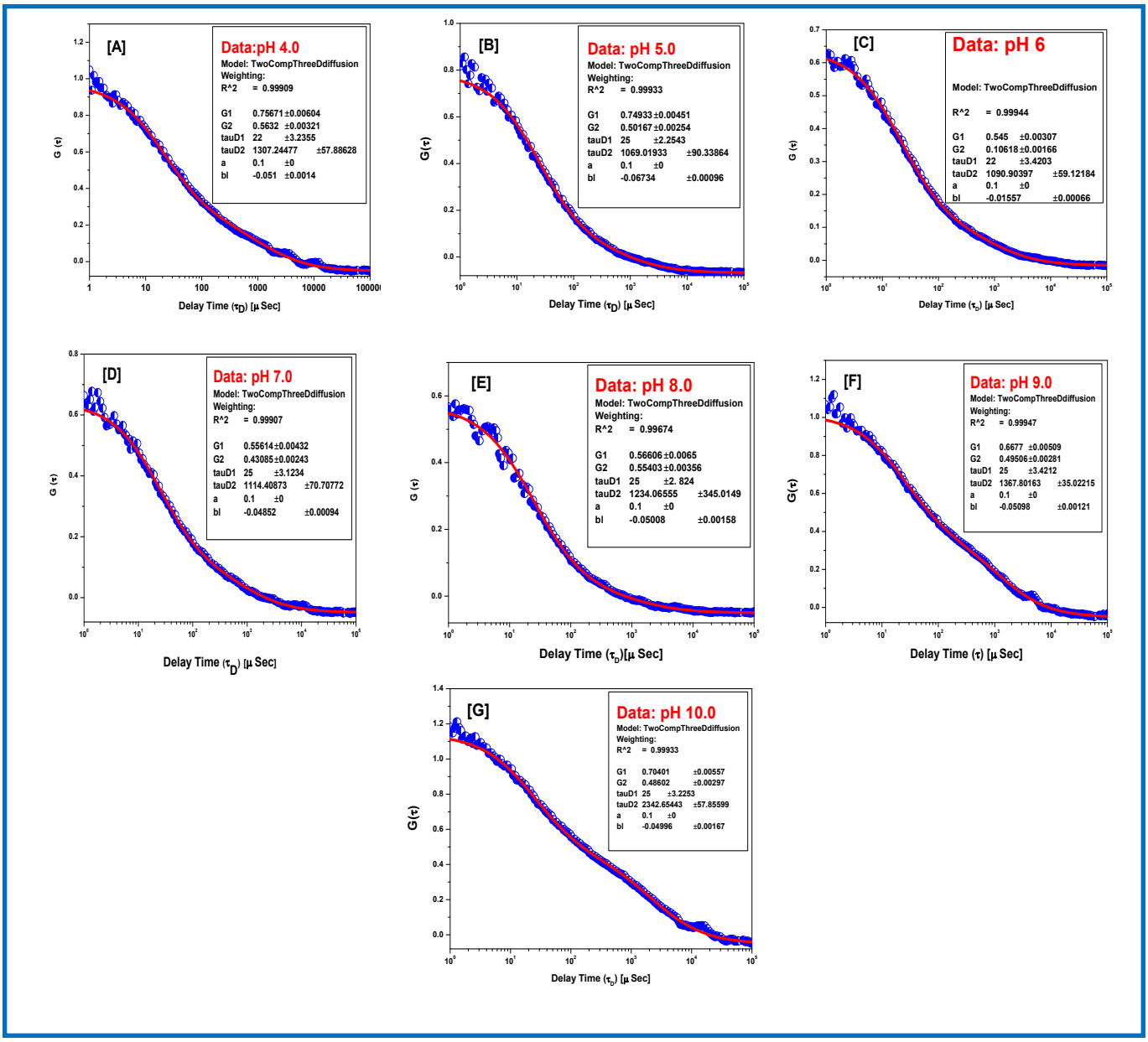



**Figure S4:** FCS of **RhB:SST-AuNPs** [10 ng/mL] at different pH [ 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0] under 543.5 He/Ne Laser in PBS.

**S8. FCS with RhB:SST-AuNPs, Before and After Lyophilization:**

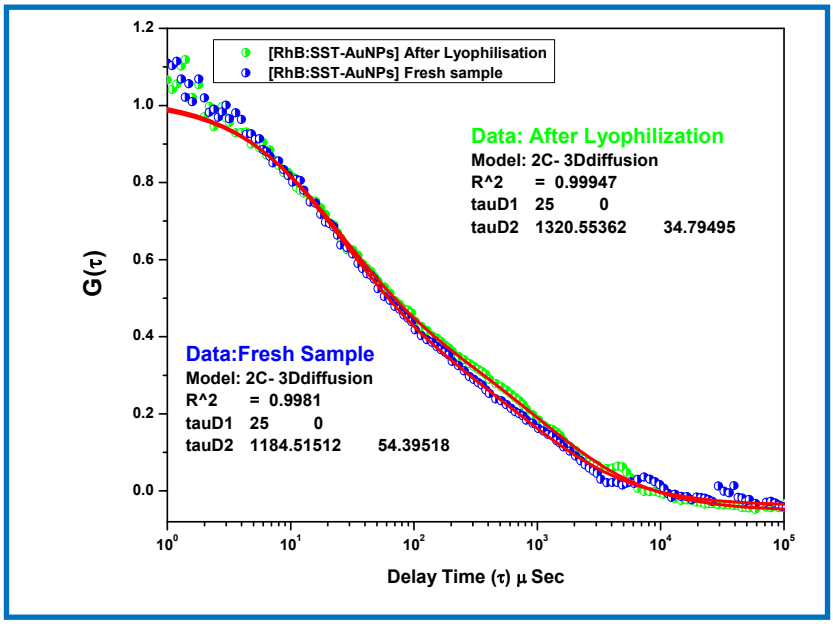
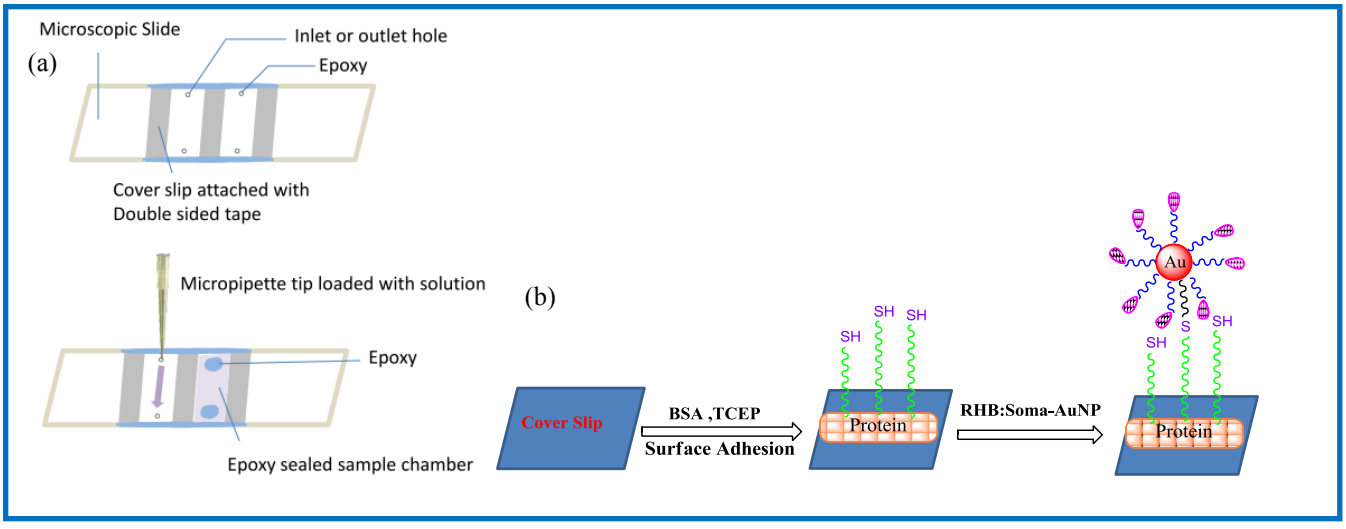



**Figure S5:** FCS data of 10 ng/mL of **RhB:SST-AuNPs**, **blue**- before lyophilization ,green- **after** lyophilization [both having the $\tau_D$ in the range of 1000- 1400 μSec].

**S9. Visualization of RhB:SST-AuNPs by Fluorescence microscopy:**

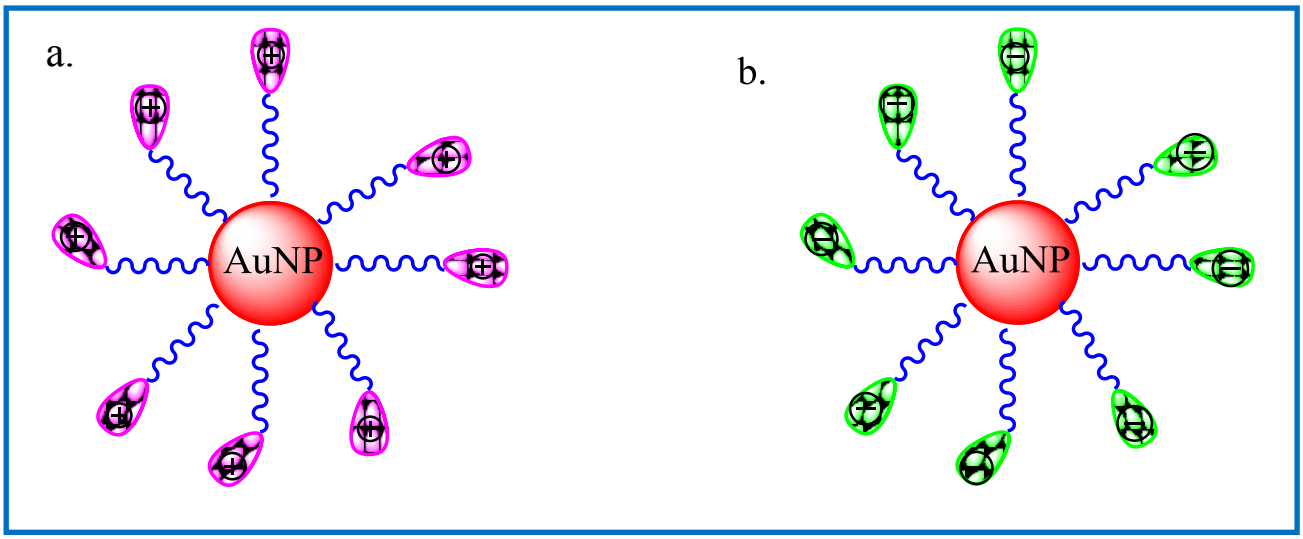
For imaging, fluorescence inverted microscopic technique was employed. Continuous LASER light of 532 nm (Ventus mpc6000, Laser Quatum, UK) was used to generate evanescent wave, which excites the fluorescent molecule, based on total internal reflection (TIR) principle. The fluorescence emission was collected through an Olympus 60x NA 1.2 water-immersion objective mounted on a inverted microscope (IX71, Olympus, Japan) and send to EMCCD camera (iXon3, Andor, UK).

Sample chamber was made by well cleaned cover slip and microscopic slid sandwiched by double sided tape leaving some gap inside. The open portion was sealed by epoxy glue to prevent any leakage. Solution was flowed through a hole made previously on the microscopic slid and the excess of the solution was drained out from the other hole and soaked with blotting/tissue paper. Finally, the holes were sealed with epoxy glue to prevent the drying of the solution and it was ready to put on the microscopic stage (Scheme S4).




**Scheme S4:** (a) Preparation of sample chamber (b) Principle of protein-assisted surface immobilization of **RhB:SST-AuNPs** and imaging.

**S10. Charge Distribution on AuNP surface:**




**Scheme S5:** Schematic representation of probable charge distribution on **RhB:SST-AuNPs** and **Fl:SST-AuNPs**.

**S11. Zeta Potential Measurements:**

Zeta potential values (ξ) were determined using a Malvern Zetasizer Nano NS particle analyser. The ξ was determined for each solution [four types of AuNPs] having concentration 1 μg/mL of NPs.

Table-S1:

| Nanoparticles | Zeta Potential (ξ) [avg. of 3 repeats] |
|---|---|
| **SST-AuNPs** | **-32.2 mV** |
| **RhB:SST-AuNPs** | **-35.3 mV** |
| **Fl:SST-AuNPs** | **-27.2 mV** |
| **RhB/Fl:SST-AuNPs** | **-31.9 mV** |

**S12. Cell Imaging:**

Neuro 2A cells were routinely grown in DMEM (HiMedia) supplemented with 10% fetal bovine serum (Biowest, USA) at 37°C in 5% $CO_2$ atmosphere under humidified condition. Imaging was performed on LSM 510 META confocal laser scanning microscope equipped with an argon-krypton laser (Carl Zeiss, Germany). Cells were grown on L-lysine coated cover slips and washed with PBS, mounted on slides and images were grabbed with 63X plan-apochromatic objective.

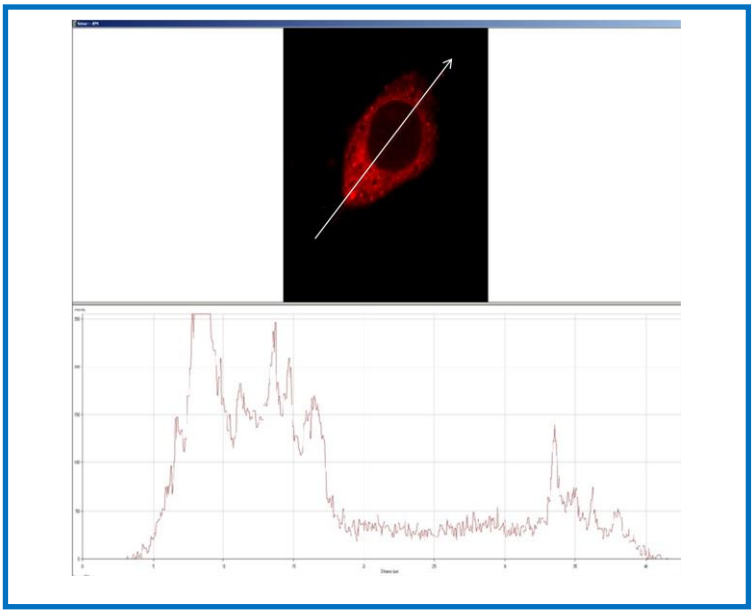


**Figure S6:** Line plot of luminescent intensity on neuro 2A cell incubated with 20 $\mu g/mL$ of **RhB:SST-AuNPs** for 2H at $37^0$C.

**S13. Control Experiments in Cells:**

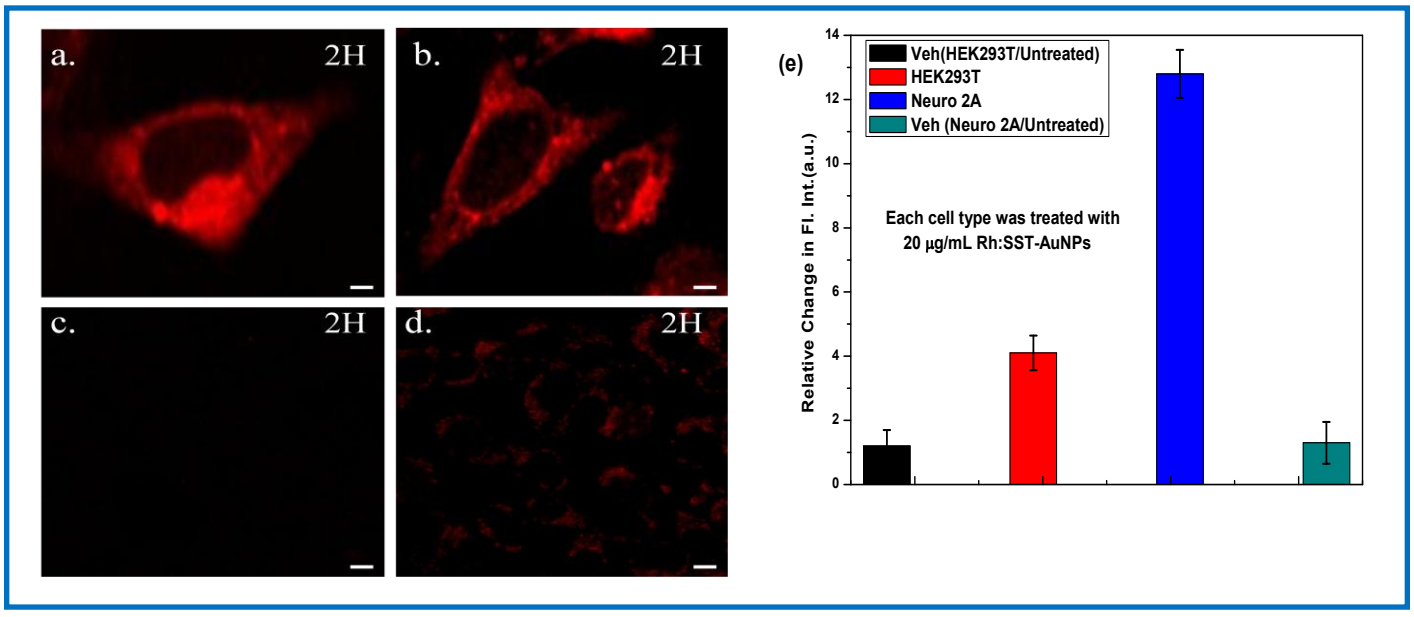



**Figure S7:** Fluorescence confocal image of Neuro 2A cell incubated with (a) 10 $\mu M$ RhB (b) 10 $\mu M$ RhB:SST (c) 20 $\mu g/mL$ AuNPs with out SST for 2H at $37^0$C [ no fluorescence signal as AuNPs (with out SST) is almost nonfluoroscent at 540 nm excitation ]. (d) Fluorescence confocal image of HEK293T cell incubated with 20 $\mu g/mL$ **RhB:SST-AuNPs** for 2H at $37^0$C [all scale bar 5 µm]. (e) Relative change of fluorescence intensity when **Rh:SST-AuNPs** [20 $\mu g/mL$] was treated separately with Neuro 2a and HEK293T cells [excitation 540 nm].

**S14. Time Dependent distribution of Fl:SST-AuNPs & CLSM of Neuro 2A cell incubated with RhB:SST.**

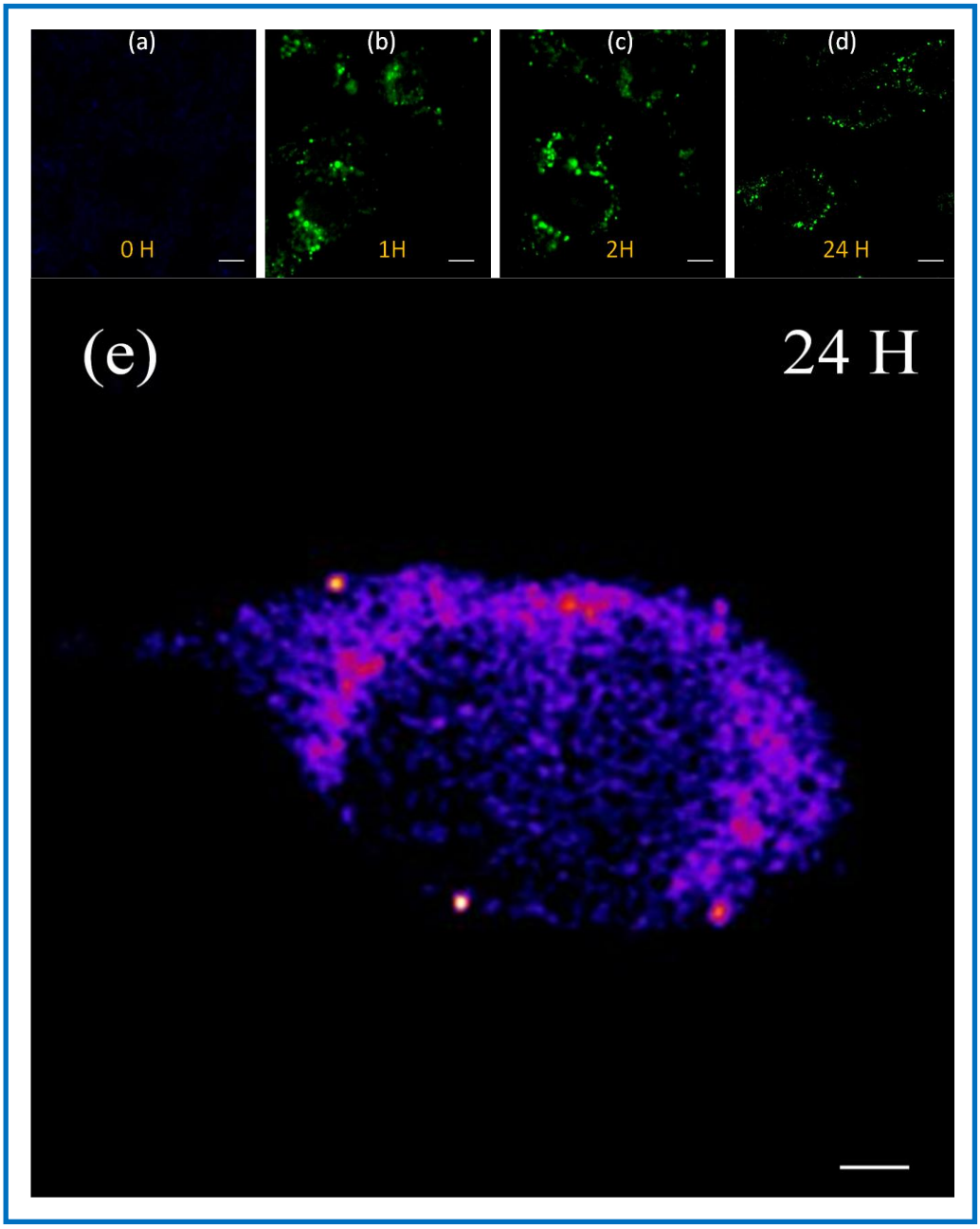



**Figure S8:** Time dependent fluorescence confocal image of Neuro-2A cell incubated with 10 $\mu g/mL$ **Fl:SST–AuNPs** for (a) 0H (b) 1H, (c) 2H [experiment a-c were carried out in same cell culture dish] and (d) 24H at $37^0$C [all scale bar 10 μm]. (e) Fluorescence confocal image of Neuro-2A cell incubated with 20 $\mu g/mL$ **RhB:SST** for 24H at $37^0$C [all scale bar 10 μm].

**S15. Toxicity Assay:**

Rat primary cortical neuronal cultures plated in 96 well plates were treated with high concentrations of AuNPs (100 µg/mL). The cells were assessed after 60 h for the extent of cell death. The cells were treated with 0.01 mg/ml concentrations of Hoechst 33342 (a DNA intercalating dye that permeates membranes and hence label all the cells present) and propidium iodide (PI, another DNA intercalating dye that does not permeate live membranes and hence labels only the dead cells with damaged membranes) in TB for 10 min followed by washing with TB. The cells were imaged for Hoechst 33342 and PI fluorescence in a confocal microscope setup (LSM-710, Zeiss, Germany) using a 20X objective. 690 nm pulsed light form a mode-locked Ti-sapphire laser (MaiTai, Spectra Physics, CA, USA) was used for the two-photon excitation of Hoechst 33342. The fluorescence was separated from the excitation using a 690 nm dichroic mirror and detected using a photomultiplier tube (385–535 nm). PI was excited using a 543 nm laser (He-Ne, Zeiss), the fluorescence was separated using a dichroic mirror and detected between 565 and 720 nm. Images were analyzed for the total number of cells (Hoechst 33342 fluorescent spots) and the number dead cells (PI fluorescent spots) using an automated particle counter in Image J. The ratio of cells which are alive (PI negative) to total cells was reported as the % viability.

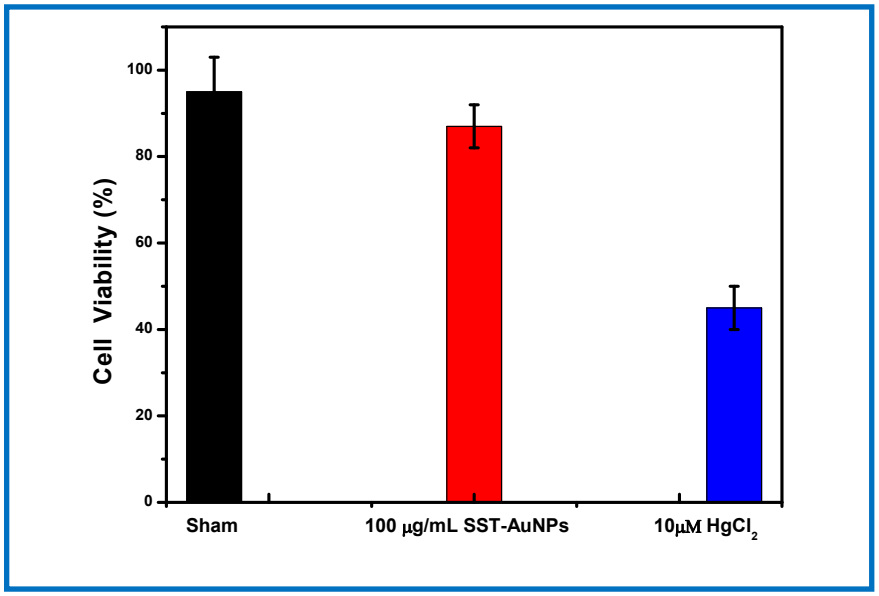



**Figure S9:** Toxicity Assay of **SST-AuNPs** (100 µg/mL) towards cortical culture *10 µM* $HgCl_2$ was used as +Ve control.

**Reference:**

1. Invitrogen: Amine-Reactive Probes (Copyright 2009, 2011, Molecular Probes, Inc.)