Electronic Supplementary Material (ESI) for Nanoscale
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Supporting Information

Optical Imaging of the Non-fluorescent Nanodiamonds in Live Cells Using Transient Absorption Microscopy

Tao Chen,[+]a Feng Lu,[+]b Aaron M. Streets,[a] Peng Fei,[a] Junmin Quan[b] and Yanyi Huang[a]*

[a] *Biodynamic Optical Imaging Center (BIOPIC), and College of Engineering, Peking University, Beijing, China. Fax: +86-010-62758323; Tel: +86-010-62744058; E-mail: yanyi@pku.edu.cn*

[b] *School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, China.*

[+] These authors contributed equally

Methods and Materials

Preparation of the nanodiamonds (NDs)

The raw, detonated NDs (NanoCarbon Institute, Japan) were acquired as colloidal particles and then dispersed in deionized water. The raw ND colloid was carboxylated in strong oxidizing conditions as previously reported.[30] Briefly, the colloid was treated in acid mixture of 68% HNO3 : 98% H_2SO_4 (1:3 in volumn) in ultrasonic bath for 24 h, followed by stirring in 0.1 M NaOH at 90 °C for 2 h. After high-speed centrifugation, an additional stirring was performed in 0.1M HCl aqueous solution at 90 °C for 2 h, and finally sent for high-speed centrifugation. The pellet was resuspended in deionized water and precipitated again by high-speed centrifugation. This procedure was repeated three times to remove excessive HCl and impurities. A minute portion of the aqueous suspension was dispensed on a silicon wafer and characterized with scanning electron microscopy.

Cell culture

The NIH 3T3 fibroblast cells were maintained in Dulbecco's modified Eagle medium (DMEM) complete media containing 10% new born calf serum (NBCS) and 1% penicillin-streptomycin mix. Cells were plated onto 6-well plates at a concentration of 10^5/cm2 and cultured in a humidified incubator at 37°C and 5% CO_2 atmosphere.

Transient absorption microscope (TAM)

The TAM was constructed from a modified two-photon scanning microscope system. Light source for TAM is a pump source integrated optical parametric oscillator, also known as one-box OPO (picoEmerald, Berlin, Germany). This one-box OPO can simultaneously provide two spatially and temporally overlapped pulse trains, with a repetition rate of 80MHz. The wavelength of one beam is 1064 nm (the pump beam in TAM), and the other is tunable between 720-990nm (the probe beam in TAM). Pulse duration is 8ps for the 1064nm beam and 6ps for the tunable laser. The two beams are sent into an inverted multiphoton microscope (IX81/FV1000, Olympus, Japan). Intensity of the 1064nm beam

is modulated with an electro-optic modulator (EO-AM-NR-C2, Thorlabs, USA) at 9.81 MHz. The two beams are co-aligned and focused by a 60X water immersion objective (UPLSAPO 60XW, Olympus, Japan), where they scan over the sample through galvo mirrors in the scanning unit. After transmitting through the sample, the probe beam is collected by a water immersion condenser (N.A. 0.9, Olympus, Japan), then focused to a large area photodiode (FDS1010, Thorlabs, USA). The photo-electric signal was sent into a lock-in amplifier (designed by Dr. Brian Saar). After demodulation, the amplified signal was sent into an A/D converter, and then used to reconstruct images. Stimulated Raman scattering signal of paraffin's CH2 stretching mode (2845 cm^{-1}) was acquired to characterize the system response and reveal the correlation between the two pulse trains.

Raman Spectra Measurement

Spontaneous Raman spectrum was acquired with a confocal microscopic Raman spectrometer (HR800, HORIBA, Japan), excited with a 532nm laser. ND suspension was spun on a slide and dried before spectrum collection.

Absorption Spectrum

Absorption spectrum was acquired with a UV-VIS spectrometer (UV2450, SHIMADZU, Japan). Spectra of aqueous solution were acquired ten times for noise suppresion.

Laser Power Tolerance

IR lasers ranging from 810nm to 1064nm were used for TAM imaging. Cells don't absorb much light in this wavelength region. Cells were irradiated with both pump and probe beams. Pump beam was set at its maximum power, 350mW. Probe beam power was changed from 400mW to 700mW. These powers indicate the output from the laser head. Throughput from the laser head to the sample was measured to be 6.7% for the pump and 19.1% for the probe beam. Irradiation time for each power combination is 220 s. Results were recorded and shown in Fig. S2-S6.

Cell uptake experiment

The ND suspension was sonicated for over 8 h and mixed with culture medium. This ND-containing mixture was then used to replace the normal medium to culture 3T3 cells for 8 h. Extracellular NDs were washed away by PBS before imaging. Transmission images and transient absorption images were acquired sequentially in the same field of view. To validate the uptake mechanism, cells were stained with the amphiphilic fluorescent dye FM4-64 (Invitrogen). The lipophilic tail of FM4-64 can insert into cellular phospholipid membrane, stain the membrane specifically and firmly [1]. Simultaneously, ND-containing medium was added to the stained cells and cultured for another 4 h before imaging. Fluorescence and transient absorption images were acquired sequentially.

TAM intrinsically has 3D imaging capability. Fig. S7 showed the distribution of NDs in 3T3 cell when changing focal plane.

To monitor the dynamics of cell uptake, we cultured the cells in petri dishes with an on-stage incubator (Chamlide TC-A, LCI, Korean). Image acquisition started right after the

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introduction of NDs. Images were acquired in three dimensions with 1 um steps in z direction, 7 sections for each stack. It took 17 s to finish a stack and ~ 1 h to finish the 3D time-lapse observation. The same batch of cells was cultured under the different conditions for test cell uptake. For each condition, cells were cultured for 3 h, and then 6 random locations in the Petri dish were picked for imaging. We also adjusted the experimental schedule to ensure that the cells were treated for the same amount of time under different conditions before the image acquisition. Three-dimensional images using both transmission and transient absorption were taken for each field-of-view. The number of particles taken up by the cells was counted in each image using FIJI [2]. For each field of view, total intensity in the whole volume was calculated, and divided by cell numbers. Particles taken per cell were used to compare uptake ability under each condition.

MTT Assay

MTT assay [3] was applied to cells to measure the viability of cells treated with NDs. Cells were co-cultured with NDs for 8 hours before the experiments. The assay was carried out under two conditions, with serum and without NBCS. For both conditions, NDs concentrations in ratio of 1:2:3 were applied. For each concentration each condition, 6~7 groups were set. This entire assay was repeated three times.

Signal Confirmation

Stimulated Emission (SE) is the process to induce fluorescence with pump-probe scheme in non-fluorescent species. To rule it out, we checked the phase of our signal, as illustrated in Fig. S10.

For clarity, we use phase of SRS as reference. The SRS we detected is the loss of pump. Transient absorption here showed the same phase with SRS. However, for SE, it has the opposite phase compared with SRS. In Fig3 C, we can see SRS and TA were both brighter than background, indicating they have the same phase. If not, those spots representing NDs should be darker than background.

References:

[1] Cochilla, A. J.; Angleson, J. K.; Betz, W. *Annu. Rev. Neurosci.* **1999**, *22*, 1-10.
[2] Schindelin, J.; Arganda-Carras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J. Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A. *Nat. Methods*, **2012**, *9*, 676-682.
[3] Mosmann, T. *J. Immunol. Methods,* **1983**, *65 (1-2)*, 55-63.

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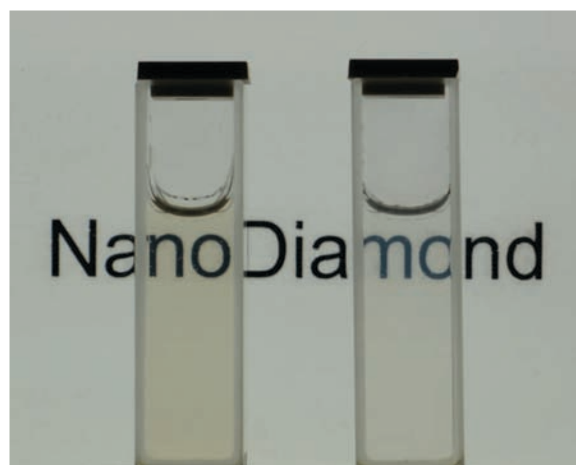

Fig. S1. A photograph of raw (left) and carboxylated (right) NDs in cuvettes.

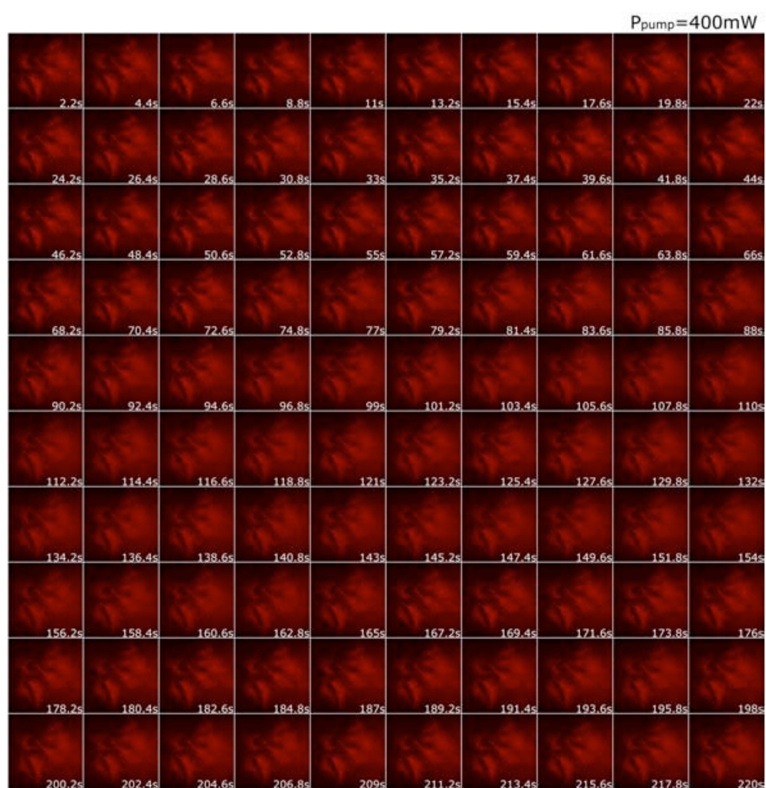

Fig. S2. Selected time-lapse TAM images of raw NDs in the live fibroblast cells. The power of the pump beam was set to 400 mW.

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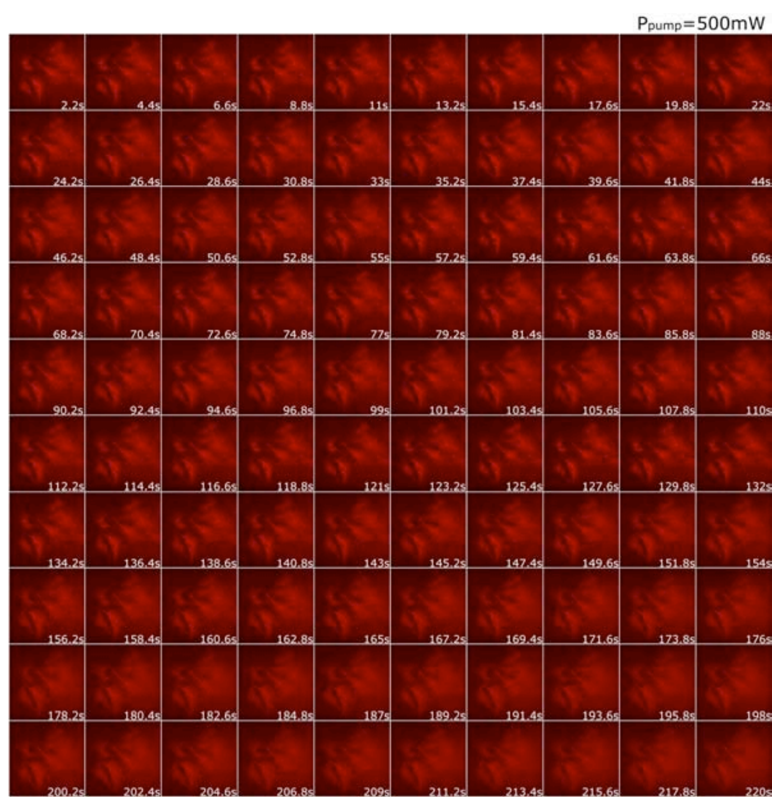

Fig. S3. Selected time-lapse TAM images of raw NDs in the live fibroblast cells. The power of the pump beam was set to 500 mW.

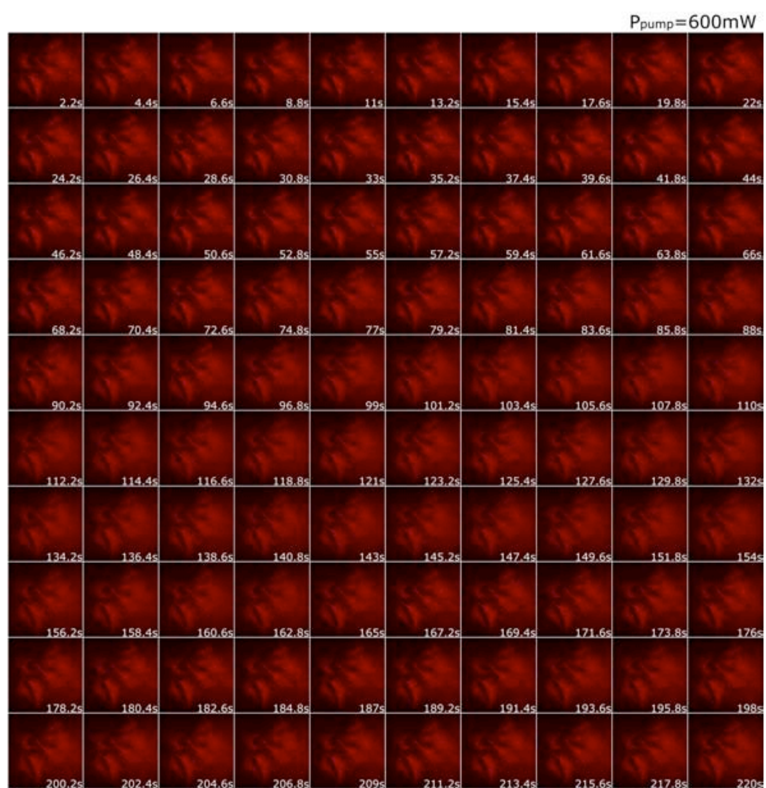

Fig. S4. Selected time-lapse TAM images of raw NDs in the live fibroblast cells. The power of the pump beam was set to 600 mW.

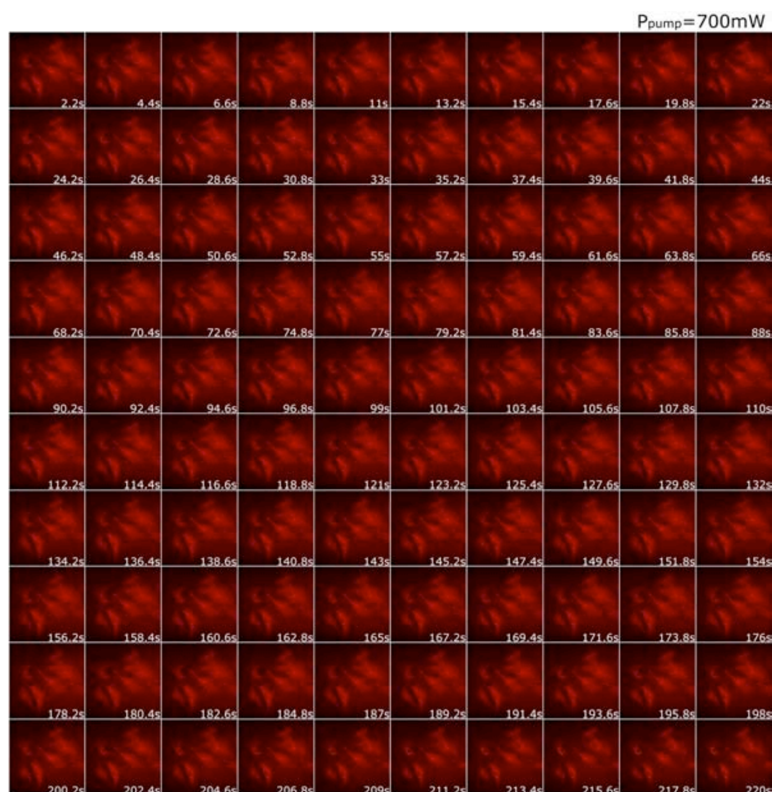

Fig. S5. Selected time-lapse TAM images of raw NDs in the live fibroblast cells. The power of the pump beam was set to 700 mW.

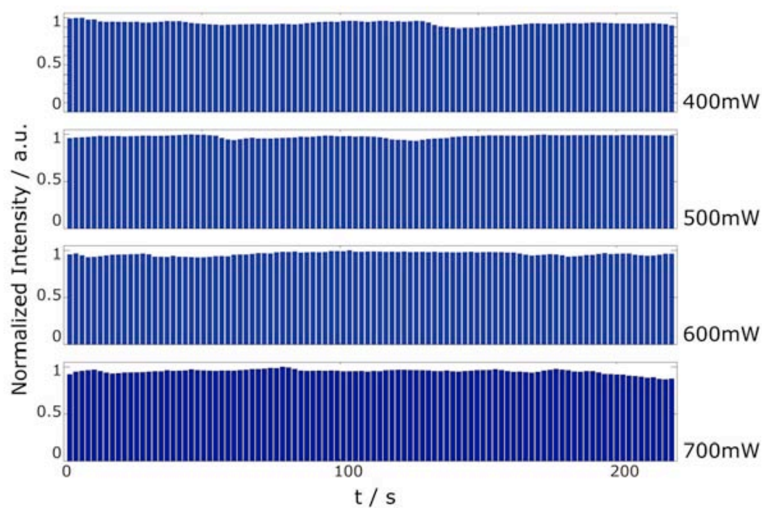

Fig. S6. The normalized TA intensity of the TA images during the continuous imaging acquired with various pump intensity (also check Fig. S2-S5).

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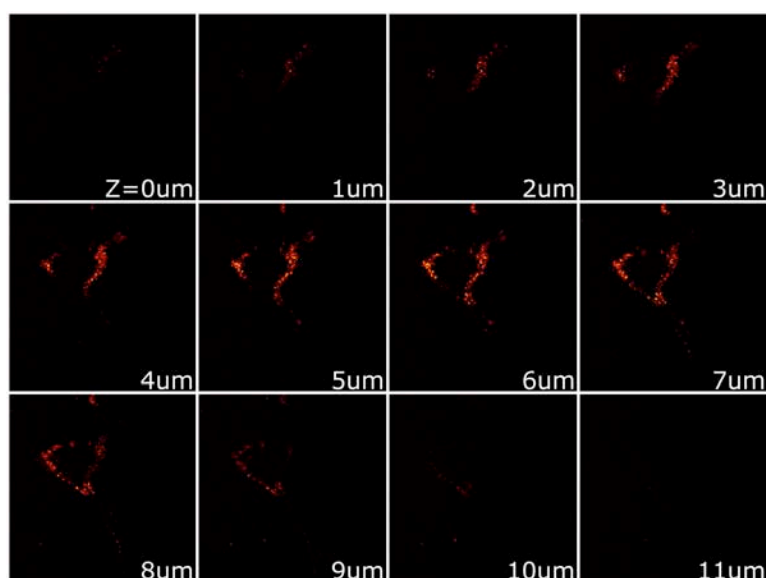

Fig. S7. A stack of TAM images taken at the different focal plane, representing the intrinsic 3D sectioning of the TAM.

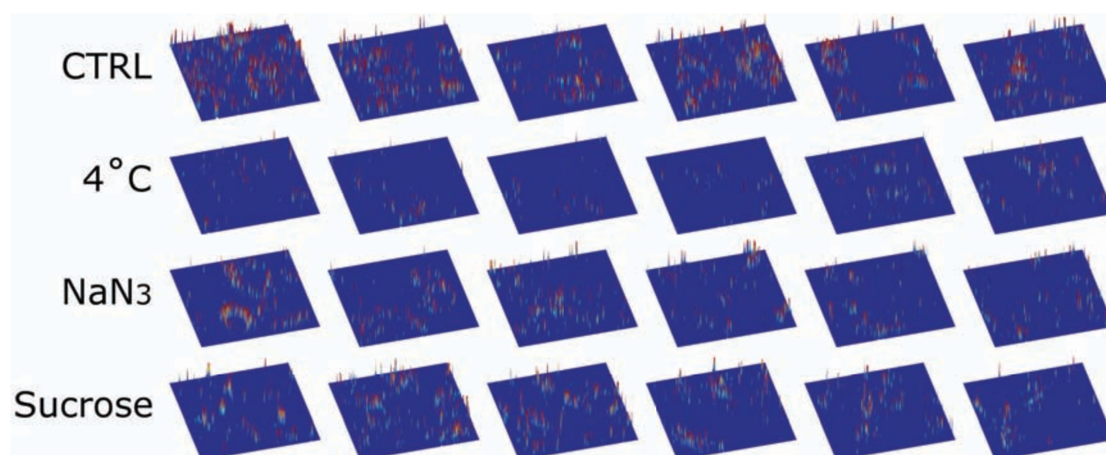

Fig. S8. Comparison of NDs in cells under different culture conditions. Results of six experiments were shown. Variation between different experiments is evident. Meanwhile, in all experiements changing the culture conditions lead to reduction of NDs uptake.

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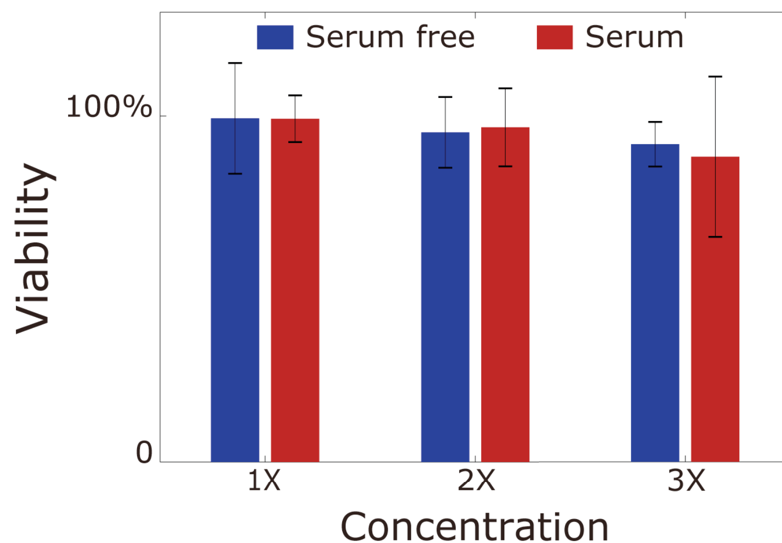

Fig. S9. MTT assay result for 3T3 cells co-cultured with NDs for 8 hours. The variation originated from cell proliferation.

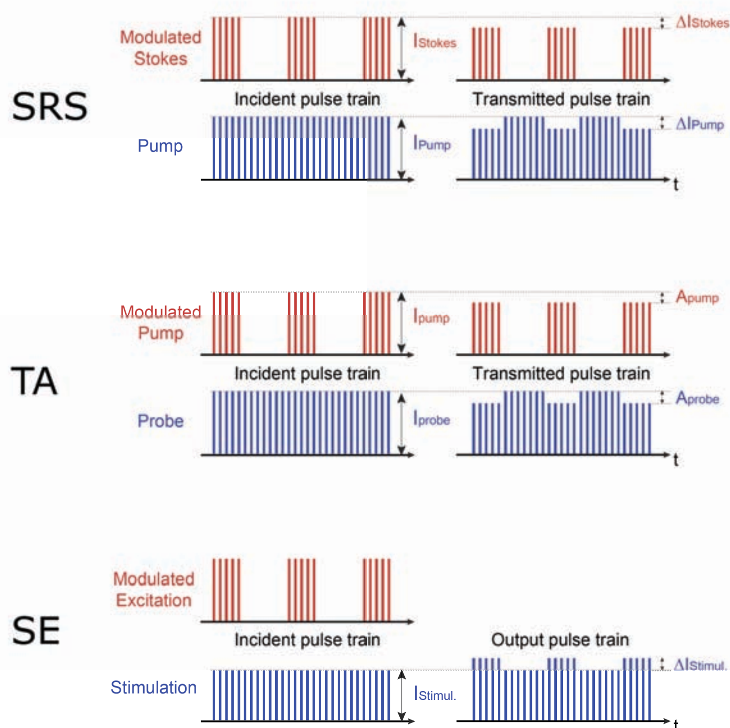

Fig. S10. Phase Comparison between stimulated Raman scattering (SRS), transient absorption (TA), and stimulated emission (SE). SRS and TA show the same phase, meanwhile SE shows the opposite phase.