SUPPLEMENTARY

Investigations of Astrocyte Calcium Signaling and Imaging with Classical and Non-Classical Light

Authors

Diletta Spennato[1,4], Josephine Leone[2], Carolyn Gundhardt[2], Oleg Varnavski[2], Roberta Fabbri[3], Marco Caprini[3], Roberto Zamboni[3], Valentina Benfenati*[3], Theodor Goodson III*[2]

	n. of experiments (n. of animals)	n. of replicates	n. of recorded cells
Standard solution -CTRL	3	4	160
Ns laser 450 nm	3	5	221
in 0 calcium solution	2	2	40
+ 2-APB	2	2	42
+ Rutenium Red	2	2	41
+ RN	2	2	39
+ HC 030031	**1**	**2**	**20**
+ Chembridge	**2**	**2**	**40**
LASER 450 nm + CPA	2	2	40

Figure S1 Table of the experiments conducted on astrocytes, in vitro, describing the number (n°) of animals used/ n° of experiments, n° of replicates and n° of recorded cells in the different conditions where we used laser stimulation and pharmacological approach.

Hoeschst Dye Information:

For entangled light experiments, live astrocyte samples were incubated with Hoescht dye to stain the nucleus of each cell. Hoechst 34580 was selected for this experiment due to its effective permeability and compatibility with astrocyte physiology. Given the Hoescht dye's ability to selectively attach to the minor groove of A-T-rich regions of double-stranded DNA, cellular

fluorescence was localized in the nucleus of the individual cells. A reported increase of 30 times the original fluorescence is shown in cells incubated in Hoechst-loaded solutions. Additionally, compared to traditionally used DAPI dyes, Hoechst lowers the risk of cytotoxicity, increasing the percentage of live cells that can be imaged. Hoescht 34580 has an excitation maximum of 390 nm with a range of 350 nm to 450 nm. Given the constant wavelength excitation of 409 nm provided by the continuous-wave (CW) laser, significant sample excitation was observed upon laser stimulation. The emission was detected at a peak of 440 nm.

Entangled Two-Photon Laser:

The quantum entangled imaging technique utilized a scanning microscope with a galvo-galvo scanning head and lenses. The entangled light source used to produce images of these cells was generated via a nonlinear type-II Beta Barium Borate (BBO) crystal through spontaneous parametric down-conversion (SPDC). This SPDC process was pumped into the type II BBO crystal by a continuous-wave laser centered at 409 nm with a bandwidth of 1 nm. The type II BBO crystal was aligned for collinear and degenerate spontaneous parametric down-conversion of the photons delivered by the CW laser. The maximum output flux from the SPDC unit was 3.6×10^7 photons/s when the CW laser was attenuated to maximum pump power.

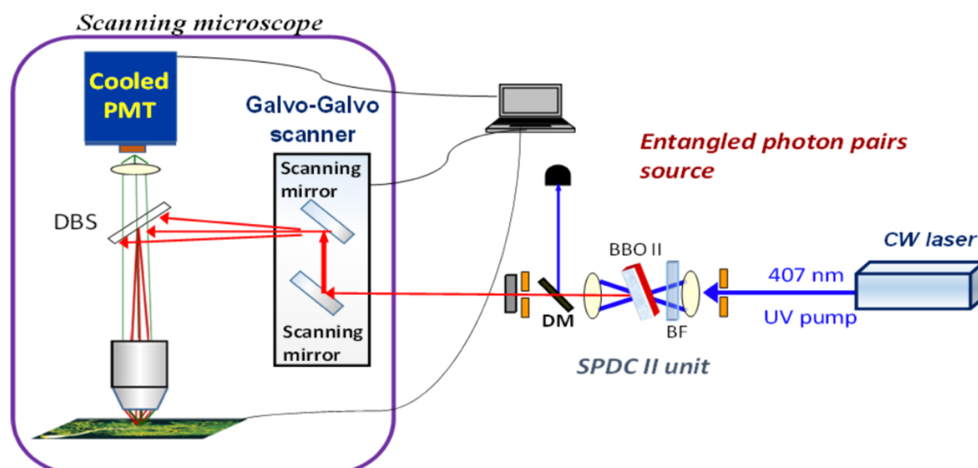

Figure S2 Schematic of the ETPA microscope. Interference filter (IFR) and dichroic mirrors (DM) separate pump light (409nm) from entangled photons produced in SPDC. Flipping mirrors SM1,SM2 direct the classical 818nm light beam to the microscope for reference experiments with classical light. Pump power references are provided by photodetector (PD). The biphoton beam or classical light undergoes raster scanning in the microscope galvo-galvo scanner. The dichroic beam splitter DBS directs the excitation beam to the objective lens. The fluorescent signal from the sample was epi-collected by the microscope objective lens and detected by the cooled PMT.

The scanning microscope utilized a galvo mirror that raster-scanned the focus area of the sample cells from the objective lens and recorded the excitation response from the entangled light source. A dichroic beam splitter with high reflection at 800 nm was used to direct the output beam from the SPDC unit to the objective lens of the scanning microscope. The fluorescence emitted by the sample upon excitation was transmitted to the photomultiplier tube (PMT, R7518P, Hamamatsu Photonics) via a beam splitter that is transparent to visible fluorescence. Three filters were employed to cut the remaining 400 and 800 nm light, ensuring the PMT selectively detected and recorded the fluorescence signal. The resultant images were produced from the detection of this

fluorescence signal, which was converted to electrical voltages via the PMT and transmitted to the computer system.

The type-II SPDC unit utilized a 1 mm thick Beta Barium Borate (BBO) crystal to convert the pumped light from 409 nm to 818 nm. The continuous-wave (CW) laser pumped this SPDC unit with 405 nm light and produced a high degree of entanglement frequency (1). A photon flux of 3.6 × 10^7 photons/s was generated with an output power of approximately 300 mW from the CW laser. The SPDC unit employed two lenses, positioned one focal length apart from the center of the BBO crystal.

For initial imaging with the classical light source, constant wavelength one-photon light set at 408 nm was directed toward fluorescently labeled astrocytes. Excited state emission was recorded to capture the landscape of the cell sample. Subsequently, cells of interest were fixed upon, selected, and magnified for entangled light imaging. The classical imaging utilized a frame size of 1024 × 1024 pixels and a pixel binning factor (PBF) of 100, while the entangled image was captured in complete darkness with a frame size of 128 × 128 pixels and a PBF of 400. The entangled image accumulated 3000 recorded frames.