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Infrared-LAMP: two-photon uncaging and imaging of gap junctional communication in three dimensions

To the editor: Our recent work demonstrated the advantages of a new imaging technique, local activation of fluorescent molecular probes (LAMP), which uses the cell-permeable caged dye NPE-HCCC2/AM to study cell gap junction coupling[1]. To extend this assay to cells in physiological preparations, we integrated two-photon uncaging and imaging (infrared-LAMP) to monitor intercellular dye diffusion in three dimensions (see **Supplementary Methods** online).

Activating caged dyes by two-photon excitation should offer superb resolution for tracking cellular and molecular movements[2]. This application requires caged probes with high two-photon uncaging cross-sections (δ_u). We measured the wavelength dependence of δ_u of our coumarin dyes, NPE-HCCC and NPE-HCC[3] (**Fig. 1a**). Both caged probes were most sensitive to photolysis around 730 nm, and their δ_u values dropped to unmeasurable above 790 nm (**Fig. 1b**). The two-photon excitation cross-sections (σ_{2PE}) of their parent fluorophores, HCCC and HCC (**Fig. 1a**), reached maxima of over 25 Goeppert-Mayer (GM; 1 GM = 10^{-50} cm^4 × s / photon) at 790 nm (**Fig. 1b**), which is sufficient for live-cell two-photon imaging based on a comparison with the σ_{2PE} of fluorescent proteins[4].

Other caged dyes have δ_u well below 0.1 GM (refs. 3,5). Even at high laser power (>40 mW), the release of fluorophores by two-photon excitation remains challenging[6]. Although two-photon excitation limits photodamage to the focal plane, the possibility of bleaching and damage remains[2,6]. To minimize phototoxicity and diffusion of photolyzed products, it is important to be able to use a low laser power but still generate a sudden jump of effector molecules in a confined volume. The high spatial selectivity of two-photon excitation (<1 μm^3) combined with a caged compound such as NPE-HCCC2/AM with a high δ_u value should be ideal for this.

To confirm the above measurements in cells, we loaded HeLa cells with NPE-HCCC2/AM and monitored the movement of released fluorophores by two-photon laser scanning microscopy (2PLSM). Before uncaging, cellular fluorescence was negligible; during continuous two-photon excitation at 790 nm, we detected no photolysis, but two-photon uncaging at 730 nm selectively activated HCCC2 (**Supplementary Fig. 1** online). We used low laser power (<11 mW) to uncage and to image.

Finally, we used mouse pancreatic acini as a model system to test infrared-LAMP in tissue. The pancreatic acinar cell is the enzyme-secreting unit of the exocrine pancreas, where junctional communication is important for stimulation-secretion coupling. After loading dissected acini (**Supplementary Fig. 2** online) with NPE-HCCC2/AM and calcein/AM, we acquired a stack of 2PLSM images (*xy*) of the calcein signal along the *z* axis (**Fig. 1c**). We achieved single-cell photoactivation by scanning the laser beam (730 nm) on a *z* plane across a region of interest encircled by the cell equator. We followed the diffusion of HCCC2 by acquiring 2PLSM z-stacks every 45 s. Post-acquisition analysis provided dynamic and quantitative information on cell-cell dye transfer in three dimensions (**Fig. 1d–g** and **Supplementary Video 1** online). Experiments with mice were carried out according to a protocol approved by the Institutional Animal Care and Use Committee.

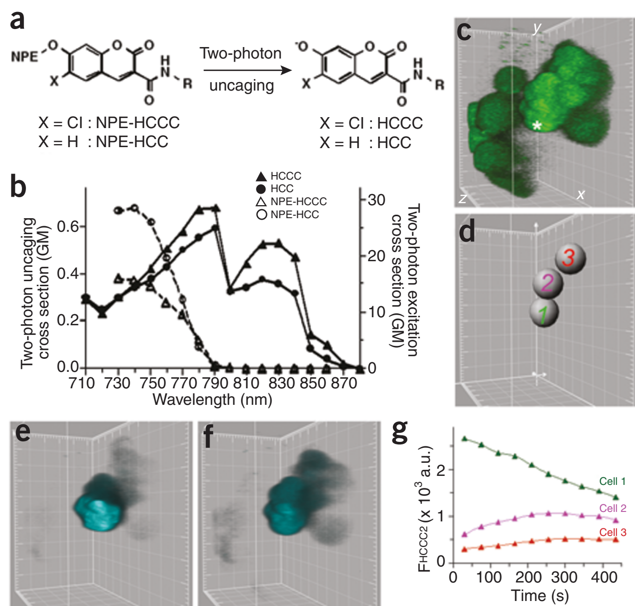

Figure 1 | Infrared-LAMP integrates two-photon uncaging and imaging to study junctional dye transfer in three dimensions. (**a**) Structures of coumarin dyes and NPE-caged forms. R, alkyl substitution. (**b**) Two-photon excitation spectra of HCCC and HCC, and wavelength dependence of two-photon uncaging efficiency of NPE-HCCC and NPE-HCC. (**c**–**f**) Volume-rendered three-dimensional images constructed from 43 slices (z-step, 1.25 μm) of 2PLSM images in the calcein channel (**c**) or coumarin channel (**e** and **f**; 30 s and 420 s after two-photon uncaging, respectively). The asterisk in **c** indicates the uncaged cell. Three spheroids in **d** outline the donor (1) and two recipient cells (2 and 3). (**g**) Time course of the average HCCC2 fluorescence intensities within the volume of each spheroid or the corresponding cells.

Note: Supplementary information is available on the Nature Methods website.

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