**Alignment of Imaging and Stimulation Paths**

Our experimental setup (Supplementary Fig 2) relies on two independent optical paths, one for imaging and one for photostimulation, which are merged into a single path with a polarizing beam splitter. For 3D imaging with point scanning two-photon fluorescence microscopy, a pair of resonant galvo-mirrors controls the position of the diffraction-limited photoexcitation spot along the x and y axis. In-plane range is limited by the maximal deflection angle of the galvo-mirrors, and by the numerical aperture of the scan-, tube-, and objective- lenses. With a 20X objective, the planar range is 600 along each axis. Depth along the optical (z) axis is determined by the adjustable focus of the electro-tunable lens (EL). The accessible depth is 250, and is distributed half way across either side of the natural focal plane of the objective. Overall, the two-photon imaging system yields fast 3D two-photon fluorescence measurements within the imaging volume of interest, and spatial location is expressed in a system of coordinates (x,y,z) corresponding to the position of galvo mirrors and to the focus depth of the electrotunable lens (EL).

For photostimulation with 3D-SHOT, precise targeting of individual neurons is made possible by computing a point-cloud hologram. The 3D locations of targeted neurons are expressed in a second set of coordinates (x’,y’,z’), defined in reference to the spatial light modulator (SLM). Diffraction efficiency and pixel size determine maximal in-plane and axial range of the holograms and are respectively given by : , and , where denotes the wavelength, f the focal length of the SLM telescope lens (L6), M, the magnification of the microscope objective, p the SLM pixel size, and h the SLM height. With our experimental setup, we find, and 900. Diffraction efficiency is significantly lower near the edges of this envelope than in the center. In practice, the operational range of the SLM is limited to a smaller operating volume: , and 300 that better matches the dimensions of the imaging window and within which diffraction efficiency is greater.

The accessible volume and spatial resolution can be enhanced by considering SLMs with a larger number of pixels. Smaller pixel size usually comes at the expense of speed (fps), damage threshold, or diffraction efficiency, which are all relevant criteria for photostimulation applications. Since our experimental setup is limited by available laser power, we prioritized diffraction efficiency and speed over spatial resolution in selecting the appropriate SLM. Finally, the microscope objective (L1) is mounted on a stage enabling mechanical displacements in a third set of ‘true’ spatial coordinates (x’’,y’’,z’’).

2 – Change of coordinates

Two-way all-optical read and write operations with individual neurons requires the ability to activate neurons that are identified and by the imaging path using the photostimulation path. A change of coordinates is therefore necessary to identify what coordinates (x’,y’,z’) for photostimulation match the corresponding location (x,y,z) of a neuron observed with the imaging path. Let denote this bijective change of coordinates.

Our system relies on optical elements historically developed for 2D imaging, yet operates in 3D in large volumes. Since the paraxial approximation for wave propagation is no longer always satisfied, M is generally a non-linear transform. Also, since M is affected by all optical elements placed before the BS in each optical path, it cannot be estimated reliably, even with high performance ray optics simulation tools (e.g. Zemax). To identify the change of coordinates, M with good approximation, we rely on a 3D calibration procedure. The aim of this calibration is to construct a set of alignment points distributed throughout the volume of operation, and for which the coordinates can be identified in both sets of coordinates, to build a polynomial interpolation. The calibration should be repeated anytime the setup is modified or realigned. We note that the proposed calibration is not affected by any modifications of the optical path happening after the BS. Mechanical displacements of the microscope objective stage, or replacing the objective to change magnification do not require a recalibration.

Before starting a calibration, the setup is finely aligned to maximize overlap between the effective volumes for imaging and for photostimulation. We adjust the respective depth of the imaging and photostimulation volumes by displacing lens L4 along the optical axis, as needed to maximize overlap. The initial calibration procedure is summarized in Supplementary Figure 11. We place a calibration glass slide under the microscope objective (Supplementary Figure 11a). The slide is coated with a thin film of fluorescent spray paint (TAMIYA fluorescent red), and intercepts the 3D imaging and/or photostimulation volume at any desired depth, z’’, which can be selected by mechanically adjusting the distance between the objective and the surface of the slide. The thin film of fluorescent paint has a broadband response and enables the direct detection of two-photon induced fluorescence, proportionally to the square of the light intensity. We record a 2D image of the fluorescence induced in the thin film with an inverted microscope and a sub-stage camera, after filtering out the remaining infrared light. The resulting setup enables high resolution visualization of 3D excitation patterns by stacking 2D slice views obtained by mechanically displacing the objective.

We first compute a set of vertical alignment data points by considering 30 holograms, each targeting one depth level z’. Targets are linearly spaced in depth, and placed in the center of the imaging window (x’=y’=0.5), or slightly off-centered to clear the 0-order block if it is already in place. We mechanically move the objective to record the CTFP at each depth and for each hologram. We measure the CTFP dimensions along each axis (Supplementary Figure 11b,c,d) and we estimate the corresponding targeted depth z’’, by locating the maximal response with a Gaussian interpolation of the two-photon response curve as a function of depth (Sup Fig. 11e, f). A similar procedure is followed to map the real coordinates (z’’) as a function of the focus of the electrotunable lens (z). The resulting data set enables the computation of the depth interpolation given by: z’ = C(z) independently of the interpolant for coordinates x’ and y’.

The second part of the calibration procedure is shown in Supplementary Figure 16. We consider a grid pattern of targets at locations (x’,y’) from which we keep half by only keeping a random selection. For each target in this randomized pattern, and for any desired depth level as selected for calibration purposes, z’, we compute a hologram targeting the location (x’,y’,z’). For each depth level, we place the calibration slide to intercept one plane of calibration points. With a high power pulse of light, we then selectively photodamage all the targets in the randomized pattern, and we finally acquire a two photon image with the imaging pathway. The procedure is repeated for as many depth levels as in the calibration, (Supplementary Fig 16 a-c). We then identify the targets, by matching the known SLM coordinates of the randomized pattern to the corresponding coordinates (x,y,z) given by the imaging path. Matching is made unambiguous recording one depth level at a time and by manually recognizing the orientation of the randomized pattern. The alignment marks are identified manually, and their precise localization is refined automatically by identifying the nearest local minimum of intensity.

The result is a set of points for which the photostimulation coordinates (x’,y’,z’) are known by construction, (see Supplementary Fig. 16d) and their corresponding imaging coordinates (x,y,z), are also known (Supplementary Fig. 16e), by measurement.

We then consider a polynomial interpolation

With ; ; and ;

We limit the order of the polynomial interpolant by enforcing:

= 0, = 0, except for.

By design, microscope objectives are designed to correct for stigmatism, even at high incidence angles. Within the volume of interest, the image of a plane orthogonal to the optical axis, is also a plane orthogonal to the same axis. This convenient property is what allows us first map coordinates along the optical axis, z, independently of the x, and y coordinates. For depth interpolation, we therefore enforce = 0, except for.

Non-zero coefficients can be added or removed to refine the model and include higher order geometric aberrations if necessary.

The non-zero coefficients , and are measured by respectively minimizing the quantities:

, , and .

We finally check the interpolation by displaying the measured coordinates () after conversion in the system of coordinates of the SLM (Supplementary Figure 16f).