Fast volumetric calcium imaging across multiple cortical layers using sculpted light


In the version of this supplementary file originally posted online, Supplementary Notes 1 and 2 and Supplementary Table 1 were missing. In addition, the link for Supplementary Video 2 directed readers to an unrelated video. The errors have been corrected as of 14 November 2016.
**Supplementary Figure 1**

**Schematic comparison of TeFo and diffraction-limited excitation.**

In conventional two-photon imaging, a neuronal cell body is excited by a set of small (diffraction-limited) PSFs, whose signals are integrated (left). In contrast, in scanned TeFo, the whole neuron is sampled with a single, enlarged sculpted PSF (right). Here V is the (volume) ratio of the diffraction limited spot compared to the enlarged, sculpted TeFo spot.
Supplementary Figure 2

Home-built ytterbium (Yb) all-fiber chirped-pulse laser amplifier (FCPA) design and characterization.

(a) Detailed sketch of the FCPA, showing the Yb-fiber seed oscillator (pulse repetition rate 58 MHz) at the top. LD – laser diode; PC – polarization controller; DCF – dispersion compensating fiber; FCAOM – fiber-coupled acousto-optic modulator; PLMA – polarization-maintaining large mode area fiber; PLMA10 – 10 µm core diameter PLMA; PLMA30 – 30 µm core diameter PLMA. The oscillator is pumped with a 600 mW single-mode 976-nm laser diode. The amplifier is pumped with 10W ~980-nm pump diodes. (b) Left: Output spectral intensity (black) as well as phase (red) of the FCPA; Right: Temporal pulse profile (black - measured FWHM 183 fs, blue - calculated Fourier limited pulse duration based on the output spectrum, FWHM 170fs) and temporal phase profile (red). (c) Measured beam profile of the FCPA, showing corresponding cross sections (white). Scale bar 1mm.
Supplementary Figure 3

Immunohistochemical assessment of photo-induced damage.

Representative images of brain sections containing the laser scanned regions that were immunostained with antibodies to detect microglial (anti-Iba1; green) and astrocyte (anti-GFAP; red) activation. All brains (with the exception of control) were illuminated for continuous 20 min under the condition stated below. Arrows indicate the approximate center of imaging site. (a) Immunolabeled sections of a mouse brain imaged with 2D s-TeFo scanning at 350 µm depth with 220 mW effective average laser power. Condition is similar to Fig. 2e. (b) Same as in a, but with 3D s-TeFo from 0-500 µm depth and 70-200 mW cycled averaged laser power. Condition is the same as in Fig. 4. (c) Control group that underwent window surgery but was not exposed to laser illumination. (d) Positive control group that was subject to 2D s-TeFo at 200 µm depth and high laser power (400 mW). (e,f) Intensity of immunolabeling, as fraction compared to control area, for mice illuminated with different laser intensities and scanning modalities, as indicated. 2P – standard two-photon microscopy. Shaded area denotes the 95% confidence interval of the control group mean.
Supplementary Figure 4

**Custom animal mount minimizes brain motion during awake imaging.**

(a) Schematics of the custom animal mount utilized in the experiments. The mouse is head restrained but can freely move on a rotating disk. The disk is suspended by springs and a damped counter weight and the animal is held up by a custom jacket (see Fig. 2b). These measures reduce and compensate for any force that is applied by the mouse’s limbs and thus minimize the vertical motion of the mouse brain during active behavior (b) Typical measured lateral drift of the images during the experiment before (blue) and after (green) motion correction in image postprocessing. In general, motion is <5µm r.m.s before and <1µm after correction. Dataset is the same as in Fig. 2c, motion correction algorithm is based on tracking the peak of the image autocorrelation using a maximum likelihood estimation algorithm and subsequent sub-pixel shifting using image interpolation. The high frame rate of s-TeFo (160 Hz) further facilitated image motion correction, as the in-frame movement during the acquisition of a single frame becomes negligible. (c,d) Representative calcium traces extracted before (blue) and after (green) motion correction in image postprocessing. Note that the curves are deliberately offset vertically from each other. (e) Histogram of correlations between corrected and uncorrected traces. On average the correlations are high (R=0.94±0.05). Frame rate is ~160Hz.
Supplementary Figure 5

Comparison of different neuron segmentation and calcium dynamic extraction approaches on s-TeFo imaging data.

ROI maps (left) and activity heat maps (right) for data analysis using PCA/ICA in (a), non-negative matrix factorization (NMF) in (b), and standard-deviation projection in (c). (d) Direct comparison of NMF vs. PCA/ICA analysis. Overlay of example activity traces extracted with PCA/ICA and NMF respectively. (e) Correlation coefficient matrix (left) and correlation coefficient histogram (right) of all neurons detected by both methods. On average the correlations are high (R=0.90±0.04). Comparison was done on data set shown in Figure 2c.
Supplementary Information

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This Supplementary Information contains:

Supplementary Note 1 Rational for using an enlarged sculpted excitation PSF and signal to noise in s-TeFo versus diffraction limited excitation

Supplementary Note 2 Design considerations in s-TeFo regarding V-FOV and laser pulse repetition rate

Supplementary Table 1 Comparison of s-TeFo to existing volumetric high-speed imaging techniques
Supplementary Note 1 Rational for using an enlarged sculpted excitation PSF and signal to noise in s-TeFo versus diffraction limited excitation

As stated in the main manuscript, one of the underlying ideas in our imaging approach is that for a given FOV and resolution, choosing the voxel size, i.e. the 3D size of the laser focus, to provide just the desired spatial resolution will result in the highest frame acquisition rate. This is because, provided no limitations in available laser power, biological damage or mechanical scan speed, the plane exposure time, $t_{\text{exp}}$, required to acquire the signal from the desired field-of-view, $A_{\text{FOV}}$, depends on the excitation area $A$ and its dwell time $\Delta t$, via $t_{\text{exp}} = (A_{\text{FOV}} / A) \cdot \Delta t$. Therefore, for a constant dwell time, therefore the largest possible value for $A$ for a desired spatial resolution will result in the minimum number of voxels that need to be scanned for a given $A_{\text{FOV}}$ and thus will result in the shortest plane exposure time $t_{\text{exp}}$.

The dwell time needs to be adequately long so that sufficient fluorescence signal can be acquired from each voxel. The fluorescence signal, $N_a$, in two-photon excitation via a pulsed laser source is proportional to the number of absorbed photons per voxel and laser pulse, and is given by:

$$N_a \sim \frac{p_0^2}{f^2\tau} \left( \frac{\lambda}{\lambda_T} \right)^2 A \delta$$

with $P_0$ being the average laser power at the sample plane, $f$ the laser's pulse repetition rate, $\tau$ the pulse length, $\lambda$ the central wavelength, and $A$ the excitation area at the sample and $\delta$ the axial confinement of excitation.

Thus, the number of emitted fluorescence photons from a TeFo (T) and diffraction-limited (D) PSF for a voxel dwell time $\Delta t$ are given by

$$N_{a,T} \sim \frac{p_0^2}{f^2\tau} \left( \frac{\lambda}{\lambda_T} \right)^2 A_T \delta_T \Delta t_T f_T$$

and

$$N_{a,D} \sim \frac{p_0^2}{f_0^2\tau} \left( \frac{\lambda}{\lambda_D} \right)^2 A_D \delta_D \Delta t_D f_D ,$$

respectively.

Irrespective of whether a TeFo or a diffraction limited excitation is used, from the equations (B) and (C) it can be seen that

$$N_a \sim \frac{p_0^2}{f^2\tau} ,$$

i.e. the fluorescence signal is inversely proportional to the pulse repetition rate which implies that for a given average power the signal is maximized for the lowest possible repetition rate and therefore for the maximum pulse energy. Given that each imaging voxel needs at least one pulse for excitation we
conclude that the fluorescence signal is maximized when one pulse per pixel is used.

Combining the one pulse per pixel excitation strategy with an enlarged sculpted PSF provides further advantages in the obtainable signal to noise ratio compared to the diffraction-limited excitation strategy.

Assuming the same power density \( (J/(\mu m^2 s)) \) of the excitation light and that in Eq. (1) the excitation volume of the TeFo spot was chosen to be a multiple (here denoted by \( V \)) of the diffraction limited excitation volume, i.e. \( A_T \delta_T = V \times A_D \delta_D \) then it follows for the TeFo case that

\[
N_{a,D} \sim \frac{P_0^2}{f_T^2} \left( \frac{\lambda}{\lambda_T} \right)^2 \frac{1}{V} \times A_T \delta_T \Delta t_T f_T,
\]

i.e., the number of absorbed photons per voxel and laser pulse in the case of TeFo excitation are \( N_{a,T} = V \times N_{a,D} \). As a consequence, the fluorescence signal will also be higher by a factor of \( V \) when a TeFo excitation is used when the emitted fluorescence signal from a diffraction limited spot is compared to that of a TeFo spot at the same power density and dwell time.

Next we compare the fluorescence signal emitted from a TeFo spot to the sum of diffraction limited spots comprising the same volume and acquired at the same total dwell time and power density as the TeFo spot. To image the same region as in the TeFo case, the diffraction-limited focus must be scanned over the neuron (illustrated in Supplementary Fig. S1) while the recorded signal is being integrated. In order to scan \( V \) voxels at the same acquisition rate, the voxel dwell time in this case must be reduced by at least \( 1/V \) even when assuming no overhead due to finite scan speed. Moreover, in this scenario given that each voxel again still needs to be excited by at least one pulse, it follows that the laser repetition rate needs to be increased by \( V \). Assuming the same average power as in the TeFo case, this leads to a decrease in pulse energy by \( V \), which however nonlinearly reduces the obtained signal from each voxel. Thus, the overall obtained signal from the sum of all voxels (denoted by subscript MD) that comprise the same volume as the TeFo PSF is:

\[
N_{a,MD} \sim V \times \frac{P_0^2}{V f_T^2} \left( \frac{\lambda}{\lambda_T} \right)^2 \frac{1}{V} \times A_T \delta_T \frac{1}{V} \Delta t_T V f_T,
\]

which is by a factor of \( V^2 \) lower than TeFo. Thus, for the same power density, average power and dwell time, the TeFo based excitation leads to a higher obtainable signal than both the diffraction limited excitation and the sum of multiple diffraction limited spots that add up to the TeFo PSF volume.

There are several contributions to noise including shot noise, fluorescence fluctuations and electronic noise related to the data acquisition such as read-out noise. Under the assumption that the noise is dominated by shot noise, we find that the noise is identical in the two excitation schemes. This is a consequence of the Bienaymé formula which states that the variance of the sum of uncorrelated random variables equals the sum of their variances.
Since the signal is proportional to \( N_a \), i.e. \( \text{Signal} \sim N_a \), the resulting signal-to-noise ratio, \( \text{SNR} = \text{Signal}/\sqrt{\text{Signal}} = \sqrt{\text{Signal}} \), for TeFo excitation is given by \( \text{SNR}_T = \sqrt{V} \) \( \text{SNR}_D \) compared to the single diffraction-limited PSF and \( \text{SNR}_T = V \text{SNR}_{MD} \) for scanned multiple diffraction-limited excitation.

The above ratios are for an idealized case and represent a conservative estimate. Noise sources such as read-out noise, which accumulate during the acquisition process, further reduce the SNR in case of diffraction-limited excitation. In the experiment shown in this work, \( V \sim 130 \) taking into account the geometry of the focal spot. Therefore, as a consequence, the SNR of TeFo can be more than one order of magnitude higher compared to the diffraction-limited case.

This increase in SNR can be used to lower the power requirements compared to conventional diffraction limited 2p laser scanning microscopy, in order to reduce photo-damage and to circumvent limitations due to the saturation of the fluorophores. Alternatively, for the same power density, this gain can be used to lower the dwell time of each voxel in order to allow image acquisition at a higher speed.

**Supplementary Note 2** Design considerations in s-TeFo regarding V-FOV and laser pulse repetition rate

As outlined above the imaging approach of s-TeFo is based on scanning an enlarged, sculpted PSF in a one-laser-pulse-per-image-voxel scheme. Therefore, in this approach the (volumetric) imaging FOV together with the desired spatial resolution determine the voxel rate and therefore the required laser repetition rate. Furthermore, overheads related to scanning in the lateral as well as axial directions have to be considered. In our work, we use a resonant scanner of frequency \( f_r \) for scanning in the x-direction with a lateral fill fraction \( t_f \) (i.e. the central portion of the sinusoidal resonant mirror trajectory during which signal is acquired), and use a galvo mirror for scanning the beam in the y-direction, allowing a galvo flyback time of \( t_g \). In the axial direction, the number of planes to be scanned, \( n_z \), and the time it takes for the piezo to return to its original position, i.e. the axial flyback time \( t_p \), also contribute to the overall volume acquisition rate. The number of pixels to be scanned in the lateral dimensions, \( n_x \) and \( n_y \), depend on the respective desired dimensions of the field-of-view and the lateral spatial resolution. With these parameters, the laser repetition rate, \( F_l \), required to achieve a desired volume acquisition rate, \( V_{ps} \), is given by

\[
F_l = \frac{n_x(n_y+2t_f f_r)n_z V_{ps}}{t_r(1-t_f V_{ps})}
\]

In our work, we have chosen \( n_x = n_y = 120 \). Furthermore, our aim was to image a volume which extends over \(~500\times500\times500\mu m\). For a desired volume rate of \( >3\text{Hz} \), this required a laser repetition rate of \( \sim 4.14\text{MHz} \), given the parameters mentioned above as well as \( t_f = 0.71 \), \( t_p = 1.1\text{ms} \), \( n_z = 51 \), and \( t_g = 30\text{ms} \) (see Methods). Consistent with this requirement our FCPA laser provided sufficient

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pulse energy (~500nJ at the output) at 4.16MHz to generate detectable GCaMP fluorescence even in deeper layers of the above volume.

**Supplementary Table 1 | Comparison of state-of-the-art multi-photon calcium imaging techniques in mouse cortex with our s-TeFo approach.** Methods based on one-photon excitation have not been included here due to their susceptibility to scattering, which makes them more suitable to a different range of imaging applications. Note that random access-scanning and 3D line-scan approaches do not image the entire volume and thus are currently not well-suited for imaging of awake mice. Furthermore, we note that speed is given in either frames (fps) or volumes per second (vol/s), depending on the actual type of demonstration (see references given). Volume speed performance of resonant two-photon microscopy is an extrapolated figure.

<table>
<thead>
<tr>
<th>Imaging Technology</th>
<th>Typical Volume/2D-FOV</th>
<th>Resolution</th>
<th>Speed</th>
<th>Typical Depth</th>
<th>Strength</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resonant two-photon scanning microscopy; Review</td>
<td>~350x350x500μm</td>
<td>diffraction limited</td>
<td>&lt;0.1 vol/s</td>
<td>&lt;1mm</td>
<td>High spatial resolution, broadly disseminated and commercially available</td>
<td>Low frame / volume acquisition rate, ultimately limited by fluorophore lifetime</td>
</tr>
<tr>
<td>3D line-scan two-photon microscopy; here: Ref 3</td>
<td>~250x250x250μm</td>
<td>diffraction limited</td>
<td>10 vol/s</td>
<td>&lt;250μm</td>
<td>Higher acquisition rate, high spatial resolution</td>
<td>Limited to ~ 200 μm in mouse cortex, susceptible to motion</td>
</tr>
<tr>
<td>Random-access two-photon microscopy 4-6, here: Ref 5</td>
<td>~400x400x500μm</td>
<td>diffraction limited</td>
<td>100 vol/s</td>
<td>&lt;600μm</td>
<td>High neuron access speed, flexible scan trajectories</td>
<td>Discontinuous scanning, requires a-priori knowledge of location, power inefficient, sensitive to motion</td>
</tr>
<tr>
<td>Statistical multi-plane demixing; here: Ref 7</td>
<td>500x500μm</td>
<td>diffraction limited</td>
<td>10 fps</td>
<td>&lt;500μm</td>
<td>Simultaneous recording from multiple planes at high speed</td>
<td>Limited scalability, requires sparse signal, limited laser power</td>
</tr>
<tr>
<td>Temporal multiplexing 8-10, here: Ref 11</td>
<td>400x400μm</td>
<td>diffraction limited</td>
<td>250 fps</td>
<td>&lt;300μm</td>
<td>Simultaneous recording from multiple sites/planes</td>
<td>No further scalability due to fluorophore lifetime, limited laser power</td>
</tr>
<tr>
<td>2D-FOV 3x5x10µm</td>
<td>3 vol/s</td>
<td></td>
<td></td>
<td>&lt;500μm</td>
<td>High speed and large FOV; flexible laser source; large population size</td>
<td>Resolution, limited to somatic activity, requires amplified laser system</td>
</tr>
</tbody>
</table>

**References:**


