**Time-lapse imaging of disease progression in deep brain areas using fluorescence microendoscopy**

Robert P. J. Barretto, Tony H. Ko, Juergen C. Jung, Tammy J. Wang, George Capps, Allison C. Waters, Yaniv Ziv, Alessio Attardo, Lawrence Recht, Mark J. Schnitzer

**Supplementary Figure 1 | Histological analysis of normal and glioma tissue studied by long-term microendoscopy.**

**a–d.** A 25–40 μm layer of glial activation arises at the CA1 surface in hippocampi studied by microendoscopy but not in control hippocampi. Coronal slices from the left (a, c) and right (b, d) hippocampus of an example *Thy1-GFP* mouse expressing GFP (green) in a subset of CA1 pyramidal and other hippocampal neurons. To reveal glial aggregation, immunostaining for glial fibrillary acidic protein (GFAP) is shown in red. The right hippocampus was followed by time-lapse microendoscopy over 14 days. The left hippocampus underwent no experimental procedures prior to sacrifice of the animal. Blue arrowheads point to the location where the cover slip of the imaging guide had been placed. Scale bars are 500 μm for (a, b), 250 μm for (c, d).
e. A close-up image of tissue that faced the tip of the imaging guide tube, showing heightened GFAP labeling at the hippocampal surface. Scale bar is 50 µm.

f. Glioma invades hippocampus following tumor cell inoculation. Coronal tissues slice of the control (left) and experimental (right) hippocampus processed with hematoxylin and eosin staining. The animal was perfused 20 days after glioma cells were inoculated into the right hemisphere during implantation of the imaging guide tube. Cancerous and normal tissues show markedly different organization. Scale bar is 500 µm.

g. A close-up of the experimental hemisphere in (f) reveals significant tumor invasion into hippocampus. Scale bar is 200 µm.
In rare instances, individual dendrites underwent extension (a) or retraction (b) during the period of examination. In (a), the red arrows point to a new segment of dendrite. In (b), the orange arrows point to the tip of a dendrite undergoing gradual retraction. The two image sequences were acquired in two different mice, on the same days following the initial implantation surgery. All panels show average intensity projections of 5 images acquired at 4.2 µm axial spacing. The scale bar applies to all panels and is 50 µm.
A time-lapse image sequence acquired in the dorsal striatum of an individual mouse by two-photon microendoscopy and intravascular injection of fluorescein-dextran, on the specified days relative to the implantation of the imaging guide tube. Each image is labeled by the day of image acquisition relative to the first imaging session and is a 2D projection of 5 images acquired at 5 μm axial increments from a stack of 180 μm total axial extent. Scale bar is 100 μm.
Automated and manual methods of blood flow speed determination yield speed values in close agreement.

Each data point represents an image pixel at which blood flow speed was determined by visually tracking erythrocyte movements and via the automated algorithm. Across the 1028 data points plotted, there is close agreement between the speed values found by the two methods ($r = 0.99$). Overall, there is a median discrepancy of only 1.5% between the two approaches and a small minority of points at which the discrepancies are $>5\%$. 
Supplementary Video Legends

Supplementary Video 1 | 3D image stack of hippocampal blood vessels acquired in a live mouse by two-photon microendoscopy and intravascular injection of fluorescein-dextran.

On day 22 following implantation of an imaging guide tube, a 1-mm-diameter microendoscope was used to create an image stack extending 660 µm in depth and containing 220 images acquired at 3 µm axial increments. The field of view is 1 mm in lateral extent. The 2D projection of this image stack is shown in Fig. 3a.

Supplementary Video 2 | Hippocampal microcirculation in normal tissue imaged by high-speed one-photon microendoscopy and intravascular injection of fluorescein-dextran.

On day 8 following bilateral implantation of two imaging guide tubes, a 1-mm-diameter microendoscope was used to image red blood cell flow in normal tissue within the control hemisphere. The field of view is 315 µm across. The frame acquisition rate was 100 Hz but playback has been slowed to 50 Hz. The movie shows the same imaging field as Fig. 3d,e. The mouse is the same as that used for Supplementary Video 3, which shows data acquired within the brain tumor in the opposing hippocampus.

Supplementary Video 3 | High-speed imaging of microcirculation in a hippocampal glioma using one-photon microendoscopy.

In the same mouse as in Supplementary Video 2 but in the opposing hippocampus, a 1-mm-diameter microendoscope was used to image red blood cell flow in glioma tissue on day 15 following the initial surgery and tumor cell implantation. The field of view is 315 µm across. The frame acquisition rate was 100 Hz but playback has been slowed to 50 Hz.
Supplementary Methods

Surgical Preparation. Mice were anesthetized by i.p. injection of ketamine (75 mg kg\textsuperscript{-1}) and xylazine (15 mg kg\textsuperscript{-1}). Body temperature was maintained using a heating blanket. The skull was exposed and cleaned with saline solution (0.9% NaCl, Baxter). Two or three stainless steel screws were implanted into the skull. For imaging studies of CA1 hippocampus a craniotomy was performed at stereotactic coordinates 2.0 ± 0.3 mm posterior to bregma and 2.0 ± 0.3 mm lateral to midline. For imaging studies of the dorsal striatum the craniotomy was performed at 1.0 ± 0.3 mm anterior to bregma and 1.8 ± 0.3 mm lateral to midline. By using a trephine to mark the skull, the clearance between the capillary and the craniotomy diameters can be limited to ~200 µm. For studies using 500-µm-diameter microendoscopes and 840-µm-diameter guide tubes the craniotomy was just over 1.0 mm in diameter. For studies using 1-mm-diameter microendoscopes and 1.8-mm-diameter guide tubes the craniotomy was 2.0 mm in diameter. The dura was removed with forceps. To prevent mechanical compression of the tissue to be imaged, a cylindrical column of neocortical matter (visual and association cortex) above the hippocampus or striatum was removed by aspiration using a 27-gauge blunt needle followed by a 29-gauge needle as the hippocampus was approached. This removal of tissue not being studied allowed the guide tube to be placed gently just above the dorsal surface of CA1 hippocampus or the dorsal striatum, without ever entering the structure being imaged. During this procedure exposed tissue was regularly irrigated with warm Ringer’s solution (Electron Microscopy Sciences).

For glioma studies, two craniotomies were performed on opposing hemispheres at symmetric coordinates. The left hemisphere served as the control, receiving no glioma cells. In the right hemisphere a few microliters of glioma cells (~100,000 cells) were placed on the hippocampus using a syringe. The injection was left undisturbed for several minutes before
inserting the capillary guide tube into the craniotomy. The washer on the guide tube was adjusted to rest on the skull. Alternatively, low melting point agarose (1.5%; Type III-A, high EEO, Sigma) was used to seal the craniotomy and stabilize the capillary over the hippocampus. A sealing of dental acrylic (Ortho-Jet, Lang Dental) was applied over exposed areas of the skull. The implanted screws provided anchors for the acrylic, permitting stable attachment the skull. Animals were given either buprenorphine (0.1 mg kg\(^{-1}\) intraperitoneal) in glioma studies, or carprofen (5 mg kg\(^{-1}\) subcutaneous) and dexamethasone (2 mg kg\(^{-1}\) intramuscular) in neuronal studies, and returned to their home cage.

**Histology.** Mice were deeply anesthetized with ketamine (100mg kg\(^{-1}\)) and xylazine (20mg kg\(^{-1}\)) and perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4). Brains were fixed overnight at 4°C. For hematoxylin and eosin (H&E) staining, the brain was cryoprotected with up to 30% sucrose in PBS. Floating sections (50 µm) were prepared on a freezing microtome, mounted on glass slides, rinsed with ethanol, and then successively stained with hematoxylin and eosin following conventional procedures.

For glial fibrillary acidic protein (GFAP) immunostaining, floating sections (100 µm) were prepared on a vibrating microtome (VT1000S, Leica) and washed with PBS buffer several times before staining. Sections were incubated in blocking solution (1% Triton X-100, 2% BSA, 2% goat serum in PBS) for 4 hours. Primary antibody (mouse anti GFAP, MAB3402 from Millipore) was diluted 1:500 in blocking solution and sections were incubated overnight in this solution. The following day sections were washed with PBS buffer and incubated in diluted secondary antibody (1:500, Alexa-594 conjugated goat anti-mouse IgG, A11005 from Molecular Probes) in blocking solution for 3 hours. All staining procedures were done at room temperature. After three washes, sections were mounted with Fluoromount-G (Southern Biotech). Histological
specimens were inspected on a stereoscope with fluorescence capabilities (Leica MZ16 FA) (Supplementary Fig. 1a–d) and a confocal fluorescence microscope (Leica SP2 AOBS) (Supplementary Fig. 1e).

**Time-lapse imaging and analysis of dendrite stability in CA1 pyramidal neurons**

GFP-M mice were imaged at 4-day intervals. Two-photon microendoscopy was performed using 920 nm illumination with 30–40 mW of average power delivered to the back aperture of the microendoscope probe. During each imaging session, the tissue volume of interest was examined by acquiring four successive image stacks. The image slices within each stack were acquired at an axial spacing of 4.2 μm and each had 512 × 512 pixels. The laser dwell time per pixel was 2.0 μs.

Image registration for our studies of dendrite stability was done using custom software written in Matlab (Mathworks). The four stacks acquired at each imaging session were aligned by automated rigid body image registration and then averaged, to improve the signal to noise ratio. To align images across multiple imaging sessions, reference neuronal features were registered at the proximal and distal ends of each image stack. These features were used to calculate 2D affine transformation matrices that accounted for slight misalignments across imaging sessions of the outer image slices in the 3D stacks. To account for slight rotations of the optical axes, the transformation matrices for the intermediate slices within an image stack were calculated by linear interpolation between the transformation matrices for the most proximal and most distal slices.

After this image pre-processing, the resulting image stacks were sub-divided into non-overlapping regions 50 μm × 50 μm × 12.6 μm in size and containing 4 image slices. There were 12,227 such regions within the total data set, many of which contained multiple dendrites. To
make the job of analysis more manageable, a subset of these regions was randomly selected for
detailed examination. This subset contained 4,241 dendrites that were tracked in detail, although
many of the randomly selected regions contained no dendrites. The images from each region
with one or more dendrites underwent a fine registration, to precisely align the images across the
days of the time-lapse series. The four slices from each region were flattened to 2D via a
maximum intensity projection.

We selected dendrites from the set of these 2D projections, at randomly chosen time
points in the experiment, and subjected them to a semi-automated image analysis. Each
dendrite’s presence at the other time points of the time-lapse series was visually checked in each
imaging session and scored as either ‘stable’, ‘absent’, or ‘unreadable’. To do this, we wrote
custom software in the open source language Processing that displayed simultaneously all
images of the time-lapse sequence side-by-side, as well as overlapped on top of one another, so
the user could easily see any dendritic alterations. The overwhelming majority of time-lapse
sequences were unambiguously stable, with no discernible changes to dendrite structure. Rarely
occurring within the data were changes in dendrite length that were maintained or progressed
across imaging sessions (Supplementary Fig. 2). These instances of dendritic extension or
retraction were among the 16 recorded instances of dendritic alteration and were scored as the
appearance or disappearance of a stable dendritic segment. Time-lapse sequences with more than
30% unreadable time points were discarded. Sequences beginning or ending with ‘absent’ or
‘unreadable’ time points were manually re-inspected using the raw 3D data to verify the presence
or absence of the dendrite in question.

Any ‘unreadable’ time points flanked on both sides by adjacent time points that were
both ‘stable’ or both ‘absent’ scores were assumed to be the same as the flanking time points.
Similarly, any segment that was ‘absent’ but later reappeared in the same spatial location was reclassified as ‘stable’. The rate at which dendrites ‘absent’ at one time point needed to be re-categorized as ‘stable’ due to the flanking time points in the series provided an approximate estimate of the reliability of our scoring procedures. This occurred in only 0.51% of all individual observations. Based on this false negative rate for an individual time point, we estimate the probability that a dendrite would be incorrectly missed at two successive time points as $2.7 \cdot 10^{-5}$. Using two successive misses as a conservative proxy for the occurrence of a falsely recorded dendritic turnover, this calculation yields an estimate of <1 falsely recorded dendritic turnover within the entire data set. This estimate of the overall false negative rate is less than the 16 instances of dendritic turnover found in the data. Note, however, that the estimate is based on the simplifying assumption that there are no temporal correlations in the ability to score a given dendrite between days — an assumption that is almost certainly false. Due to the very small numbers associated with these rare occurrences, our interpretation is that the recorded rate of turnover approaches the reliability limits of our scoring procedure but does reflect a few instances of unmistakable dendritic alteration (Supplementary Fig. 2). Overall, comparison of dendritic segments across multiple time points seems to provide a more reliable measurement of dendritic stability, as discrimination indices comparing only two time points are more sensitive to differences in the quality of the two imaging sessions.

To estimate the daily rate of loss of dendritic stability, the total number of dendrites that had a change in structure (losses plus gains, including the few instances of dendritic length changes) were divided by the total elapsed time of our observations (expressed in units of dendrites-days, so as to yield a rate in units of day$^{-1}$). The standard error in this rate was
calculated using binomial statistics. Mean dendritic lifetime was calculated under the simplifying assumption that the rate of dendritic change is uniform in time.

**Analysis of erythrocyte flow speed.** Speed determinations were carried out using one-photon fluorescence image sequences captured at fast frame rates (typically 1,000 frames acquired at 100 Hz or 84.6 Hz, but in some cases up to 1.2 kHz). To correct for motion artifacts due to respiration and heartbeat, every frame was aligned to the first frame of the image stack. Alignment relied on a two-dimensional cross-correlation to find the maximum overlap in image structures.

It has been shown previously that since pixels lying within vessels generally exhibit significant temporal fluctuations due to erythrocyte flow, the image computed at each pixel as the standard deviation across a sequence of image frames enhances the visualization of blood vessel structure\(^{40}\). Thus, after image alignment, only pixels likely to be in a vessel were selected for study by restricting the analysis following image alignment to pixels with high standard deviation values above a minimum threshold.

To compute the average speed of erythrocytes passing through a specific pixel, we computed the cross-correlation between the intensities over all the frames in the stack at that pixel and at neighboring pixels for relative temporal delays, \(\tau\), of \(-1\), 0, and +1 images frames\(^{40}\). Those pixels that did not show any significant cross-correlation with neighboring pixels were deemed to be outside of a vessel and eliminated from analysis. After setting to zero the values of all pixels lying outside vessels or exhibiting cross-correlations with the reference pixel below a minimum value, we computed the centroid of each of the three cross-correlograms. The average speed of erythrocytes passing through the reference pixel was determined as the mean distance
traveled between the \( \tau = -1 \) and the \( \tau = +1 \) centroids, divided by the time between two image frames.

For the analysis of Fig. 5, we sought an unbiased sampling of vessels by dividing the field of view into a grid of squares about 25 \( \mu \)m in width, roughly the width of the largest vessels present. In each grid square only one pixel with the highest standard deviation was chosen for analysis. If the selected pixel did not have a standard deviation above a minimum threshold or show any significant cross-correlation with neighboring pixels, the entire square was eliminated from analysis. At pixels exhibiting cross-correlations with neighboring pixels above a minimum value, average erythrocyte speed was calculated from the cross-correlograms.

To assess the degree of potential bias on speed determinations incurred by our grid-based procedure for sampling pixels, as a control experiment we compared mean flow speeds estimated via this procedure to those estimated by sampling all pixels identified within vessels by morphological filtering based on the vessels’ geometric properties (see next section), rather than on pixels’ standard deviations in time. Mean speeds estimated by the two approaches differed by 10%, indicating there is potentially a modest bias, but at a level insufficient to account for the substantial differences seen between flow speeds at control and tumor tissue sites.

**Presentation of erythrocyte flow speed maps**

Maps of erythrocyte flow speeds were used for display purposes (Figs. 3e, 4d) and were not used in the statistical analyses of vessel diameter and flow speed (Fig. 5b). To create speed maps, image pixels lying within blood vessels were first identified within the one-photon movie data via morphological filtering. To do this, the average image across the entire move was first calculated, to emphasize vessels and reduce background temporal fluctuations. A high-pass spatial filter was then applied to reduce gradual spatial variations in background fluorescence.
intensity across the imaging field. Small neighborhoods of 16 pixels × 16 pixels within the resulting image were then thresholded in an automated manner, using Otsu’s method to pick a local threshold value that separated vessel structure from local background fluorescence. A sliding window of such neighborhoods were processed in this way, and all thresholded images were summed with a normalization that accounted for border effects. A final thresholding was performed on the composite image to yield a more sensitive detection of blood vessel morphology. Once the vessel structure had been identified, flow maps for display (Figs. 3e, 4d) were created by determining the flow speed at each pixel identified within the vasculature, using the cross-correlogram methods described above.

**Comparison of manual and automated determinations of flow speeds**

Erythrocyte speeds were manually determined from raw movie data to permit quantitative comparisons to speed values determined in an automated manner. The two methods of speed determination proved to be in close agreement ($r = 0.99$); median differences between the two were 1.5% and there were only a small minority of points at which the discrepancies were >5% (Supplementary Fig. 4).

For manual determinations, the trajectories of visually identifiable blood cells were tracked over the time each cell spent moving along a chosen blood vessel segment. Image areas in which visual tracking was untrustworthy, such as at vessel branching points or in out of focus regions, were not used for manual tracking. For a typical vessel segment, 20–80 erythrocytes were tracked manually. Erythrocyte speed was determined by a linear interpolation of the cell’s manually assessed positions in successive frames, *i.e.* a cell’s flow speed was assumed constant between consecutive frames and its trajectory was assumed to be a straight line between consecutive positions. In this way, speed values were assigned to every pixel along a vessel for
each erythrocyte. Mean speeds were then calculated at each pixel. The values obtained in this way from 1028 pixels, with a wide distribution of speeds, were compared to those provided by the automated computation of flow speed (Supplementary Fig. 4). To give large and small diameter vessels equal weight per unit length in Supplementary Fig. 4, pixels were taken from vessels’ midlines, as determined via skeletonization. Pixels located at branch points or at the boundaries of the image were discarded.

**Analysis of blood vessel diameter.** All statistical analyses of vessel diameters used values measured from two-photon image stacks. To compute a vessel’s diameter, a 2D projection of the two-photon 3D image stacks was created for each imaging session. Image contrast was approximately normalized across days at the time of acquisition. The projection images were converted to a binary image to highlight the blood vessel’s dimensions. The intensity distributions of background pixels and pixels within vessels were generally sufficiently well separated that the binary image showed a vascular structure that closely matched by eye the vessels’ structure in the raw data, in a manner that was relatively independent of the threshold value. A distance transform was then applied to the binary image, by computing the distance from every nonzero pixel inside of a blood vessel to the nearest zero-valued pixel outside the vessel. To seek an unbiased sampling of vessels, the field of view was divided into a grid of equally sized squares approximately 50 µm wide, about twice the widths of the largest vessels, to minimize double counting. In each square only one pixel with the highest distance value was selected for analysis. If the selected pixel was only one pixel away from the nearest pixel of zero value, this was deemed an artifact of binary conversion and no vessels were analyzed in that square. Otherwise, vessel diameter was determined to be twice the distance value of the selected
pixel. The constancy of control vessel diameters determined in this way (Fig. 5b) confirms the reliability of this approach at the level of population statistics.

For scatter plots of flow speed versus vessel diameter for individual vessel segments (Fig. 5a), diameter estimations were performed at the same vessel locations within one-photon video sequences as flow speed determinations. To examine the diameter of a blood vessel at a chosen pixel, we examined one-dimensional intensity profiles of the vessel along lines that cut through the chosen pixel at various angles. These line profiles were computed at two-degree angular intervals and extended from the pixel of maximum intensity (the center of the vessel) to the nearest local minima (the vascular boundary). These profiles were fit to Gaussian functions, and the blood vessel diameter was defined as the $1/e^2$ width of the narrowest Gaussian fit that met minimum goodness of fit and minimum amplitude criteria. We also confirmed that mean blood vessel diameters as determined from the one-photon data sets matched those from the two-photon data sets.

**Analysis of vascular branching ratios.** 3D image stacks acquired by two-photon microendoscopy were examined using commercial image analysis software (Imaris; Bitplane A.G., Switzerland). Vessels were visually identified and manually traced. Individual vessels were defined as having no interruption in their physical continuity within the 3D stack. The branching ratio for each vessel was determined as the vessel’s total physical length divided by the number of branch points.

**Statistical Analysis.** We used non-parametric statistical testing to avoid assumptions of normality. Comparisons of unpaired distributions employed either the two-tailed Mann-Whitney U-test, for comparisons of two sample sets, or the Kruskal-Wallis ANOVA, the generalization of the Mann-Whitney test for multiple comparisons. Comparison of cell counts from paired
hippocampal tissue slices, taken from opposing cerebral hemispheres of the same animal, were performed using a one-tailed Wilcoxon signed rank test to check for cell loss in the hemisphere in which microendoscopy was performed as compared to the hemisphere not studied by microendoscopy.

Supplementary Reference

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