

Journal of Visualized Experiments

Visualizing Protein Kinase A Activity In Head-Fixed Behaving Mice Using In Vivo Two-Photon Fluorescence Lifetime Imaging Microscopy --Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59526R2
Full Title:	Visualizing Protein Kinase A Activity In Head-Fixed Behaving Mice Using In Vivo Two-Photon Fluorescence Lifetime Imaging Microscopy
Keywords:	Two-photon fluorescence lifetime imaging microscopy (2pFLIM); A-Kinase Activity Reporter (AKAR); tAKAR α ; cAMP; protein kinase A (PKA); neuromodulation; Dopamine; norepinephrine; in vivo imaging; craniotomy; single-photon counting; adeno-associated virus
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Portland, Oregon, USA

Dear Xiaoyan and Philip,

Thank you for the opportunity to revise our manuscript entitled “*Visualizing protein kinase A activity in head-fixed behaving mice using in vivo two-photon fluorescence lifetime imaging microscopy*” (JoVE59526R1). We have now made all changes following the editorial comments. We hope that our manuscript is now appropriate for publication in JoVE. Please advise how we should proceed.

Thank you very much for your consideration!

On behalf of all of the authors,

Bart and Haining

TITLE:

Visualizing Protein Kinase A Activity In Head-Fixed Behaving Mice Using In Vivo Two-Photon Fluorescence Lifetime Imaging Microscopy

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KEYWORDS:

neuromodulation, cAMP-dependent protein kinase/protein kinase A (PKA), A-kinase activity reporter (AKAR), Förster resonance energy transfer (FRET), tAKAR α , in vivo two-photon fluorescence lifetime imaging microscopy (2pFLIM), craniotomy, locomotion

SUMMARY:

A procedure is presented to visualize protein kinase A activities in head-fixed, behaving mice. An improved A-kinase activity reporter, tAKAR α , is expressed in cortical neurons and made accessible for imaging through a cranial window. Two-photon fluorescence lifetime imaging microscopy is used to visualize PKA activities in vivo during enforced locomotion.

ABSTRACT:

Neuromodulation exerts powerful control over brain function. Dysfunction of neuromodulatory systems results in neurological and psychiatric disorders. Despite their importance, technologies for tracking neuromodulatory events with cellular resolution are just beginning to emerge. Neuromodulators, such as dopamine, norepinephrine, acetylcholine, and serotonin, trigger intracellular signaling events via their respective G protein-coupled receptors to modulate neuronal excitability, synaptic communications, and other neuronal functions, thereby regulating information processing in the neuronal network. The above mentioned neuromodulators converge onto the cAMP/protein kinase A (PKA) pathway. Therefore, in vivo PKA imaging with single-cell resolution was developed as a readout for neuromodulatory events in a manner analogous to calcium imaging for neuronal electrical activities. Herein, a method is presented to visualize PKA activity at the level of individual neurons in the cortex of head-fixed behaving mice. To do so, an improved A-kinase activity reporter (AKAR), called tAKAR α , is used, which is based on Förster resonance energy transfer (FRET). This genetically-encoded PKA sensor is introduced

into the motor cortex via in utero electroporation (IUE) of DNA plasmids, or stereotaxic injection of adeno-associated virus (AAV). FRET changes are imaged using two-photon fluorescence lifetime imaging microscopy (2pFLIM), which offers advantages over ratiometric FRET measurements for quantifying FRET signal in light-scattering brain tissue. To study PKA activities during enforced locomotion, tAKAR α is imaged through a chronic cranial window above the cortex of awake, head-fixed mice, which run or rest on a speed-controlled motorized treadmill. This imaging approach will be applicable to many other brain regions to study corresponding behavior-induced PKA activities and to other FLIM-based sensors for in vivo imaging.

INTRODUCTION:

Neuromodulation, also known as slow synaptic transmission, imposes strong control over brain function during different behavioral states, such as stress, arousal, attention, and locomotion¹⁻⁴. Despite its importance, the study of when and where neuromodulatory events take place is still in its infancy. Neuromodulators, including acetylcholine, dopamine, noradrenaline, serotonin, and many neuropeptides, activate G protein-coupled receptors (GPCRs), which in turn trigger intracellular second messenger pathways with a wide window of timescales ranging from seconds to hours. While each neuromodulator triggers a distinct set of signaling events, the cAMP/protein kinase A (PKA) pathway is a common downstream pathway for many neuromodulators^{1,5}. The cAMP/PKA pathway regulates neuronal excitability, synaptic transmission, and plasticity⁶⁻⁹, and therefore, tunes the neuronal network dynamics. Because different neurons or neuronal types express different types or levels of neuromodulator receptors¹⁰, the intracellular effects of the same extracellular neuromodulator may be heterogeneous across different neurons, and thus, have to be studied with cellular resolution. To date, it remains challenging to monitor neuromodulatory events in individual neurons in vivo during behavior.

To study the spatiotemporal dynamics of neuromodulation, a suitable recording modality is required. Microdialysis and fast-scan cyclic voltammetry are frequently used to study release of neuromodulators, but they lack the spatial resolution to monitor cellular events^{11,12}. Analogous to calcium dynamics being used as a proxy for neuronal electrical activity in population imaging¹³, PKA imaging may be used to read out neuromodulatory events across a neuronal population at cellular resolution. The present protocol describes the use of an improved A-kinase activity reporter (AKAR) to monitor PKA activities in vivo during animal behavior. The method described here allows for simultaneous imaging of neuronal populations at subcellular resolution with a temporal resolution that tracks physiological neuromodulatory events.

AKARs are composed of a donor and an acceptor fluorescent proteins linked by a PKA phosphorylation substrate peptide and a forkhead-associated (FHA) domain that binds to the phosphorylated serine or threonine of the substrate^{14,15}. Upon activation of the PKA pathway, the substrate peptide of AKAR is phosphorylated. As a result, the FHA domain binds to the phosphorylated substrate peptide, thereby bringing the two fluorophores into close proximity, referred to as the closed state of AKAR. The closed state of a phosphorylated AKAR results in increased Förster resonance energy transfer (FRET) between the donor and acceptor fluorophores. Since the proportion of phosphorylated AKARs is related to the level of PKA

activity¹⁶, the amount of FRET in a biological sample can be used to quantify the level of PKA activity^{16–20}.

Early versions of AKARs were primarily designed for two-color ratiometric imaging¹⁴. When imaging deeper into brain tissue, the ratiometric method suffers from signal distortion due to wavelength-dependent light scattering^{17,18,21}. As discussed below, fluorescence lifetime imaging microscopy (FLIM) eliminates this problem because FLIM only measures photons emitted by the donor fluorophore^{18,21}. As a result, FLIM quantification of FRET is not affected by the tissue-depth¹⁷. In addition, a “dark” (i.e., low quantum yield [QY]) variant of the acceptor fluorophore can be used. This frees a color channel to facilitate multiplexed measurement of orthogonal neuronal properties via simultaneous imaging of a second sensor or a morphological marker^{17,19,20}.

FLIM imaging quantifies the time that a fluorophore spends in the excited state, i.e., the fluorescence lifetime¹⁸. The return of a fluorophore to the ground state, thus the end of the excited state, often concomitates with the emission of a photon. Although the emission of a photon for an individual excited molecule is stochastic, in a population the mean fluorescence lifetime is a characteristic of that particular fluorophore. When a pure population of fluorophores are excited simultaneously, the resulting fluorescence will follow a single exponential decay. The time constant of this exponential decay corresponds to the mean fluorescence lifetime, which typically ranges from one to four nanoseconds for fluorescent proteins. The return of an excited donor fluorophore to the ground state can also occur by FRET. In the presence of FRET, the fluorescence lifetime of the donor fluorophore is reduced. The unphosphorylated AKARs exhibit a relatively longer donor fluorescence lifetime. Upon phosphorylation by PKA, the sensor exhibits a shorter lifetime because the donor and acceptor fluorophores are brought near each other and FRET is increased. The quantification of the fluorescence lifetime in a population of AKARs therefore represents the level of PKA activity.

Early versions of AKARs have not been successfully used for in vivo imaging at single-cell resolution. This is mainly due to the low signal amplitude of the AKAR sensors to physiological activations¹⁷. Recently, by systematically comparing available AKAR sensors for two-photon fluorescence lifetime imaging microscopy (2pFLIM), a sensor called FLIM-AKAR was found to outperform alternative sensors. Furthermore, a series of FLIM-AKAR variants called targeted AKARs (tAKARs) were developed to visualize PKA activity at specific subcellular locations: microtubules (tAKAR α), cytosol (tAKAR β), actin (tAKAR δ), filamentous actin (tAKAR ϵ), membrane (tAKAR γ), and postsynaptic density (tAKAR ζ). Among tAKARs, tAKAR α increased the signal amplitude elicited by norepinephrine by 2.7-fold. This is consistent with the knowledge that the majority of PKA in neurons are anchored to microtubules at the resting state^{22,23}. tAKAR α was the best performer among existing AKARs for 2pFLIM. Furthermore, tAKAR α detected physiologically-relevant PKA activity elicited by multiple neuromodulators, and the expression of tAKAR α did not alter neuronal functions¹⁷.

Recently, tAKAR α was successfully used to visualize PKA activities in head-fixed behaving mice¹⁷. It was shown that enforced locomotion triggered PKA activity in the soma of superficial layer

neurons (layer 1 through 3, up to a depth of ~300 μm from pia) in the motor, barrel, and visual cortices. The locomotion-triggered PKA activity was in part dependent on signaling via β -adrenergic receptors and D1 dopamine receptors, but was not affected by a D2 dopamine receptor antagonist. This work illustrates the ability of tAKARs to track neuromodulation events in vivo using 2pFLIM.

In the current protocol, the entire method for PKA activity imaging in head-fixed awake mice during an enforced locomotion paradigm is described in six steps. First, the addition of 2pFLIM capabilities to a conventional two-photon microscope (**Figure 1**). Second, the construction of a motorized treadmill (**Figure 2**). Third, the expression of the tAKAR α sensor in the mouse cortex by in utero electroporation (IUE) of DNA plasmids, or stereotaxic injection of adeno-associated virus (AAV). Excellent protocols for surgeries for IUE^{24,25} and stereotaxic injection of viral particles²⁶ have been previously published. The key parameters we used are described below. Forth, the installation of a cranial window. Excellent protocols have been previously published for cranial window surgery^{27,28}. Several steps that have been modified from the standard protocols are described. Fifth, performing in vivo 2pFLIM. Sixth, the analyses of 2pFLIM images (**Figure 3** and **Figure 4**). This approach should be readily applicable to many other head-fixed behavioral paradigms and brain areas.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Oregon Health and Science University.

1. 2pFLIM microscope setup

1.1. Install a photon timing counting module (PTCM, **Table of Materials**) and connect to the computer (**Figure 1**) according to the manufacturer's manual.

NOTE: The PTCM is typically a computer board that receives a "sync" input for the laser pulse timing and a photon input from the photomultiplier tube (PMT). It also receives clock timing for pixels, lines, and frames, from two-photon imaging control software. The PTCM uses the clock signals to separate individual photons into different pixels and frames.

1.2. Add a photodiode with > 200 MHz bandwidth to measure the laser timing. Place a standard glass coverslip in the light path to reflect a small fraction of the laser light into the photodiode placed perpendicular to the light path (**Figure 1**). Connect the photodiode output to the "sync" input of the PTCM.

NOTE: Many modern lasers also output the laser timing. For these lasers, the photodiode is not necessary, and one can directly connect the laser timing output to the sync input of the PTCM.

1.3. Exchange the PMT, in case of the tAKAR α , the green channel PMT, with a low-noise, fast GaAsP PMT (**Figure 1**). Connect the PMT output to the signal input of the PTCM.

1.3.1. Add an optional signal splitter (**Figure 1**) if simultaneous acquisition of intensity through the conventional two-photon imaging channel is desired. Connect the PMT output to the signal splitter and connect the splitter output to the PTCM and the conventional two-photon imaging module.

NOTE: GaAsP PMTs give faster single-photon signals than conventional bialkali PMTs and allow for a more precise determination of the photon timing. Certain models of GaAsP PMTs can be cooled to 10–35 °C below ambient temperature, allowing the suppression of dark counts to a level below a few hundred per second (typically ≤ 200 counts/s). This low noise level is important for the precise measurement of fluorescence lifetimes because noise photon counts cannot be easily distinguished or subtracted from the fluorescence lifetime curve.

1.4. Add a band-pass fluorescence emission filter that minimizes the spectral contamination, if any, from the acceptor fluorophore. For example, for tAKAR α , a 500 nm \pm 20 nm barrier filter for the green channel is used to reduce the contamination from the acceptor sREAcH, which is a dark (QY \sim 0.07) yellow fluorescent protein (YFP)^{29,30}. Connect timing signals, such as the clocks for individual image pixels, lines, and frames, as appropriate to the control software and described in the PTCM user manual. Install the appropriate data control and acquisition software.

NOTE: Some PTCM manufacturers (**Table of Materials**) provide their software for 2pFLIM imaging. Here, custom software called FLIMimage is used, which was developed by the Yasuda Lab (Max Planck Florida, via personal communication). This software functions as an add-on user function to certain two-photon acquisition software (**Table of Materials**). It controls and communicates with the PTCM at the appropriate timing during two-photon imaging to acquire 2pFLIM images.

2. Construction of a motorized treadmill

NOTE: The design of the custom-built motorized treadmill is shown in **Figure 2**.

2.1. Cut a foam roller (Φ = 200 mm) to 150 mm in length with a fine hacksaw. Alternatively, glue the two halves of a foam ball together and place tape over the seam. Optionally, glue a rubber mat with profile on the roller to increase traction on the roller.

2.2. Drill a ¼ inch diameter hole through the center of the roller at the flat side of the roller or drill a ¼ inch diameter hole through the middle of each half of the ball if the foam ball is used.

2.3. Install a ¼ inch diameter steel axle through the hole. Glue the foam roller/ball to the axle using foam-compatible glue. Optionally, modify two flexible shaft couplings (¼ inch inner diameter) to strengthen the coupling of the axle to the foam roller/ball.

NOTE: Please note that many common glues may dissolve foam.

2.3.1. For each shaft coupling, position the shaft coupling on its flat side and at the center of the

rectangle metal plate (0.7 mm x 15 mm x 76 mm). Weld the plate to the shaft coupling. Drill a ¼ inch hole at the center of the plate to allow for installation of the modified shaft coupling on to the axle and two screw holes into the metal plate lateral from the center.

2.3.2. Install the shaft coupling onto the axle against the foam roller/ball. If using the latter, slightly bend the plate to fit the curvature of the ball. Place screws into the lateral holes to fix the roller/ball on the axle.

2.4. Drill and tap a 3/8-32 thread at the center of a cage plate and install the rotation sensor. Attach both the rotation sensor and the motor to the end of the axle using a flexible shaft coupling. Drill a screw hole into the base of the right-angle motor bracket to allow attachment of the motor onto the post holder.

2.5. Install the assembled treadmill on an aluminum bread board using posts for the motor and rotation sensor (**Figure 2**). Connect the motor inputs to the speed controller, and the rotation sensor output to an analog input of the computer data acquisition (DAQ) board.

NOTE: The rotation angular speed is encoded by the rotation sensor as voltage and is digitized using custom software called AnimalTracker written in MATLAB.

2.6. Install the headplate-compatible holder to an adjustable mounting post bracket. Install a solid post on the bread plate in front of the treadmill and install the assembled headplate holder with the post bracket on the post (**Figure 2**). Ensure that the headplate holder bars are aligned with the axle such that the mouse can adopt an adequate and comfortable walking position on the treadmill (**Figure 2C**).

3. Expression of tAKARα sensor in the mouse cortex

3.1. In utero electroporation

3.1.1. Prepare a DNA solution for IUE by adding 0.2% final concentration of fast green dye (for visualization during injection) to a plasmid DNA (3–4 µg/µL; the sensor constructs containing a CAG promotor, sensor sequence, and a woodchuck hepatitis virus post-transcriptional response element [WPRE] translational enhancer) dissolved/diluted in water or Tris-EDTA.

3.1.2. Prepare a timed pregnant female mouse (e.g., C57BL/6) for IUE at E16²⁴. Cut open the abdominal cavity with a scalpel and a pair of scissors and carefully expose the uterine horns.

3.1.3. Inject 1 µL of DNA solution per embryo in the lateral ventricle of one hemisphere, as previously described²⁴.

3.1.4. Perform regular IUE²⁴ for cortical neurons by placing the positive electrode end foot at the cortex and using five 100-ms square pulses (38 V) at 1 Hz with an electroporator.

NOTE: Different cortical regions can be targeted for electroporation by changing the placement of the electrode end foot relative to the lateral ventricle.

3.2. Stereotaxic injection

3.2.1. Prepare a mouse at postnatal day 30 for stereotaxic surgery²⁶.

3.2.2. Dilute AAV serotype 2/1 (AAV2/1) expressing hSyn-tAKAR α -WPRE to an empirically determined titer ($\sim 1 \times 10^9$ – 1×10^{10} genomes/ μ L) in syringe-filtered (0.2 μ m cellulose acetate membrane) phosphate-buffered saline.

3.2.3. Drill a ~ 500 μ m diameter hole using a handheld drill under a stereomicroscope at the following coordinates for the motor cortex: 0.5 mm anterior to bregma, 1.2 mm lateral to midline.

3.2.4. Mount an injector (e.g., oil hydraulic manipulator, with custom-made plunger/glass pipette holder) to a motorized manipulator. Place the injection needle at a 15° angle relative to the bregma-lambda plane. Program a diagonal movement across the x- and z- axes equivalent to a 700 μ m and 200 μ m progression along the anterior-posterior and dorsal-ventral axes, respectively.

NOTE: To avoid damage to the tissue directly above the intended imaging field, AAV particles are injected at an angle relative to the bregma-lambda plane.

3.2.5. Position the tip of the injection needle at the pia in the center of the drill hole and slowly execute the diagonal movement (~ 25 μ m/s) described above. This procedure will position the center of injection at 1.2 mm anterior to bregma, 1.2 mm lateral to midline, 0.2 mm below the pia.

3.2.6. Inject 20 nL of diluted viral particles (~ 10 nL/minute). Wait at least 10 min and slowly retract the injection needle (~ 12.5 μ m/s).

3.2.7. Finish the stereotaxic injection procedure and glue/suture the skin²⁶.

4. Installation of the cranial window

4.1. Perform the placement of the cranial window on mice expressing tAKAR α via IUE (section 3.1) or stereotaxic injection of viral particles (section 3.2), between postnatal days 30 and 60. For mouse infected with viral particles, implement the cranial window at least two weeks after the virus injection. Install the cranial window as previously described^{27,28}, with the following details.

4.2. Remove the periosteum and retract the neck muscle. Glue the edge of the skin to the skull with tissue adhesive to avoid exposure of the neck musculature after surgery.

4.3. Dry and remove any periosteum from the skull by gently scraping using a scalpel. Place the imaging headplate (8 mm inner diameter) to surround the intended imaging field. Glue the headplate to the skull using cyanoacrylate-based glue, followed by dental acrylic cement. For optimal adhesion, ensure that the headplate rests on the exposed and dried skull. Glue accelerator can be used to accelerate the hardening.

4.4. Draw a circle of 5 mm in diameter above the intended imaging field (coordinates as specified in step 3.2.3) using a dental drill and expose the dura mater.

4.5. Apply a thin layer of transparent polymer, also called artificial dura, to the dura surface to cover the entire cranial window. The polymer will protect and stabilize the dura mater. Place a sterile circular coverslip (5 mm diameter) on the dura mater. Secure the coverslip with cyanoacrylate glue applied around the edges of the window followed by dental acrylic cement.

5. In vivo two-photon fluorescence lifetime imaging microscopy

5.1. Commence 2pFLIM imaging at or beyond 2 weeks post-installation of the cranial window (section 4). Minimize experimental interference due to stress by frequent handling and scruffing of the mouse prior to the start of the imaging study to habituate the mouse.

5.2. Set the two-photon excitation laser wavelength to 960 nm using the software that controls the two-photon laser.

5.3. Anaesthetize the mouse using 4% isoflurane. Confirm proper anesthetization by tail-pinch and observing breathing rates. That is, there should be no response to the tail-pinch and the breathing rate should be reduced to ~1 breath per second.

5.4. Transfer the anaesthetized mouse to the motorized treadmill (**Figure 2C**) and mount the headplate of the mouse to the headplate holder of the treadmill setup (see **Figure 2** for details). Clean the surface of the cranial window coverslip on the mouse with 70% ethanol.

5.5. Place the motorized treadmill with the mounted mouse under the 2pFLIM objective. Apply a drop of distilled water between the cranial window coverslip and the objective.

5.6. Let the mounted mouse wake up from anesthesia and become acclimated to the treadmill and microscope environment for at least 10 min.

5.7. Locate tAKAR α -positive neurons using epifluorescence. Document fiducial features (i.e., blood vessels) under brightfield to aid imaging of the same region of interest (ROI) during subsequent imaging sessions.

5.8. Activate the 2pFLIM PMT by switching on the hardware command voltage control. Eliminate any incoming light other than the emitted light from the brain tissue. Switch off the epifluorescence light source and close the enclosure of the 2pFLIM rig.

5.9. Acquire a z-stack 2pFLIM image using the 2pFLIM acquisition software FLIMImage with the following recommended settings for imaging tAKAR α -positive somata in awake mice. Set frame averaging to 3 frames, scanning speed to 2 ms/line, image size to 128 x 128 pixels, and field of view to 90–100 μ m. Adjust imaging settings based on the preparation and hardware configuration.

5.10. Inspect the acquired image in FLIMview (in-house developed custom software; see section 6). Adjust imaging settings following step 5.9 to optimize photon count and minimize photobleaching.

NOTE: A workable integrated photon count in an ROI for lifetime imaging of a tAKAR α -positive soma in vivo is ~1,000–10,000 photons depending on the signal amplitude that results from a particular stimulus (see DISCUSSION).

5.10.1. Where needed, use a decreased field of view, decreased scanning speed, increased laser power, and increased number of frames to be averaged to increase the integrated photon counts and reduce the lifetime estimation error. At the same time, be sure to use the minimal essential laser power, frame averaging, and scanning speed to minimize photobleaching.

5.11. Image at a regular time interval (e.g., every 30–60 s) by repeating the z-stack acquisition using settings determined in step 5.9. Acquire baseline 2pFLIM images for at least 15 min at zero treadmill speed.

5.12. Set the treadmill rotation speed to ~15 cm/s for 15 min while acquiring 2pFLIM images. Continue imaging for \geq 20 min after switching off the treadmill rotation, to assess the duration of PKA activity after cessation of forced locomotion.

6. Analysis of 2pFLIM images

6.1. Open the acquired images in FLIMview and set the following parameters in the FLIMview.

NOTE: Parameter details are described in DISCUSSION.

6.1.1. Click on the single photon counting (SPC) minimum and maximum range fields in FLIMview. Enter the appropriate minimum and maximum SPC range value, typically ranging between 1.2–2 and 10–12 ns, respectively.

6.1.2. Click on the t_0 value field in FLIMview and enter the t_0 value (typically ~2 ns). Click on the lifetime luminance minimum threshold value field in FLIMview and enter the desired threshold value to 5–30 photons.

6.2. Click on the new group button (**N**) and assign an experiment group name. This will generate a group that combines data from each added FLIM image.

6.3. Click on the **ROI** button in the **Roi Controls** module of FLIMview and draw an ROI around a tAKAR α -positive soma. Reduce the z-stack range, by moving the lower and upper z-limit in the **z-stack Control** sliders in FLIMview, to minimize signal contamination originating from background photons in other z depths.

6.4. Click on the **+** button to add the FLIM image to the group (step 6.2). Click on the **Calc** button to calculate the mean lifetime (LT, also called mean photon emission time [MPET]), for the ROI and the lifetime estimation error ($\delta\tau$).

6.5. Open the next file in the chronological 2pFLIM imaging series. Repeat step 6.4. Be sure to adjust the position of the ROI and z-stack range to measure the same tAKAR α -positive soma over time, because there can be tissue drift over time.

6.6. Select the **deltaMPET/MPET₀** in the drop-down menu of the **Group Controls** module. Click on the **baseline#** field and enter the index(es) (e.g., **1 2 3 4 5** for the first five images in the group created in step 6.3). This will define the image used to calculate baseline lifetime (LT₀).

6.7. Click on **Plot** to generate a graph containing the FLIM response ($\Delta LT/LT_0$) of tAKAR α during the experiment in the defined ROIs. Normalized changes in lifetime (ΔLT) of individual ROIs by the corresponding baseline lifetime (LT₀) allow for comparison of PKA activity during locomotion across different ROIs.

REPRESENTATIVE RESULTS:

FRET-FLIM sensors allow for the visualization of many different signaling pathways, including the cAMP/PKA pathway involved in neuromodulation. The current protocol utilizes the recently-developed tAKAR α sensor in combination with 2pFLIM to visualize PKA activities in head-fixed behaving mice. Most existing two-photon microscopes can be upgraded with 2pFLIM capabilities by adding three to four components, as illustrated in **Figure 1** (see also section 1). To visualize FRET in 2pFLIM-acquired images, quantification of mean lifetime was performed on histogram plots of photon timing collected per pixel (**Figure 3A,B**). Mean lifetime was visualized using a pseudo-colored image, in which high (cold color) and low (warm color) mean lifetimes represent low and high PKA activities, respectively, since PKA activation leads to the decrease of lifetime. Care must be taken to set the SPC range correctly; this range should be set within the laser pulse interval (e.g., 12.5 ns of a pulse rate of 80 MHz) with minimized hardware edge artifacts (see also section 6 and DISCUSSION). Calculation of PKA activity within ROIs was performed by combining the LT of all pixels within a given ROI (**Figure 3C,D**). In head-fixed awake mice basal lifetimes ranged between 1.3 and 1.8 ns (**Figure 3E**). Imaging of tAKAR α in the motor cortex in head-fixed awake mice allowed for the real-time quantification of PKA activity with cellular resolution during basal and enforced locomotion (**Figure 4**). The experiment can be repeated over days and months. Enforced locomotion triggers PKA activity in a population of neurons within the superficial layers of the mouse motor cortex¹⁷. This PKA activity is dependent on neuromodulation via activation of β -adrenergic and D1 receptors¹⁷.

FIGURE LEGENDS:

Figure 1: Schematic of a 2pFLIM system. 2pFLIM can be implemented on a conventional two-photon microscope by the addition of the yellow highlighted hardware components: a photon timing counting module, a low-noise fast photomultiplier tube (PMT), a photodiode (only needed if the laser does not have an output signaling for laser timing), and an optional signal splitter. This figure has been modified from Ma et al.¹⁷.

Figure 2: Design of a custom-built motorized treadmill. (A) Schematic of the treadmill design from front (top left), side (top right), and top views (bottom left). The treadmill (foam ball) axle is connected to a rotation sensor and a motor that are collectively mounted on two posts on a solid aluminum bread plate. The headplate-compatible holder on an adjustable mounting post bracket is fixed to a solid post and positioned above the treadmill. Schematic drawings are not to scale. Front (B) and side (C) view photographs of the treadmill. Proper positioning of the mouse on the treadmill is shown in panel C.

Figure 3: The quantification of 2pFLIM data. (A) A FLIM image with each pixel pseudo-colored to represent the mean lifetime (LT), relative to the laser timing, of all photons in that pixel. (B) The photon arrival times within a single pixel (purple square in panel A) were plotted in a histogram (left panel). Integration boundaries were set to determine the single photon counting range (SPC, gray). Within the SPC range, the integral of photon timing was divided by the total number of photons and then subtracted by the t_0 (1.65 ns, dashed line), resulting in a mean lifetime (LT, distance between dashed and dotted lines) of 1.74 ns. Quantification of the mean lifetime of the entire field of view (light blue square in panel A) involved the integration of photon timing collected in all pixels (right panel), resulting in a mean lifetime of 1.7 ns. Insets show the same data in semi-log scale. (C and D) Quantification of mean lifetime per region of interest (ROI). (C) Representative example of a 2pFLIM image. Two ROIs were drawn around two somata in layer 2/3 of the motor cortex. (D) Photon timing distributions integrated across all pixels within each ROI (left panel). Cell ROIs were color-coded as shown in panel C: red, cell 1; blue, cell 2. Normalized photon counts allow for comparison of photon timing distributions between the two ROIs (right panel, mean lifetime; cell 1, 1.33 ns; cell 2, 1.73 ns). Insets show the same data in semi-log scale. (E) Distribution plot of mean basal lifetimes from 254 imaged cells in the superficial layers of the motor cortex. L1 cells ($n = 186$ cells/11 animals, left panel), residing within 100 μm below pia, expressed tAKAR α after a stereotaxic injection of AAV2/1-hSyn-tAKAR α -WPRE, and L2/3 pyramidal cells ($n = 68$ cells/4 animals, right panel), residing at least 150 μm below pia, expressed tAKAR α after IUE of a CAG-tAKAR α -WPRE DNA construct.

Figure 4: tAKAR α tracks enforced locomotion-induced PKA activities in the motor cortex. (A) Representative intensity (left panel) and lifetime (middle and right panels) images of three L1 cells in the motor cortex. Cell ROIs were color-coded: orange, cell 1; blue, cell 2; yellow, cell 3. (B) Photon timing distributions measured in cell 1 (upper panel) during the basal condition (orange trace, as measured in middle panel A) and enforced locomotion (loco., light orange trace, as measured in right panel A). Normalized photon counts allowed for direct comparison of photon timing distribution (lower panel, mean lifetime: basal, 1.72 ns; locomotion, 1.42 ns). Insets show

the same data in semi-log scale. (C) Δ lifetime/lifetime₀ (Δ LT/LT₀) traces of the corresponding cells (upper panel, see panel A) with enforced locomotion speed (lower panel).

DISCUSSION:

This protocol demonstrates the use of FRET-FLIM sensor tAKAR α to visualize neuromodulation-triggered PKA activity in head-fixed behaving mice. This application is based on extensive testing and characterizations of tAKAR α in vitro and in vivo to demonstrate that the FLIM signal obtained is relevant to physiological neuromodulatory events¹⁷. Here, one in vivo application, locomotion-induced PKA activity in the motor cortex, is used to describe the procedures for delivering the sensor to the brain, animal surgery for imaging, hardware and software requirements for behavior and imaging data acquisition, and software and algorithms for imaging data analyses.

The tAKAR α sensor is introduced to the cortex by IUE of DNA plasmids or stereotaxic injection of AAV particles. Depending on the electroporation parameters and DNA concentrations, IUE results in various labeling density of cortical neurons over a relatively large area in the cortex²⁵. The cortical layer labeled using IUE is determined by the embryonic stage when the surgery is performed. Stereotaxic injection of AAV particles is used when it is desired to image many cells within a defined brain subregion. It typically results in densely labeled neurons at the injection center and increasingly sparse labeling further from the center. Importantly, infection efficiency of cells within the brain is dependent on the AAV serotype used. AAV2/1 offers great efficiency in cortical, thalamic, and striatal neurons with relatively low retrograde labeling activities. It is advised to empirically establish which AAV serotype is most efficient for the targeted brain region and cell type. Both transfection methods have successfully expressed tAKAR α . The “sweet spot” for the expression level is empirically determined.

Enforced locomotion results in increased PKA activities in layer 2/3 neurons of the motor cortex. Currently, 2pFLIM limits the range of testable behaviors due to head-fixation of the mouse. However, an ever-growing list of behavioral paradigms have been successfully implemented within this constraint, ranging from reporting stimuli in go/no-go tasks to spatial orientation in virtual reality^{31–33}. In addition, improved methods may enable imaging in deep brain regions, such as the striatum, amygdala, and hippocampus, via a needle-like gradient index (GRIN) lens¹³ (unpublished observations). Therefore, the present protocol detailing the use of tAKAR α and 2pFLIM for in vivo visualization of neuromodulation events should be readily applicable to many brain regions in the context of head-fixed behavioral paradigms.

Calculation of lifetime per pixel or ROI using curve fitting is computationally time consuming, and the limited total photon count per pixel often results in fitting errors. Hence, mean lifetime (LT) is arithmetically calculated as an approximation for the lifetime (τ)^{17,34} (Equation 1):

$$LT = \frac{\int_{SPC_{min}}^{SPC_{max}} tF(t)dt}{\int_{SPC_{min}}^{SPC_{max}} F(t)dt} - t_0 \quad (\text{Equation 1})$$

where SPC_{min}, and SPC_{max} are the measurement window (SPC) borders and F(t) is the fluorescence

lifetime decay curve. In other words, for each calculated volume (pixel or ROI, **Figure 3A**) the photon timing distribution is plotted in a histogram (**Figure 3B**). Within the SPC range the weighted integral (with time being the weight) of this distribution is divided by the total photon count to result in an averaged emission time. This time is then corrected for t_0 . To generate a lifetime image (**Figure 3A**) this procedure is performed for each pixel, whereas calculation of lifetime per ROI (**Figure 3C,D**) integrates all photons from all pixels that are above threshold within the ROI volume. The lifetime estimation error ($\delta\tau$) is calculated using the integrated intensity (N_{photon} : total photon count) (Equation 2):

$$\delta\tau \approx \frac{\tau}{\sqrt{N_{\text{photon}}}} \quad (\text{Equation 2})$$

To minimize lifetime estimation error, $\delta\tau$, and yield proper signal detection FRET-FLIM requires the acquisition of enough photons per ROI. In order to achieve a desired signal-to-noise ratio (SNR), $\delta\tau$ also have to meet the following equation (Equation 3):

$$\delta\tau \leq \frac{\Delta LT}{SNR} \quad (\text{Equation 3})$$

For example, typical measurement during locomotion in a neuronal soma in the motor cortex (LT = 1.57 ns, $N_{\text{photon}} = 9075$, $\Delta LT = 0.15$ ns; **Figure 4**, cell 1) yields a lifetime estimation error of (Equation 4):

$$\delta\tau \approx \frac{1.57}{\sqrt{9075}} \approx 0.016 \text{ ns} \quad (\text{Equation 4})$$

which results in a signal-to-noise ratio of (Equation 5):

$$SNR \approx \frac{\Delta LT}{\delta\tau} = \frac{0.15}{0.016} \approx 9.4 \quad (\text{Equation 5})$$

If a desired SNR is only 5, given $\Delta LT = 0.15$ ns a $\delta\tau$ is allowed of (Equation 6):

$$\delta\tau \approx \frac{\Delta LT}{SNR} = \frac{0.15}{5} \approx 0.03 \text{ ns} \quad (\text{Equation 6})$$

which requires a minimum total photon count of (Equation 7):

$$N_{\text{photon}} \approx \left(\frac{LT}{\delta\tau}\right)^2 = \left(\frac{1.57}{0.03}\right)^2 \approx 2739 \text{ photons} \quad (\text{Equation 7})$$

As outlined above, lifetime quantification requires appropriately setting several parameters, such as SPC range, t_0 , and lifetime luminance minimum threshold. The SPC range determines the measurement window of emitted photons within the hardware measurement window (Equation 1; hardware measurement window is typically 0–12.5 ns, as the laser repeats at 80 MHz). This is necessary because the PTCM used in this protocol has edge artifacts. The SPC range is set to incorporate most of the donor photon lifetime distribution without including the edge artifacts.

To calculate mean lifetime, the mean photon timing from the measurement window is subtracted by t_0 , which corresponds to the timing of the laser pulse within the window (Equation 1, **Figure 3A,B**)^{17,34}. t_0 can be adjusted by changing the signal cable lengths or the PTCM settings, and is typically adjusted to be ~2 ns from the start of the hardware measurement window. After initial characterization of the system, typically carried out under near ideal imaging conditions (e.g., when imaging 5 μ M fluorescein solution), both the SPC range and t_0 are set as fixed parameters of a given hardware configuration. Lifetime luminance minimum threshold is set so that only pixels with a total photon count equal or higher than the threshold will be included in display and analysis. This effectively reduces the noise due to background photon counts, including autofluorescence, ambient light, and spontaneous PMT dark counts. This threshold is empirically determined.

Successful FRET-FLIM sensors for in vivo 2pFLIM imaging have at least three common features. First, regarding the selection of fluorophores, the photon collection efficiency is usually low under the challenging in vivo imaging environment in part due to severe light scattering in brain tissue. At the same time, a high number of detected photons is required to achieve a desirable SNR ($\geq 1,000$ photons would be required to achieve a SNR of 1 for a $\Delta LT/LT_0$ of 0.03; see Equations 2 and 3). Therefore, a donor fluorophore with a high photon budget (i.e., the maximal number of detectable photons before a fluorophore is bleached) is favored. Currently, there is no systematic comparison of different donor fluorophores in terms of their photon budget under two-photon excitation. Empirically, eGFP is relatively bright while being more photostable compared to many other fluorophores in the green/yellow spectrum, making it a great donor fluorophore for in vivo use of FRET-FLIM sensors. In addition, for optimal quantification of FRET, donor fluorophores with a single-exponential fluorescence lifetime decay are favored. Many commonly-used donor fluorescent proteins for ratiometric imaging, such as eCFP, have multi-exponential fluorescence lifetime decays, suggesting that they consist of mixed populations of fluorophores. These fluorescent proteins are therefore not ideal for FRET-FLIM²¹. Contrary to the donor fluorophore, a low quantum yield of the acceptor fluorophore can be beneficial for FRET-FLIM sensors. The “dark” low-irradiant fluorophore sREACH is used for tAKARs. Low quantum yield of the acceptor fluorophore minimizes photon contamination in the donor fluorophore emission spectrum and frees one fluorescence detection channel for simultaneous imaging of a second fluorescent sensor or morphology marker in the red spectrum in the case of tAKAR α .

Second, to obtain sufficient SNR across the binding fraction range, an optimal FRET-efficiency of ~0.5–0.7 is favored²¹. The signal, i.e., the mean lifetime change under a given donor-acceptor binding ratio change, is dependent on the efficiency of FRET. This relationship between FRET efficiency and mean lifetime change is, however, non-linear. If the FRET efficiency approaches one, the donor fluorophores in bound-state are effectively emitting nearly no photons. Therefore, unless the binding ratio is 100% (this is never the case because no acceptor fluorophore matures to 100%²⁹) the mean lifetime approaches the lifetime of the donor fluorophore in open-state, and the ability of detecting bound-state sensors decreases. The FRET-efficiency for tAKAR α is estimated to be ~0.7, within the favorable range.

Third, FRET-FLIM sensors should report signals with a sensitivity and kinetics that are relevant to

animal physiology. Sensor sensitivity and kinetics should be extensively tested in vitro prior to its use in vivo, and, if necessary, can be tuned using a variety of approaches, such as adjusting substrate-binding domain affinity and kinetics, linker-optimization, and subcellular targeting of the sensor. In previous work, it was established that tAKAR α can detect PKA activity elicited by the releases of endogenous dopamine, and that the kinetics and sensitivity of the sensor align with a known PKA-dependent biological process, which is norepinephrine-induced inactivation of the slow after-hyperpolarization current¹⁷. Furthermore, the expression of tAKAR α does not appear to alter neuronal functions, as assayed by electrophysiology¹⁷ and measurement of structural plasticity of individual spines (unpublished observations).

Current technical limitations of 2pFLIM imaging are related to data handling and photon counting throughputs. First, FLIM requires the storage of photon arrival times for each pixel. The memory size of the PTCM limits the obtainable pixel resolution. For the PCTM described here, up to 256 x 256 pixels per image frame with a 64-point time resolution can be achieved. In addition, the transfer speed of FLIM image data from board to computer storage is relatively slow, again, putting practical limits on the resolution and sampling frequency. Continuous technological improvement of memory capacity and data handling may resolve these limitations in the future. Second, commonly-used PTCMs are analog-to-digital systems and are limited by their photon detection reset times (i.e., “dead time”). This means that after the detection of one photon the PTCM will not register the arrival of any subsequent photon(s) for the next 100–125 ns^{18,21}. Furthermore, the lifetime measurement is biased towards the first arrived photon after a laser pulse (so called “pile-up”). These limit the photon counting rates to <10⁷ photons per second. Although in most typical two-photon imaging regimes this is not a major problem, care should be taken not to exceed the photon counting rate limits. Newer PTCMs that have shorter dead time or a gigahertz continuous data acquisition system can alleviate this limitation (for the latter see Yellen and Mongeon¹⁸).

Fluorescent sensors for signaling pathways, such as cAMP/PKA, Akt/PKB, PKC, and ERK, are continuously being generated and optimized^{16,35}. For most of the current sensors, further characterization and optimization are needed to excel in the challenging in vivo imaging environment. In particular, increased signal amplitude is important, as any increase in the signal amplitude reduces the demand on photon budgets with a square relationship. For tAKAR α , its response amplitude to endogenous neuromodulators, such as norepinephrine, was improved by 2.7-fold compared to the previous best sensor. This translated to a ~7-fold decrease in required photons. In practice, this greatly reduced the number of false negatives (i.e., non-responders) in animals during behavior¹⁷. The maximum tAKAR α signal observed is ~30% ($\Delta LT/LT_0$). To date, this is the largest FLIM signal reported for similar classes of FRET sensors. Further improvement may also be possible by optimizing the acceptor fluorophore and the affinity of the FHA to the phosphorylated threonine. In addition, the use of sensors that monitor different aspects of the same signaling pathway may provide a powerful approach to mechanistically investigate the regulation of the signaling pathways in vivo. In the future, the successful application of FLIM sensors to visualize neuromodulatory signaling pathways in vivo will provide important insights regarding where and when neuromodulation takes place in intact neuronal networks of behaving mice.

ACKNOWLEDGMENTS:

We thank Ms. Tess J. Lameyer, Ms. Ruth Frank, and Dr. Michael A. Muniak for edits and comments, and Dr. Ryohei Yasuda at Max Planck Florida for 2pFLIM acquisition software. This work was supported by two BRAIN Initiative awards U01NS094247 (H.Z. and T.M.) and R01NS104944 (H.Z. and T.M.), an R01 grant R01NS081071 (T.M.), and an R21 grant R21NS097856 (H.Z.). All awards are from the National Institute of Neurological Disorders and Stroke, United States.

DISCLOSURES:

The authors have nothing to disclose.

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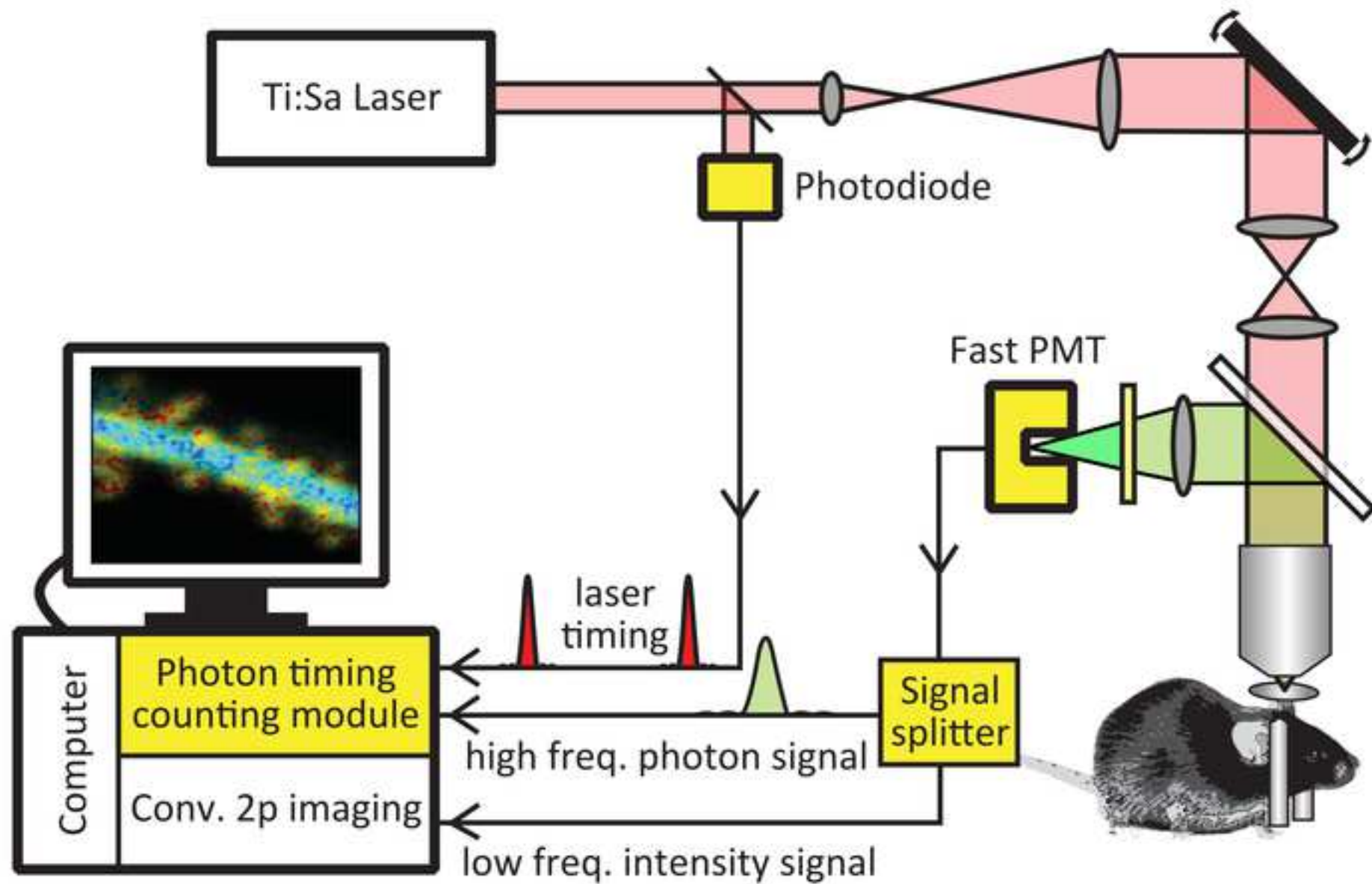
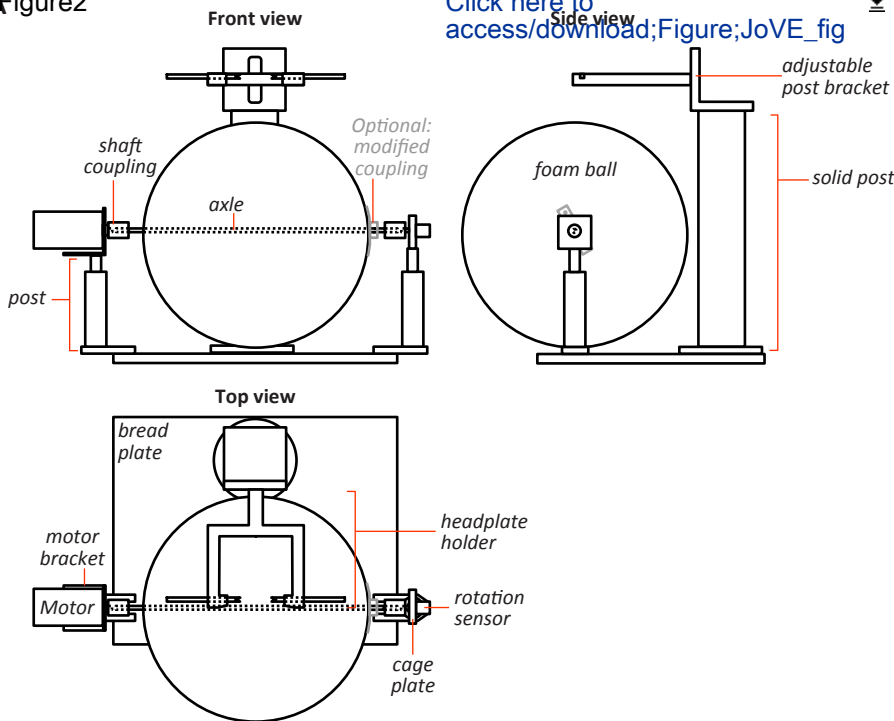
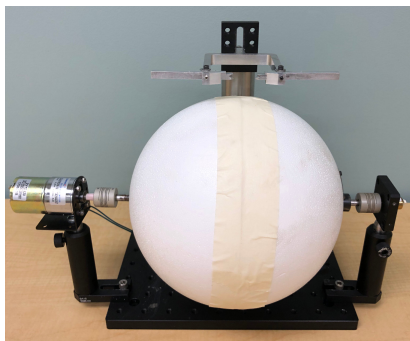


Figure2

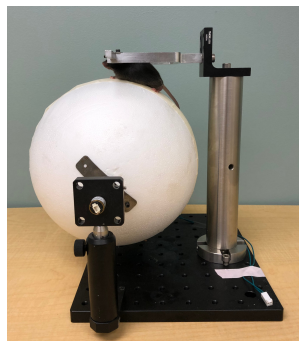
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B



C



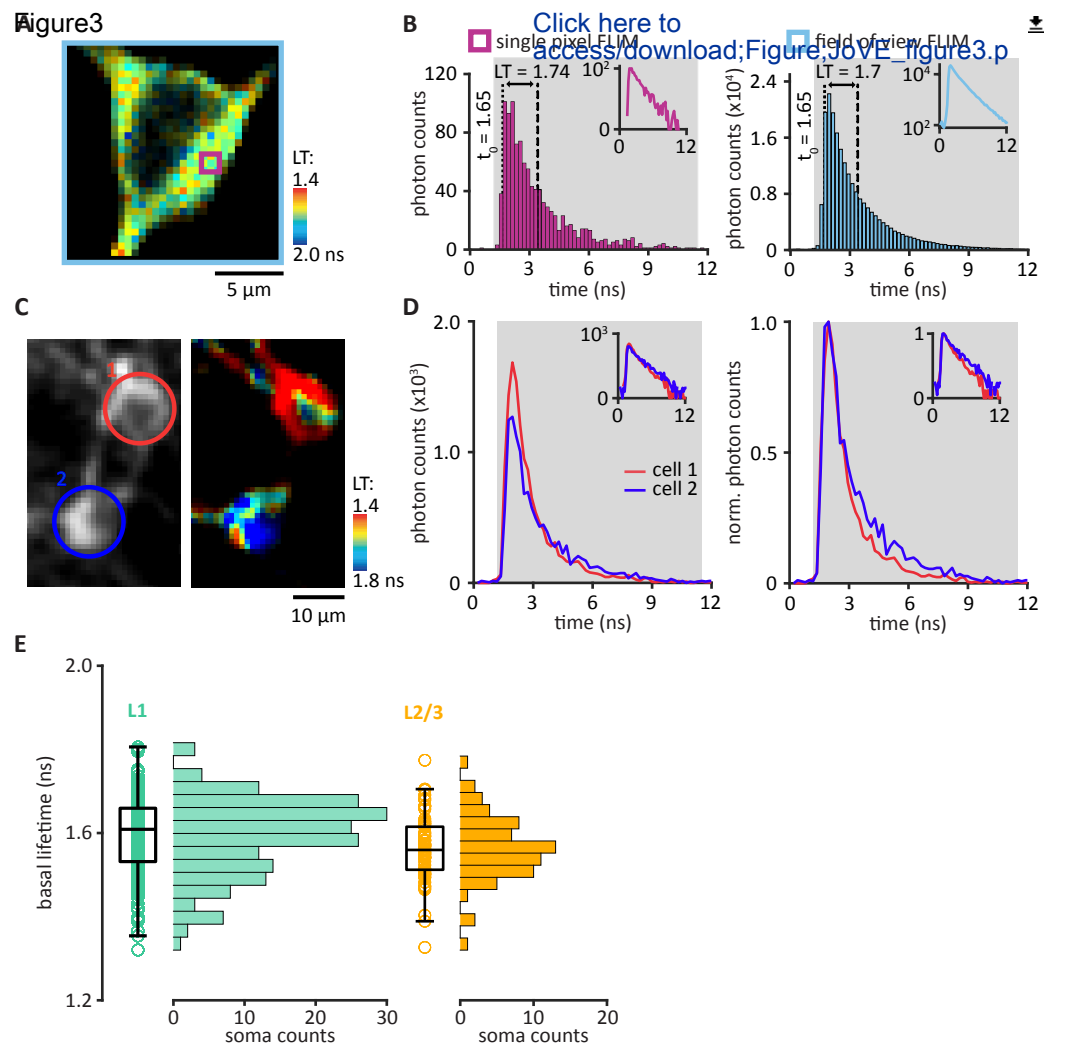
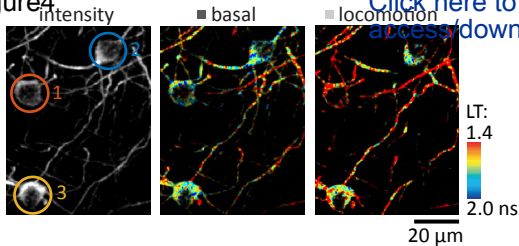
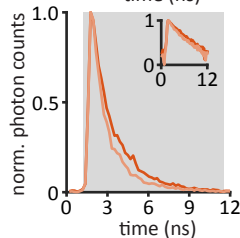
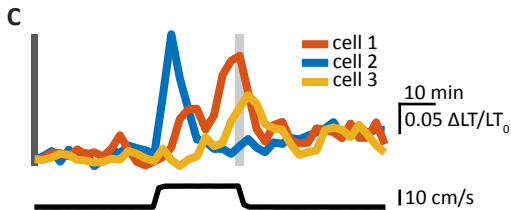
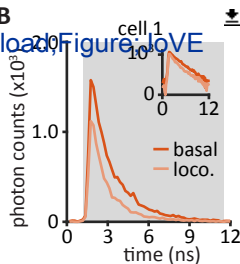


Figure 4



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Name of Material/ Equipment	Company	Catalog Number
0.2 µm cellulose acetate syringe filter	Nalgene	190-2520
16x 0.8 NA water-immersion objective	Nikon	MRP07220
3-pin cable	US digital	CA-MIC3-SH-NC
Aluminum bread board	Thorlabs	MB1012
AnimalTracker MATLAB software	N/A	N/A
Band-pass barrier filter	Chroma	ET500-40m
Cage plate	Thorlabs	CP01
Carbon steel burrs for micro drill, 0.5 mm tip diameter	FST	19007-05
Circular coverslip (5mm diameter)	VWR	101413-528
Custom-made injection needle holder	N/A	N/A
Dental acrylic	Yates Motloid	44114
Dental drill; Microtorque ii	Ram products	66699
Dowsil transparent polymer	The Dow Chemical Company	3-4680
Electroporation electrode	Bex	LF650P5
Electroporator	Bex	CUY21
Fast green FCF	Sigma-aldrich	F7258-25G
FLIMimage MATLAB software	N/A	N/A
FLIMview MATLAB software	N/A	N/A
Foam-compatible glue (Gorilla White Glue)	Gorilla	5201204
Headplate	N/A	N/A
Headplate holder	N/A	N/A
Hydraulic micromanipulator	Narishige	MO-10
Krazy glue	Krazy glue	KG82648R
Low-noise fast photomultiplier tube	Hamamatsu	H7422PA-40 or H10769PA-40
MATLAB 2012b	Mathworks	N/A
Motor	Zhengke	ZGA37RG
Motor speed controller	Elenker	EK-G00015A1-1
Motorized micromanipulator	Sutter	MP-285
Mounting base	Thorlabs	BA1S
Mounting post	Thorlabs	P14

Mounting post base	Thorlabs	PB2
Mounting post bracket	Thorlabs	C1515
Optical post	Thorlabs	TR2
Phosphate-buffered saline	N/A	N/A
Photodiode	Thorlabs	FDS010
Photon timing counting module	Becker and Hickl	SPC-150
Plasmid: tAKAR α (CAG-tAKAR α -WPRES)	Addgene	119913
Post holder	Thorlabs	PH4
Right-angle bracket	Thorlabs	AB90
Rotation sensor	US digital	MA3-A10-250-N
Rubber mat	Rubber-Cal	B01DCR5LUG
Shaft coupling (1/4 inch x 1/4 inch)	McMaster	6208K433
ScanImage 3.6	Svoboda Lab/Vidrio Technology	N/A
Signal splitter	Becker and Hickl	HPM-CON-02
Stainless steel axle (diameter 1/4 inch, L = 12 inch)	McMaster	1327K66
Stereotaxic alignment systsem	David kopf	1900
Two-photon microscope	N/A	N/A
Vetbond tissue adhesive	3M	14006
Virus: tAKAR α (AAV2/1 hSyn-tAKAR α -WPRES)	Addgene	119921
White PE foam roller (8 x 12 inch)	Fabrication enterprises INC.	30-2261
White polystyrene fom ball halves	GrahamSweet	200mm diameter 2 hollow halves
Zipkicker	PACER	PT29

Comments/Description

Step 3.2.2.

Step 5.5.

Step 2.5. To connect rotation sensor to the DAQ input of the microscope

Step 2.5.

Step 2.5 and sections 5 - 6. Will be provided upon request to the lead author

Step 1.4.

Step 2.4. Used as mount for rotation sensor

Steps 3.2.3. and 4.4.

Step 4.5.

Step 3.2.4. Technical details provided upon request to the lead author

Steps 4.3. and 4.5.

Steps 3.2.3. and 4.4.

Step 4.5. Artificial dura

Step 3.1.4.

Step 3.1.4.

Step 3.1.1.

Section 5. Kindly provided by Dr. Ryohei Yasuda, Max Planck Florida

Sections 5. and 6. Will be provided upon request to the lead author

Step 2.3.

Step 4.3. Technical details provided upon request to the lead author

Step 2.6. Technical details provided upon request lead author, used in combination with mounting post bracket and right-angled bracket

Step 3.2.4.

Step 4.3. Cyanoacrylate-based glue

Step 1.3.

Steps 2.6, and sections 5, and 6. Used to run microscope acquisition and data analysis software

Step 2.4.

Step 2.5.

Step 3.2.4.

Step 2.5. Used for posts for motor and sensor in combination with PH4 and TR2

Step 2.6. Used for headplate holder post in combination with PB2

Step 2.6. Used for headplate holder post in combination with P14

Step 2.6. Used in combination with right-angle bracket and headplate holder

Step 2.5. Used for posts for motor and sensor in combination with BA1S and PH4

Step 3.2.2. Protocol: Cold Spring Harbor Protocols 2006, doi: 10.1101/pbd.rec8247

Step 1.2.

Step 1.1.

Step 3.1.3.

Step 2.5. Used for posts for motor and sensor in combination with BA1S and TR2

Step 2.6 Used in combination with mounting post bracket and headplate holder

Step 2.4.

Step 2.1.

Steps 2.3. and 2.4.

Steps 5.9. and 6.1.

Step 1.3.1.

Step 2.3.

Steps 3.2. and 4.1. modified; Sutter micromanipulator, custom-made injection needle holder, hydraulic micromanipulator

Section 5. Built based on Modular *in vivo* multiphoton microscopy system (MIMMS) from HHMI Janelia Research Campus (<https://www.janelia.org/>)

Step 3.2.6.

Step 3.2.2.

Step 2.1.1.

Step 2.1.1.

Step 4.3. Hardening accelerator

ia.org/open-science/mimms)

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Author(s):

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Below please find point-by-point responses to the editorial comments/requests.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

Thank you. We have used the updated manuscript to incorporate the changes.

2. Please address specific comments marked in the attached manuscript.
a) Please consider moving this section to immediately follow section 1, which makes the protocol flow better.

As suggested, we have now moved the previous section 4, the construction of the motorized treadmill, to immediately following section 1 (current section 2).

a) Which software? Please specify and include it in the Table of Materials.

We have now included the name (AnimalTracker) of the custom-written software (current step 2.5) and added this information to the Table of Materials.

b) This step does not have sufficient details to replicate. Is the embryo harvested and how? Please provide a relevant reference.

We have now added a reference to the DNA injection protocol for IUE in current step 3.1.3., and included additional information on how the embryos are exposed for IUE (current step 3.1.2).

c) Is this done for the mouse that has received stereotaxic injection as described in section 2.2?

We have now specified which mice are used for cranial window installation in current step 4.1.

d) Is mouse placed on the treadmill before imaging for acclimation?

We have now added a step (current step 5.6) to describe the acclimation and recovery from anesthesia of the mouse. We write: *"5.6. Let the mounted mouse wake up from anesthesia and acclimate to the treadmill and microscope environment for at least 10 minutes."*

e) Is this set via the imaging acquisition software?

We have now added where the laser wavelength is set (current step 5.2).

f) How is the mouse fixed on the treadmill? Headplate holder of the treadmill? on mouse? Please specify.

We have now specified that the headplate of the mouse is mounted to the treadmill headplate holder (current step 5.4).

g) Headplate holder of the treadmill?

Yes. We have now clarified the details (current step 5.4). We write: “... *mount the headplate of the mouse to the headplate holder of the treadmill setup (see Figure 2 for details).*”

h) on mouse? Please specify.

Yes. This is now specified in current step 5.4.

i) Please describe how.

We have now added detail how to activate the PMT (current step 5.8).

j) Is this FLIMview?

We now include the name of the 2pFLIM acquisition software (FLIMImage; current step 5.9).

k) The mouse is anaesthetized in step 5.4. When does it wake up?

We have now added a step (current step 5.6) to describe the acclimation and recovery from anesthesia of the mouse.

l) In the JoVE Protocol format, “NOTE” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

We have now moved all NOTE sections in section 6 to the discussion.

m) Are these misplaced? $\delta\tau$ is described in line 419. If this is important, please make the calculation of $\delta\tau$ a separate step.

We have now moved the calculations to the discussion. $\delta\tau$ here is not misplaced. The current equation (equation 3) is a requirement resulted from the desired signal-to-noise ratio.

n) Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g., button clicks or menu selections for software actions, numerical values for settings, etc.).

We have now edited all software steps (current section 6) to explicitly explain how to execute the steps.

o) Please specify the steps that are being repeated here. 6.2-6.4?

We have now specified the step that is repeated (i.e. step 6.5; described in current step 6.6).

p) How is this value obtained?

We have now added information regarding how to obtain this LT_0 in the newly added step 6.7.

3. In the JoVE Protocol format, “NOTE” should be concise and used sparingly. They should only

be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

We have now moved the NOTES in section 6 to the discussion.

4. Please order the sections properly so that the protocol can be followed in chronological order.

As suggested, we have now re-ordered the sections as follows: 1) 2pFLIM microscope setup, 2) construction of a motorized treadmill, 3) Expression of tAKAR α sensor in the mouse cortex, 4) installation of the cranial window, 5) *in vivo* two-photon fluorescence lifetime imaging microscopy, 6) analysis of 2pFLIM images.

5. Please note that the editor has made some minor changes (e.g., breaking a long step into shorter steps). Please review for accuracy.

We have checked the minor editorial changes. The changes are accurate.

6. Please review the highlighted content for filming to ensure that it is continuous and contains essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have reviewed the highlighted contents. We feel that they are appropriate for the video.

7. Please update the comments/description column in the Table of Materials, specifying the exact step number. Step 2, step 5, step 14, etc. are not clear.

We have updated the comments/description column in the Table of Materials.

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Title of the article	Visualizing protein kinase A activity in head-fixed behaving mice using in vivo two-photon fluorescence lifetime imaging microscopy
Publication new article is in	Journal of Visualized Experiments
Publisher of the new article	MyJoVe Corp.

Author of new article Bart C Jongbloets, Lei Ma, Tianyi Mao, Haining Zhong

Expected publication date May 2019

Estimated size of new article 20
(number of pages)

Requestor Location Dr. Bart Jongbloets
3548 SE Belmontstreet

PORTLAND, OR 97214
United States
Attn: Dr. Bart Jongbloets

Publisher Tax ID 98-0397604

Total 0.00 USD

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