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Corresponding Author:	takashi tominaga Institute of Neuroscience. Tokushima Bunri University Sanuki, Kagawa JAPAN
Corresponding Author's Institution:	Institute of Neuroscience. Tokushima Bunri University
Corresponding Author E-Mail:	tominagat@kph.bunri-u.ac.jp
Order of Authors:	Yoko Tominaga
	Makiko Taketoshi
	Naoko Maeda
	Takashi Tominaga
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TITLE:

Wide-Field Single-Photon Optical Recording in Brain Slices Using Voltage-Sensitive Dye

AUTHORS & AFFILIATIONS:

Yoko Tominaga[1], Makiko Taketoshi[1], Naoko Maeda[1], Takashi Tominaga[1]

[1]Laboratory for Neural Circuit Systems, Institute of Neuroscience, Tokushima Bunri University, Sanuki, Japan

Corresponding Author:

Takashi Tominaga

Email Address: tominagat@kph.bunri-u.ac.jp

E-mail Addresses of Co-authors:

Yoko Tominaga: tominagay@kph.bunri-u.ac.jp

Makiko Taketoshi: makiko_taketoshi@kph.bunri-u.ac.jp

Naoko Maeda: naoko828matsui@gmail.com

KEYWORDS:

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SHORT ABSTRACT:

We introduce a reproducible and stable optical recording method for brain slices using voltage-sensitive dye. The article describes voltage-sensitive dye staining and recording of optical signals using conventional hippocampal slice preparations.

LONG ABSTRACT:

Wide-field single photon voltage-sensitive dye (VSD) imaging of brain slice preparations is a useful tool to assess the functional connectivity in neural circuits. Due to the fractional change in the light signal, it has been difficult to use this method as a quantitative assay. This article describes special optics and slice handling systems, which render this technique stable and reliable. The present article demonstrates the slice handling, staining, and recording of the VSD-stained hippocampal slices in detail. The system maintains physiological conditions for a long time, with good staining, and prevents mechanical movements of the slice during the recordings. Moreover, it enables staining of slices with a small amount of the dye. The optics achieve high numerical aperture at low magnification, which allows recording of the VSD signal at the maximum frame rate of 10 kHz, with 100 × 100-pixel spatial resolution. Due to the high frame rate and spatial resolution, this technique allows application of the post-recording filters that provide sufficient signal-to-noise ratio to assess the changes in neural circuits.

INTRODUCTION:

Wide-field single photon voltage-sensitive dye (VSD) imaging of bulk-stained brain slice preparations has become a useful quantitative tool to assess the dynamics of neural circuits[1–4]. After the analysis of the changes in optical properties due to membrane excitation[5–7], VSD

imaging was first described in early 1970s by Cohen et al.[5,6,8,9]; it is a suitable method to monitor the brain functions in real-time as the dye directly probes the membrane potential changes (i.e., the primary signal of the neurons).

The earliest VSDs possessed the desirable characteristics to understand the brain system, such as a fast time-constant to follow the rapid kinetics of neuronal membrane potential events, and linearity with the change in membrane potential[9–15]. Similar to other imaging experiments, this technique requires a wide range of specific tunings, such as the cameras, optics, software, and slice physiology, to accomplish the desired results. Because of these technical pitfalls, the expected benefits during initial efforts did not necessarily materialize for most of the laboratories that did not specialize in this technique.

The primal cause of the technical difficulty was the low sensitivity of the VSD toward the membrane potential change when applied to bulk staining of slice preparations. The magnitude of the optical signal (i.e., the fractional change in fluorescence) is usually 10^{-4}–10^{-3} of the control (F0) signal under physiological conditions. The time scale of membrane potential change in a neuron is approximately milliseconds to few hundreds of milliseconds. To measure the changes in the membrane potential of the neuron, the camera being used for the recording should be able to acquire images with high speed (10 kHz to 100 Hz). The low sensitivity of VSD and the speed needed to follow the neural signal requires a large amount of light to be collected at the camera at a high speed, with a high signal-to-noise ratio (S/N)[2, 16].

The optics of the recording system are also a critical element to ensure collection of sufficient light and to improve S/N. The magnification achieved by the optics is often excessively low, such as 1X to 10X, to visualize a local functional neural circuit. For example, to visualize the dynamics of the hippocampal circuit, a magnification of approximately 5 would be suitable. Such low magnification has low fluorescence efficiency; therefore, advanced optics would be beneficial for such recording.

In addition, the slice physiology is also essential. Since the imaging analysis requires the slices to be intact, careful slice handling is needed[17]. Furthermore, measures taken to maintain the slice viability for a longer time are important[18].

The present article describes the protocol for preparation of slices, VSD staining, and measurements. The article also outlines the improvements to the VSDs, imaging device, and optics, and other additional refinements to the experimental system that have enabled this method to be used as a straightforward, powerful, and quantitative assay for visualizing the modification of the brain functions[19–25]. The technique can also be widely used for long-term potentiation in the CA1 area of hippocampal slices[1]. Moreover, this technique is also useful in optical recording of membrane potentials in a single nerve cell[26].

PROTOCOL:

All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of Tokushima Bunri University. The following protocol for slice preparation is

almost a standard procedure[27] , but the modifications have been the protocols of staining and recording with VSD.

1. Preparation before the day of experiment

1.1 Prepare the stock A (**Table 1**), stock B (**Table 2**), and stock C (**Table 3**) solutions and store in a refrigerator.

1.2 Prepare 1 L of artificial cerebrospinal fluid (ACSF) (**Table 4**, see step 3) and keep it in the refrigerator.

1.3 Prepare 1 L of Modified ACSF (**Table 5**) and keep it in the refrigerator.

1.4 Dispense 500 µL aliquots of fetal bovine serum (FBS) in 2 mL vials and store in a freezer.

1.5 Dissolve 4% of agar powder in ACSF (ca. 120 mL) in a microwave and pour it in a 90 mm disposable Petri dish. The agar plate should be refrigerated before further use.

1.6 Place the following items in a freezer on the day before the experiment: a surgical tray, a slicer container and an aluminum cooling block (120 x 120 x 20 mm).

1.7 Ensure that there are sufficient Plexiglass rings with membrane filters for slice handling system[17,28] (see step 6.12).

1.8 Dissolve 2% of agar powder in 50 mL of 3 M KCl in a microwave. Take around 85 µL of the warm agar-KCl dissolvent in 200 µL tips using a micropipette for the grounding electrode. Detach the tip into the still warm agar 3 M KCl gel. Repeat the step to fill about 20-40 tips with 2% agar.

2. Preparation of VSD (di-4-ANEPPS) stock solution

2.1 Prepare 1 mL of 10% polyethoxylated castor oil solution with ultra-pure water.

2.2 Add 1 mL of ethanol to a vial of di-4-ANEPPS (5 mg vial), vortex and sonicate for 10 min. The solution will turn into a deep red color with possible small residues of the di-4-ANEPPS crystals.

NOTE**:** The ethanol used in this step should be freshly opened.

2.3 Transfer the solution to a 2 mL microtube with an O-ring. Spin down the solution and add 500 µL of 10% polyethoxylated castor oil solution.

NOTE: The dye is highly lipophilic. DMSO and poloxamer can also be used to dissolve di-4-ANEPPS but in terms of the optical signal upon change in membrane potential, we found that the use of ethanol – polyethoxylated castor oil gives a better signal to noise ratio. This could be related to transfer rate of solvent to the cell membrane.

2.4 Vortex and sonicate until the dye has completely dissolved.

2.5 Avoid exposure to light and keep it in a refrigerator. Do not store in a freezer. The stock can last for a few months.

NOTE: On the day of the experiment, follow the steps 3-9.

3. Daily preparation of ACSF (1 L) (Table 4)

3.1. Weigh NaCl, $NaHCO_3$, and glucose in a flask.

3.2. Add 950 mL of distilled water to the flask and start bubbling with 95% O_2/5% CO_2 gas.

3.3. Add 2.5 mL of stock A solution to the flask and incubate for approximately 10 min at room temperature.

3.4. Add 2.5 mL of stock C solution to the flask.

3.5 Add distilled water to make the solution to 1 L.

4. Daily preparation of the staining VSD solution

4.1. Sonicate a 500 µL vial of FBS and VSD stock solution (step 2) in an ultra-sonicator for 5 min.

4.2. Add 500 µL of freshly prepared ACSF into the vial of FBS.

4.3. Add 20 (in case of mice) or 40 (in case of rats) µL of VSD stock solution to the vial.

4.4. Ultra-sonicate and vortex the vial till the solution becomes pale orange.

5. Preparation for surgery

5.1. Take 100 mL of chilled ACSF separately in a 300 mL stainless steel container, a 300 mL beaker, and a plastic container, and place them in a freezer. Pour 150 mL of chilled modified ACSF (**Table 2**) in another beaker and place it in the freezer. Wait till the solutions are chilled; the time taken should be measured and determined beforehand.

5.2. Fold to break a razor blade (carbon steel, industrial grade 0.13 mm thick, blade on both sides) into half for the slicer.

NOTE: The other half can be used for dissection with a proper blade holder.

5.3. Prepare a block from the ACSF 4% agar plate with an adjusting jig (**Figure 1**).

5.4. Prepare a moist incubation chamber (an interface type chamber; a modified 1.2 L tight sealed box with a silicone packing) for keeping the brain slices physiologically alive (**Figure 2**); add ACSF in a small container and carbonate with 95% O_2/5% CO_2 gas, and fill a 90 mm x 20 mm Petri dish with ACSF in to the top.

NOTE: A smaller Petri dish (60 mm x 20 mm) should be placed in the center of the 90 mm dish to support a filter paper on the dish.

5.5 Put the box on a heating device and wait for 20 min to warm it up to 28 ˚C.

5.6. Add crushed ice into the container of the slicer. Place the following instruments in a stainless-steel vat (small) on ice: scalpel, blade holder, ring tweezers, agar block, and a stage of slicer. Keep frozen ACSF and modified ACSF on ice and bubble with 95% O_2/5% CO_2 gas (aka. carbogen).

5.7. Place the following instruments in a vat (large): scissors (large, small), tweezers, a spatula, a spoon, and diagonal pliers.

6. Surgery (mice)

6.1. Anesthetize the mouse using isoflurane in a fume hood. Assess the level of the anesthesia by checking the pedal reflex of the animal upon toe pinch.

6.2. Decapitate the mouse and immerse the head in ice-cold ACSF in a stainless-steel surgical tray.

6.3. Extract the brain within 1 min and place it in a beaker containing chilled ACSF for 5 min.

6.4. Take the brain out of the beaker and, using a scalpel, trim the brain block (**Figure 3A**).

6.5. Place the brain block onto a 4% agar block (step 5.3, **Figure 3B**). Both hemispheres can be mounted on an agar block. Wipe the excess ACSF from the block with a filter paper.

6.6. Apply thin adhesive (super glue) to the slicer table. Place the agar block on it and wipe the excess adhesive using a filter paper.

6.7. Gently apply a small amount of ice-cold ACSF (~5 mL) using a pipette from the top of the brain-agar block. This will help solidify excess super glue and prevent the glue from covering the brain and disturbing the slicing.

6.8. Fix the slicer table to the slicer tray (**Figure 3C**) and pour the modified ACSF.

6.9 Set the slicer to a slow speed, with the blade frequency at maximum.

6.10 Set the slice thickness to 350–400 µm and start slicing (**Figure 3C**). Place the slices on the corner of the slice tray in a sequence, so that the depth of the slices can be easily distinguished. Usually three to five slices can be obtained from one hemisphere.

6.11 Cut off the brain stem portion using a 30-G needle (**Figure 3D**).

NOTE: Microsurgery on the brain slice such as a cut between the CA3-CA1 border should be done at this stage under a binocular microscope, if necessary.

6.12 Place the slice on the center of the Plexiglass ring[17] (15 mm outer diameter, 11 mm inner diameter, 1 mm thickness) with a membrane filter (0.45 µm pores, PTFE-membrane, 13 mm diameter, **Figure 3E**) and small tipped paint brush. Place the ring in the moist recovery chamber (**Figure 2**) and secure the cover to keep the inner pressure high.

NOTE: The slices will stick to the membrane within 30 min and can be handled with the rings in the subsequent steps in the recording chamber. There is no need to use weights or other measures to keep the slice in place.

6.13 Adjust the direction and position of the slice in the ring to ensure it is well centered and has a consistent direction (see step 9.3).

6.14 Leave the specimen at 28 °C for 30 min, and then at room temperature for at least 10 to 30 min for the recovery of slices.

NOTE: The slices can now retain good physiological condition at least for 15 h.

7. Staining and rinsing of the slices (mice)

7.1. Gently apply 100–110 µL of the staining solution (Step 4) onto each slice on the ring using a micropipette. Eight to nine slices can be stained with one staining solution prepared in step 4. Leave the slices for 20 min for staining.

7.2. Prepare 50–100 mL of ACSF in a container and put the ring with sliced specimen in it to rinse the staining solution.

7.3 Store the rinsed slice to another incubation chamber. Wait more than 1 h for recovery before the experiment.

NOTE: The incubation chamber can be detached from the gas and moved to the place of recording in a tight sealed condition. The slice can remain alive for at least 20 min without gas supply. This is useful in case you need to move the slice to another place for recording.

8. Daily preparation of experimental apparatus

8.1. Turn on the amplifier, computer, and camera system, and check that the software is running.

8.2. Place ACSF in a 50 mL tube and bubble with carbogen.

8.3. Use a peristaltic pump to circulate the ACSF. Adjust the flow rate to approximately 1 mL/min.

8.5. Adjust the height of the suction pipette so that the liquid level inside the experiment chamber is always constant.

NOTE: The level of the solution is important to obtain a stable recording, therefore, the adjustment should be done using a micromanipulator.

8.6. Install the ground electrode made up of yellow chip filled with 3 M KCl agar (2%) (step 1.8) into a holder with an Ag-AgCl wire with small amount of 3 M KCl solution.

8.7. Fill a small amount of ACSF (approximately two-third of the volume) into the glass electrode (1 mm outer diameter, 0.78 mm inner diameter pulled with a micropipette puller) using a tapered thin tubed yellow tip and place it in the electrode holder.

8.8 Attach the holder to the rod installed in the manipulator. Ensure using an amplifier that the electrode resistance is approximately 1 MΩ.

NOTE: The long-shank wide opening (4-8 µm opening) patch type electrode should be good for field recording and as a stimulating electrode.

9. Starting a recording session

9.1. Take a slice preparation from the moist chamber with forceps.

9.2. Quickly place the slice onto an experimental chamber under the microscope (**Figure 4**).

9.3. Push the edge of the ring firmly into the silicone O-ring. Be careful not to break the membrane or the bottom of the experiment chamber.

NOTE: The direction of the slice should be taken into consideration with respect to the direction of the stimulating and recording electrodes in the field of view. The healthy slice should stick to the membrane filter so there is no need to use other devices to fix the slices such as weights and nylon meshes.

9.4 Place the tip of the stimulating electrode and the field potential recording electrode onto the slice under the microscope with transmitted light.

9.5 Use the electrophysiological recording system to check the response. Confirm the usual (non-stained) electrophysiological recording with given configuration.

NOTE: The recording electrode can be omitted but is useful to check the physiology of the slice.

9.6 Adjust the excitation light intensity to approximately 70–80% of the maximum capacity at the camera that corresponds to 13-15 mW/cm^2 at the specimen when sampling at 10 kHz with 5X water immersion objective lens and 1X PLAN APO tube lens. The excitation light wavelength is 530 nm, and the emission filter must be > 590 nm.

NOTE: Use a shutter to minimize the amount of excitation light. Continuous light exposure may deteriorate the slice physiology. The possible harmful effect of the light depends on the intensity and duration of the light. Use electrophysiological recording to judge the effect of light. In case of the strength of 13-15 mW/cm^2, about 1 s exposure should be the upper limit of the tolerance.

9.7 Adjust the focus with the acquisition system using the fluorescent light source because the focus may be different depending on the wavelength and start the acquisition.

9.8 Examine the data in an image acquisition software.

NOTE: We used original microprogramming package of numerical analysis software for detailed analysis.

REPRESENTATIVE RESULTS:

Figure 5 shows the representative optical signal upon electrical stimulation of the Schaffer collateral in area CA1 of a mouse hippocampal slice. The consecutive images in **Figure 5A** show the optical signal before any spatial and temporal filters were applied, while **Figure 5B** shows the same data after applying a 5 × 5 × 5 cubic filter (a Gaussian Kernel convolution, 5 × 5 spatial- and 5 to temporal-dimension) twice. Due to the high frame rate (0.1 ms/frame) and high spatial resolution (100 pixels × 100 pixels), the application of the filter did not change the signal but filtered out the noise, which can also be observed in the time course recorded in pixels in a single trial (**Figure 5C**, no filter; **Figure 5D**, with filter; and **Figure 5E**, superimposed).

Figure 6 compares the typical response in area CA1 of a hippocampal slice between mouse (**Figure 6A**) and rat (**Figure 6B**). As is evident in the figure, hyperpolarizing response due to inhibitory inputs is apparent in the rat hippocampal slice upon applying the same stimulus to the Schaffer collateral near the CA1/CA3 border. The small hyperpolarizing response is observed in the distal side of the CA1 after 24 ms in the mouse hippocampal slice, but more massive hyperpolarizing response overtook the depolarizing response in the rat hippocampal slice. The VSD imaging can clearly demonstrate the difference between mouse and rat hippocampal slices.

FIGURE AND TABLE LEGENDS:

Figure 1: An illustration of the agar block used to mount brain tissue for slicing and a jig to make the block. (A) A schematic illustration of the brain block and the 4% agar block **(B)**. **(C, D)** Photograph of an adjustable jig made by a Plexiglass plate (5-mm thick each) to make the agar block. When preparing the agar block, the upper and the lower plates should be stacked as shown

in D. **(E)** By inserting a blade into the long thin slots (1) and (2), the triangular part is cut out, the slot (3) is used to trim the entire depth of the block. (4) Removal of the upper plate enables slicing out of the non-necessary parts. Using the slot 1 and 2, make only 5-mm deep cuts so that resulting the block is as shown in (A) and (B).

Figure 2: An illustration of the interface type incubating chamber used to maintain slice physiology. (A) Overview of the system. **(B)** Interior details. **(C)** Illustration of the recovery chamber. Specimen should be placed on a filter paper positioned on an ACSF-filled 90-mm and 60-mm Petri-dish. The latter dish is to support the filter paper. The dishes and ACSF bubbling container are kept in place with a Plexiglass plate. The filter paper should not touch the wall of the air tight box nor the container. The air is supplied through a bubbling bottle that incorporates moistened gases in the chamber.

Figure 3: Hippocampal slice preparations from a mouse brain. (A) The isolated mouse brain should be cut first at the dashed line (a), then (b). Finally, the cut should be made along the line (c). The cut should be perpendicular to the bottom. **(B)** The brain block should be mounted on an agar block. **(C)** The agar block should be placed on the slicer. **(D)** Resulting slice. The excess tissue should be cut at the dashed line. **(E)** The slice should be placed at the center of the membrane filter with a Plexiglass holder (Outer diameter 15 mm, Inner diameter 11 mm, the PTFE membrane filter of 13 mm).

Figure 4: Recording system for optical signals from slice preparations. (A) A photograph of the microscope used to image the slices in the current manuscript. The optics consist of an objective lens (x5 NA0.60), a mirror box for dichroic filter (580 nm), and a projection (tube) lens (PLAN APO x1.0). A high-speed camera is attached on the top of the projection lens through a c-mount. There is another usual USB camera for observation. Excitation light is introduced using fiber optics. **(B)** Photograph of the lenses that are compatible with the mirror box. **(C)** Schematic diagram of the recording system. The imaging system and electrophysiological recording system are controlled by a PC. LED illumination system with a photo-diode feedback control system was used as a light source.

Figure 5: Representative optical signal in area CA1 of a mouse hippocampal slice in a single trial. (A) The consecutive images show the propagation of the neuronal signal acquired at the frame rate of 0.1 ms/frame along the Schaffer collateral pathway before applying any spatial and temporal filters (every 0.2 ms). Excitation was 530 nm and emission was > 590 nm. **(B)** The same data after an application of a three-dimensional Gaussian kernel of 5 × 5 × 5 twice. **(C, D)** The traces of optical signals in the representative pixels [each two pixels (36 μm) along a line in the middle of the CA1 is shown in the square in A, * denotes the pixel in the stratum pyramidale] from the data shown in A and B. **(E)** The superimposed signal from the optical signals in C and D.

Figure 6: Comparison of optical signal between mouse and rat hippocampal slices. The representative consecutive images upon electrical stimulation of the Schaffer collateral pathway near the CA3/CA1 border of a mouse **(A)** and rat **(B)** hippocampal slice. Video 1 shows the mouse hippocampal slice and Video 2 shows the rat hippocampal slice. The time-course of the optical

signal in the middle of the CA1 at the stratum pyramidale (st. pyr.) and stratum radiatum (st. rad.) is shown on the right of the figure.

Table 1: Stock A.

Table 2: Stock B.

Table 3: Stock C.

Table 4: Daily preparation of ACSF.

Table 5: Modified ACSF (cutting solution).

DISCUSSION:

The slice physiology is vital for collecting the right signal. The use of the ring-membrane filter system in this protocol ensures that the slice remains healthy and un-distorted throughout the procedure [2,16,17]. Other systems can be used to retain slice physiology during the recording, but the slice should not get deformed at any time as the imaging needs every part of the slice to be healthy. The ring-membrane filter system is also better for staining, as this helps us minimize the volume of the staining solution required. It is also important to control the intensity of excitation light, as it should be low with respect to the time-fraction. The continuous illumination can damage the specimen; therefore, appropriate use of the shutter is necessary.

The toxicity of the VSD has been often discussed[29], but it is a result of non-linear multiplication of the dye concentration, excitation light intensity, and duration of exposure to the light. The staining procedure shown in this protocol did not cause any measurable changes in the physiological parameters of the slice such as the input-output relationships of the field-potential recordings and those on the long-term potentiation (LTP), paired-pulse facilitation (PPF). The deterioration of the slice physiology is sizable especially under continuous illumination[16], but it can be managed by monitoring the field potential. By using these precautions, we can record LTP with continuous optical recording using VSD for more than 12 h[24], which is comparable to the best conditioned in vitro experiments.

During the recording, the air table might be useful, but insulation from other devices should be allowed because of the low magnification of the optics. However, mechanical disturbances are one of the significant causes behind poor imaging. If the considerable amount of false signal in image consists of opposite signs at the edge of the object, thus the difference of the brightness, it is the most probably caused by the mechanical disturbances. The movements of the specimen and fluid are the most frequent causes of motion noise, and hence, should be avoided or minimalized.

The VSD signal (fractional change in the light intensity) is small (10^{-3} to 10^{-4} of the initial fluorescence). To detect such a small change, the fluorescence should exceed 10^5 to 10^6 photons at the detector in the fraction of time to overcome the effect of photon-shot noise. Furthermore,

to follow the neuronal activity, the frame rate should be fast, close to the time constants needed to perform electrophysiological recording such as that around the kHz range. A combination of these two conditions requires the amount of fluorescence that is far more extensive than other kinds of fluorescent imaging. This requires a high numerical aperture in the whole optics, and the usual microscope is not the best option. Larger pupil and aperture are needed as shown in **Figure 4**.

The recoding system should match with the larger photon well depth, fast frame rate, and low noise. The choice mostly depends on the speed of neural system. The faster signal such as the hippocampal signal transduction needs specialized ultrafast, low-noise system. However, the slow signal such as the slow spread of activity in the cortices might be detected using the usual but scientific grade cameras.

The selection of the light source is also critical. The choice of the light depends on its intensity, stability, and the area of illumination. In the case of low-magnification wide-field imaging, point light source such as arcs and lasers need to expand, which makes it difficult to use these sources. Arcs, such as mercury and Xenon lamps, are the bright light source but usually are not stable. However, the recent development of Xenon light might overcome the problem. The halogen lamp is stable and has a larger area of filament that can easily match with wide-field imaging, but is limited in the strength especially at 530 nm. The recent development of power LED has enabled us to use it as the potential light source, but it must have the feedback stabilizer because of the temperature dependency. Lasers can be used but the high coherency results in a speckled noise, which is usually unacceptable for wide-field imaging.

The VSD imaging protocol presented in this article measures a value relative to the resting condition. Absolute measurement of the membrane potential cannot be performed using the current technique. Ratiometric imaging and fluorescence lifetime measurements can be used to assess the absolute membrane potentials.

The imaging of brain slices bulk-stained with VSD at low magnification can demonstrate the sub-threshold membrane potential changes in the micro-circuitry interactions of the brain. Such functional scope regarding the connection between the micro-circuitry at real-time resolution will be useful in many areas of brain research, especially to analyze the pathological aspects most likely caused by such excitatory and inhibitory functional connections between different brain areas. This application will be critical to investigate the changes in neural circuits related to certain types of neuropsychiatric diseases[30–32].

The development of the genetically encoded voltage indicator[33,34] is the future direction for optical membrane potential recordings that will pave the way for the attractive applications of cell type-specific analysis of neural circuit-level events.

There is much room for improvement in the optics, especially for visualizing the wide-field functional connections. Our novel confocal optics[35] will enable high-speed and high-S/N ratio recording of the VSD signal.

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DISCLOSURES:

The authors have nothing to disclose.

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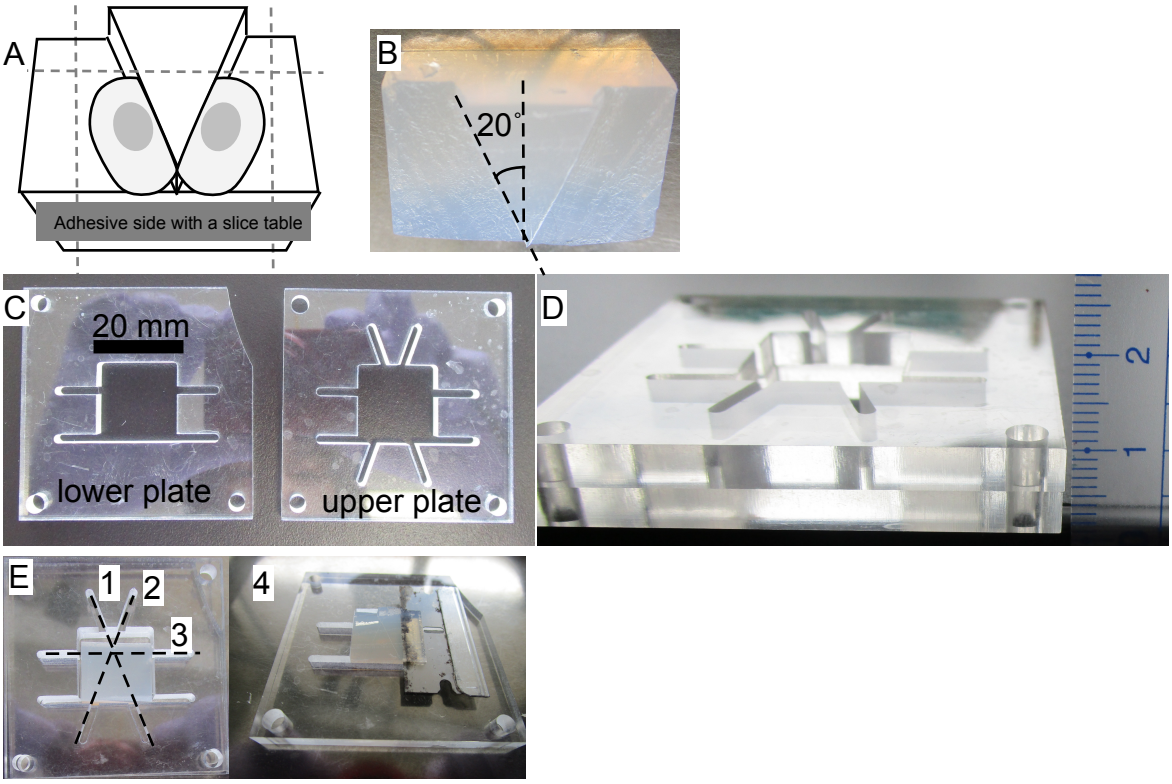

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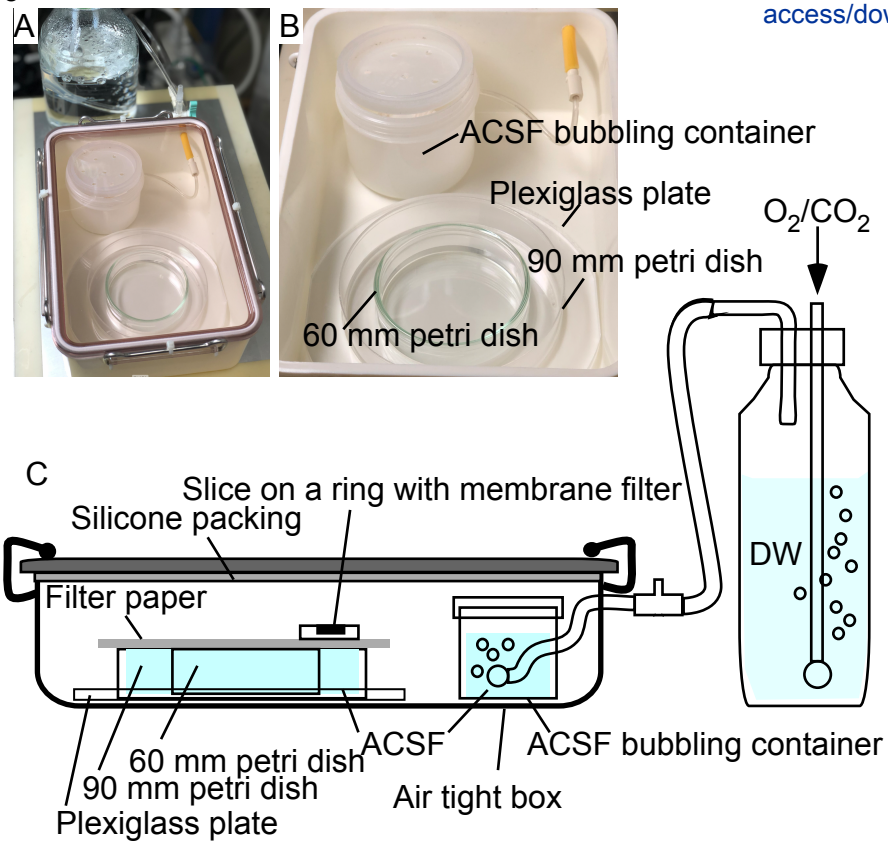
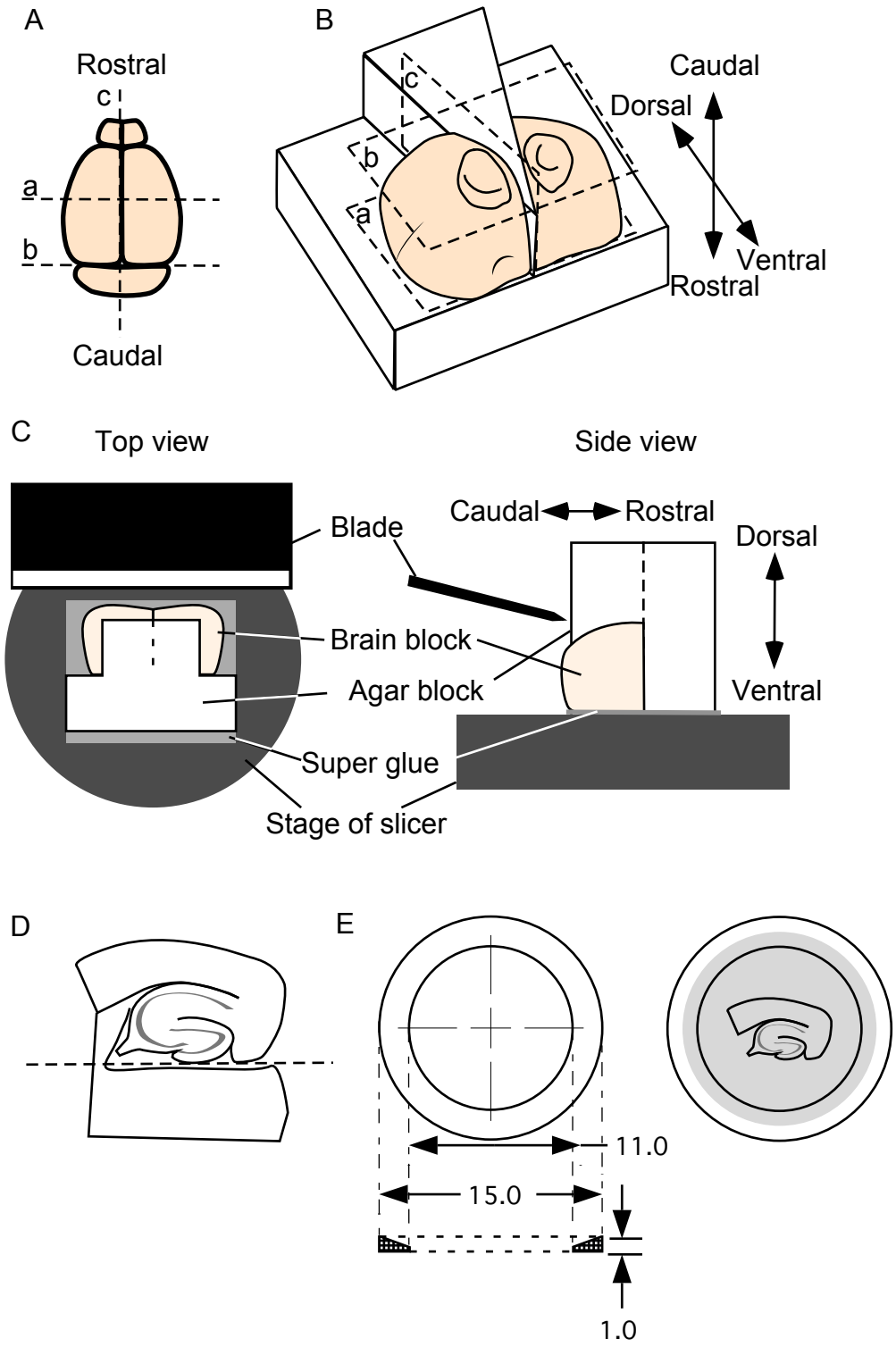


Figure 2 JoVE Tominaga et al



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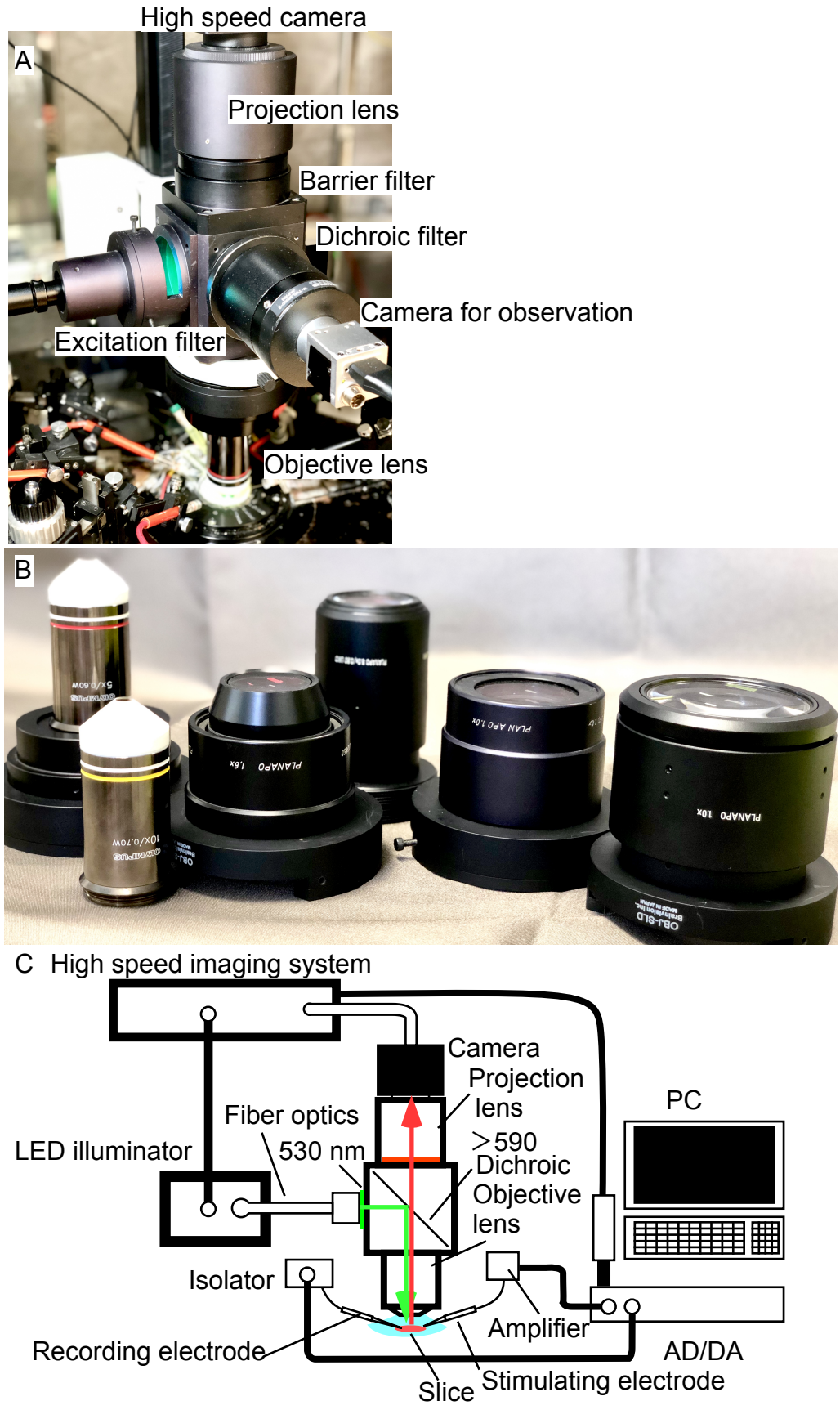

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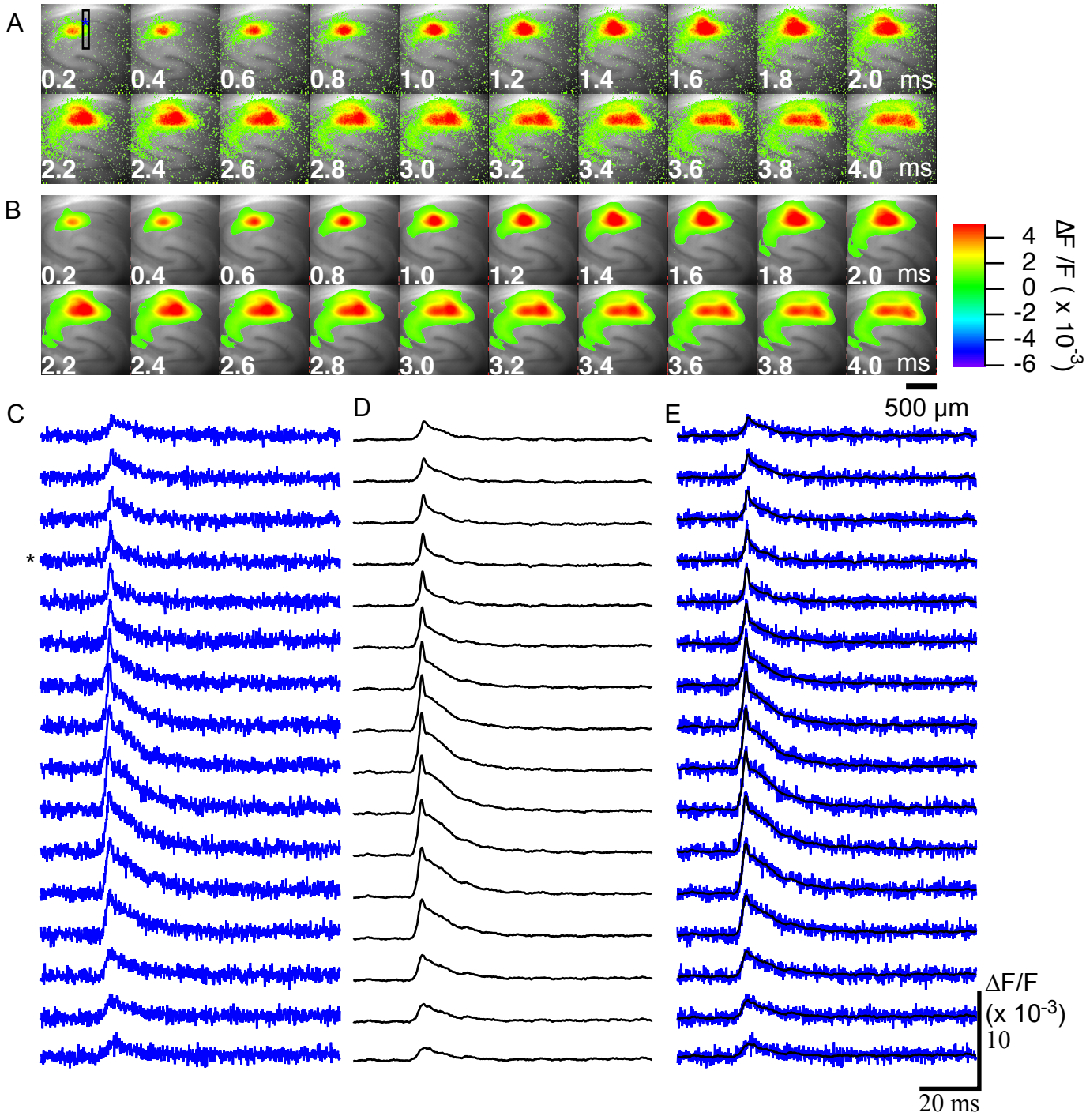

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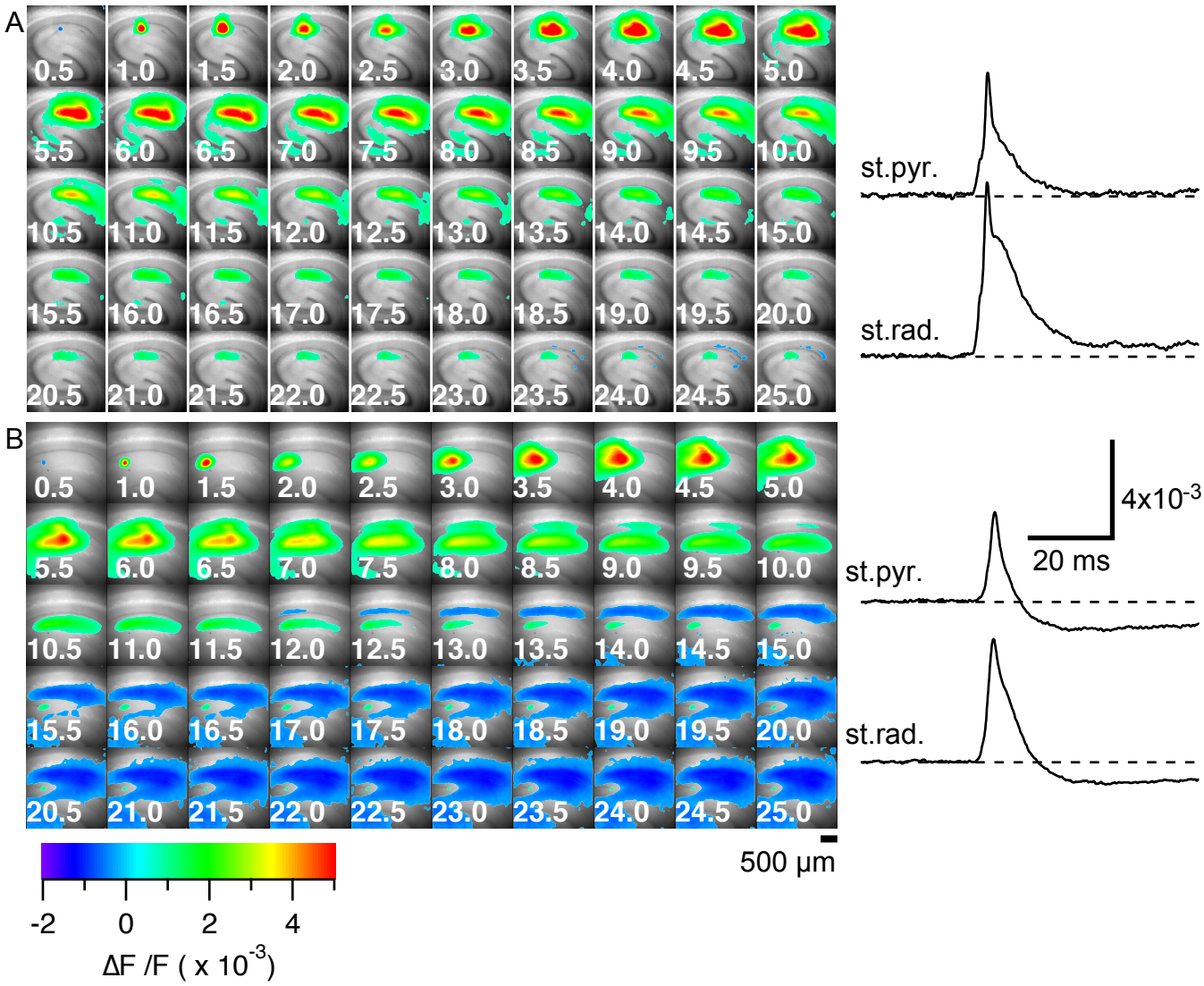

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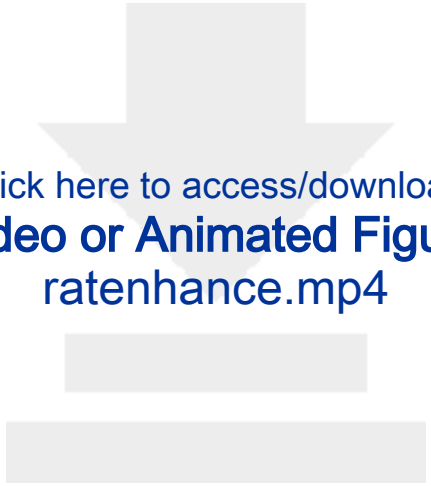

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Table1

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	Final mM	Weight
NaH2PO4・2H2O	1.25 mM	3.90 g
MgSO4•7H2O	2 mM	9.86 g
KCl	2.5 mM	3.73 g
Add H_2O to make 50 mL		

Table2

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	Final mM	Weight
MgSO4•7H2O	2 mM	12.32 g
Add H_2O to make 50 mL		

Table3

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	Final mM	Weight
CaCl2•2H2O	2 mM	5.88 g
Add H_2O to make 50 mL		

Table4

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	Final (mM)	weight
NaCl	124 mM	7.25 g
$NaHCO_3$	26 mM	2.18 g
Glucose	10 mM	1.8 g
Stock A		2.5 mL
Add about 950 mL of H_2O and bubble with mixed gas (95 % O_2/5 % CO_2) (10 minutes)		
Stock C ($CaCl_2$)	2 mM	2.5 mL
Add H_2O to make 1000 mL		

Table5

Click here to access/download;Table;NewTable5Jove_modACSF_tominaga.xl

	Final (mM)	Weight
NaHCO3	26 mM	2.18 g
Sucrose	205.35 mM	70.29 g
Glucose	10 mM	1.8 g
Stock A		2.5 mL
Stock B		2.0 mL
Add about 900 mL of H_2O and bubble with mixed gas (95 % O_2/5% CO_2)（１０minutes）		
Stock C	0.4 mM	0.5 mL
Add H_2O to make 1000 mL		

Name of Material/ Equipment	Company
High speed image acquisition system	Brainvision co. Ltd.
High speed image acquisition system	Brainvision co. Ltd.
Macroscepe for wide field imaging	Brainvision co. Ltd.
High powere LED illumination system \	Brainvision co. Ltd.
Image acquisition software	Brainvision co. Ltd.
Multifunctional electric stimulator	Brainvision co. Ltd.
Slicer	Leica
Slicer	Leica
Blade for slicer	Feather Safety Razor Co., Ltd.
Membrane filter for slice support	Merk Millipore Ltd., MA, USA
Numerical analysis software	Wavemetrics Inc., OR, USA
Stimulation isolator	WPI Inc.
AD/DA converter	Instrutech
Voltage sensitive dye Di-4-ANEPPS	Invitrogen, Thermo-Fisher Scientil
poloxamer	Invitrogen, Thermo-Fisher Scientil
polyethoxylated castor oil	Sigma-Aldrich

Catalog Number	Comments/Description
MiCAM - Ultima	Imaging system
MiCAM 02	Imaging system
THT macroscope	macroscope
LEX-2G	LED illumination
BV-ana	image acquisition software
ESTM-8	Stimulus isolator+AD/DA converter
VT-1200S	slicer
VT-1000	slicer
#99027	carbon steel razor blade
Omnipore, JHWP01300, 0.45 µm pores,	membrane filter/ 0.45 13
IgorPro	analysing software
A395	Stimulus isolator
ITC-18	AD/DA converter
catalog number: D-1199	VSD: Di-4-ANEPPS
Pluronic F-127 P30000MP	poloxamer / Pluronic F-127 (20% solutior
Cremophor EL C5135	polyethoxylated castor oil

ɹ in DMSO)

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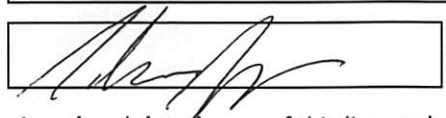
Institution:	Institute of Neuroscience, Tokushima Bunri University
Title:	Professor
Signature:	
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