High-Speed Multineuron Calcium Imaging Using Nipkow-Type Confocal Microscopy

Naoya Takahashi,[1] Shigeyuki Oba,[2] Naoto Yukinawa,[2] Sakiko Ujita,[1] Mika Mizunuma,[1] Norio Matsuki,[1] Shin Ishii,[2] and Yuji Ikegaya[1]

[1]Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan,
[2]Laboratory for Integrated Systems Biology, Graduate School of Informatics, Kyoto University, Kyoto, Japan,

ABSTRACT

Conventional confocal and two-photon microscopy scan the field of view sequentially with single-point laser illumination. This raster-scanning method constrains video speeds to tens of frames per second, which are too slow to capture the temporal patterns of fast electrical events initiated by neurons. Nipkow-type spinning-disk confocal microscopy resolves this problem by the use of multiple laser beams. We describe experimental procedures for functional multineuron calcium imaging (fMCI) based on Nipkow-disk confocal microscopy, which enables us to monitor the activities of hundreds of neurons en masse at a cellular resolution at up to 2000 fps. *Curr. Protoc. Neurosci.* 57:2.14.1-2.14.10.

Keywords: imaging • microscopy • calcium • neuron • spike

INTRODUCTION

Functional multineuron calcium imaging (fMCI) provides a unique opportunity to observe spatio-temporal patterns of neuronal activity. This technique is based on the fact that an action potential causes a transient calcium influx into the cell body of the neuron. Among many imaging devices, Nipkow-type spinning-disk confocal microscopy is a powerful tool for fMCI (Fig. 2.14.1A). In this system, an array of pinholes on a rotating disk splits a laser beam into multiple lines and focuses them on the specimen (Fig. 2.14.1B). This enables the fast parallel scanning of the entire field, promising a high spatio-temporal resolution of fMCI. This unit describes protocols for fMCI using Nipkow-type spinning-disk confocal microscopy. Described here are techniques for loading of a large neuronal population with calcium-sensitive dyes and appropriate machinery settings to monitor its activity at fine temporal resolution up to millisecond time-scale. Although this protocol focuses on brain slices, the same technique is applicable to calcium imaging in other types of living tissues and plated cell cultures, even of nonneuronal cells.

LOADING NEURONS WITH CALCIUM INDICATOR (IMMERSION-LOADING) AND IMAGING

BASIC PROTOCOL

This protocol describes procedures for recording neuronal calcium signals using Nipkow-type confocal microscopy. The procedures consist of two steps: (i) dye loading and imaging, and (ii) post-hoc image processing. There are two ways to incorporate calcium indicators into neurons: immersion loading and spot loading. Figure 2.14.1C outlines these methods. The immersion-loading method is convenient, useful to load cells on the slice surface, and more suitable for younger tissues, such as organotypic cultures on early days in vitro (DiV) and acute slices prepared from juvenile animals. The spot-loading

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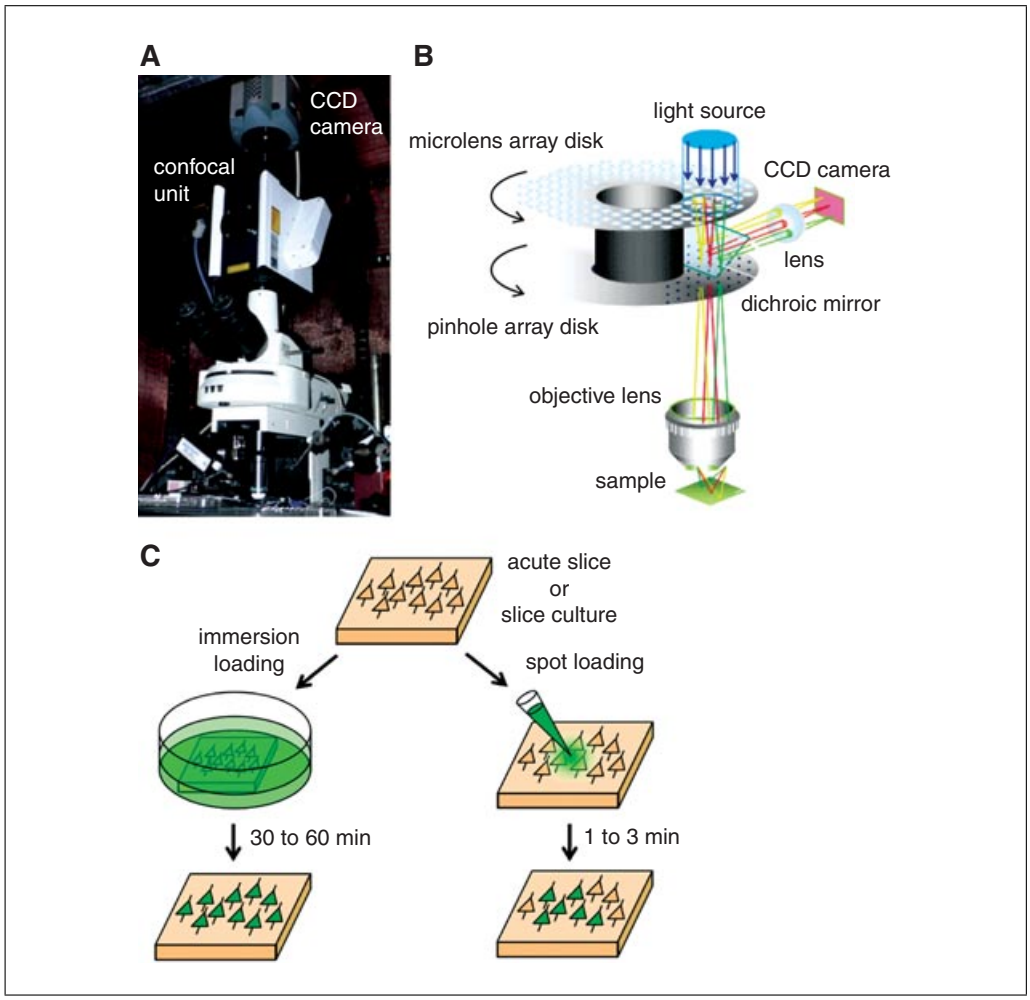

Figure 2.14.1 Overview of Nipkow-type spinning-disk confocal microscopy and experimental procedures. (**A**) Photograph of a Nipkow-type spinning-disk confocal microscope. The high-speed EM-CCD camera and the Yokogawa CSU-X1 confocal unit are mounted on a Nikon FN1 upright microscope. (**B**) Schematic illustration of the Nipkow-type confocal system. A laser source passes through a microlens-array disk and a rotating pinhole-array disk and splits into multiple beams before reaching the specimen. The microlens-array disk is used to minimize light loss by concentrating each laser beam on the corresponding pinhole. (**C**) Schematic illustration for the in vitro loading of neuronal populations with calcium indicators. The immersion-loading method (left) is suitable for slice cultures and acute slices prepared from juvenile animals. The spot-loading method (right) is more versatile.

method is applicable to aged tissues, including organotypic slices on late DiV and acute slices from adult animals (see the Alternate Protocol). In addition, related protocols for the dye loading are available in *UNIT 2.3* and *UNIT 2.5*. Appropriate machinery settings are required to attain high-speed imaging. Post-hoc image processing is to reconstruct the timing of spikes in individual cells, based on calcium fluorescence transients.

Materials

Oregon Green 488 BAPTA-1 (OGB-1) acetoxymethyl ester (AM) (Invitrogen)
Dimethyl sulfoxide (DMSO)
Pluronic F-127 (Invitrogen)
Extracellular artificial cerebrospinal fluid (aCSF; see recipe), oxygenated with carbogen gas (95% O_2/5% CO_2)
Organotypic brain slices on early DiV or acute slices from young animals
Carbogen gas (95% O_2/5% CO_2)

1.5-ml microtubes
35-mm dishes
37°C incubator
Submerged recording chamber (1.5 ml volume)
Temperature controller
Upright microscope and high-aperture (>0.8) water-immersion objective lens (e.g., Nikon or Zeiss)
High-speed CCD camera: e.g., iXon EM+ DU897 (512 × 512 pixels) for <200 frames per second (fps) or iXon EM+ DU860 (128 × 128 pixels) for 100–2000 fps (Andor)
Nipkow-type spinning-disk confocal unit, e.g., CSU-X1 (Yokogawa Electric)
488-nm laser diode
ImageJ software

Perform dye loading and imaging

1. Dissolve OGB-1 AM in DMSO to yield a concentration of 1 mM. After dispensing into 1.5-ml microtubes, the solution can be stored up to 3 months at −20°C. Mix 10 μl of the solution with 2 μl of 10% Pluronic F-127/DMSO and 2 ml aCSF.

   The final concentration of OGB-1 AM is 5 μM.

   Unused dye can be stored for >1 month at −20°C.

2. Submerge the brain slices in the dye solution (from step 1) in a 35-mm dish for 30 to 60 min. For acute slices, the solution is continuously supplied with carbogen at room temperature. For organotypic slice cultures, return the dish to the CO_2 incubator at 37°C.

3. Wash the slices with 2 ml carbogenated fresh aCSF three times to terminate dye loading. Maintain them in aCSF for >30 min at room temperature for acute slices or at 37°C for slice cultures.

4. Transfer a slice into an imaging chamber perfused (2 to 3 ml/min) with aCSF warmed to 32° to 35°C.

5. Search and focus on the dye-loaded cell layer with an objective lens. For searching the area of interest, we usually image the specimen at ∼10 fps in full-frame mode.

   In acute slices, cells near the preparation surface may be damaged or dead. Cells located at a depth of more than 20 μm should be selected for imaging.

6. Set the parameters for image acquisition.

   Camera selection is a critical part of attaining a high-quality image and should be considered with the experimental purpose (see Materials).

   Faster scanning requires an image binning of 2 × 2 or more (resolution reduction) and a reduction of the imaged area (image cropping).

   The scanning speed of a CCD camera depends on the total number of the horizontal (but not vertical) lines in an image area. Therefore, only the vertical cropping of the imaged area is valid for faster data acquisition.

7. Adjust the rotational speed of the spinning disk and illuminate a focused region using a 488-nm laser.

   Disk RPM should be set at five times more than the acquisition speed in fps (i.e., acquiring at 1000 fps requires >5000 RPM of disk speed).

   Low laser power is recommended to prevent bleaching; first start with the lowest power, and then adjust to obtain the sufficient signal-to-noise ratio (Fig.2.14.3).

Fast scanning movies often include periodic artifact, such as moving striped waves. This noise might be caused by differential interaction between the Nipkow-disk scanning and the camera readout. Increase or decrease the rotational speed of the disk to reduce the noise. Offline noise removal is also available after the experiment (see "Post-hoc image processing and analysis" below).

8. Acquire images for a time period appropriate for the purpose of the experiment.

Post-hoc image processing and analysis

9. When an acquired video contains wave-like periodic noise, apply our custom-written program (Fig. 2.14.2A,B), which extracts and removes any periodic spatio-temporal noise with a minimal setting determined by the user. The program "denoise.m" works in the MATLAB environment and is accessible online. To access the denoise file (denoise.zip), see the supplemental file at *http://www.currentprotocols.com/protocol/ns0214*.

10. Convert the video file into the multi-TIFF image file using the freely available ImageJ software; only TIFF files can be read by our MATLAB routine.

11. Run "denoise.m" and load the video file.

Two new video files, "signal.tif" (signal time-series) and "artifact.tif" (dissociated artifact time-series) will be automatically generated in multi-TIFF format. These files can be opened with ImageJ and converted into any other movie format. For an example of the filter

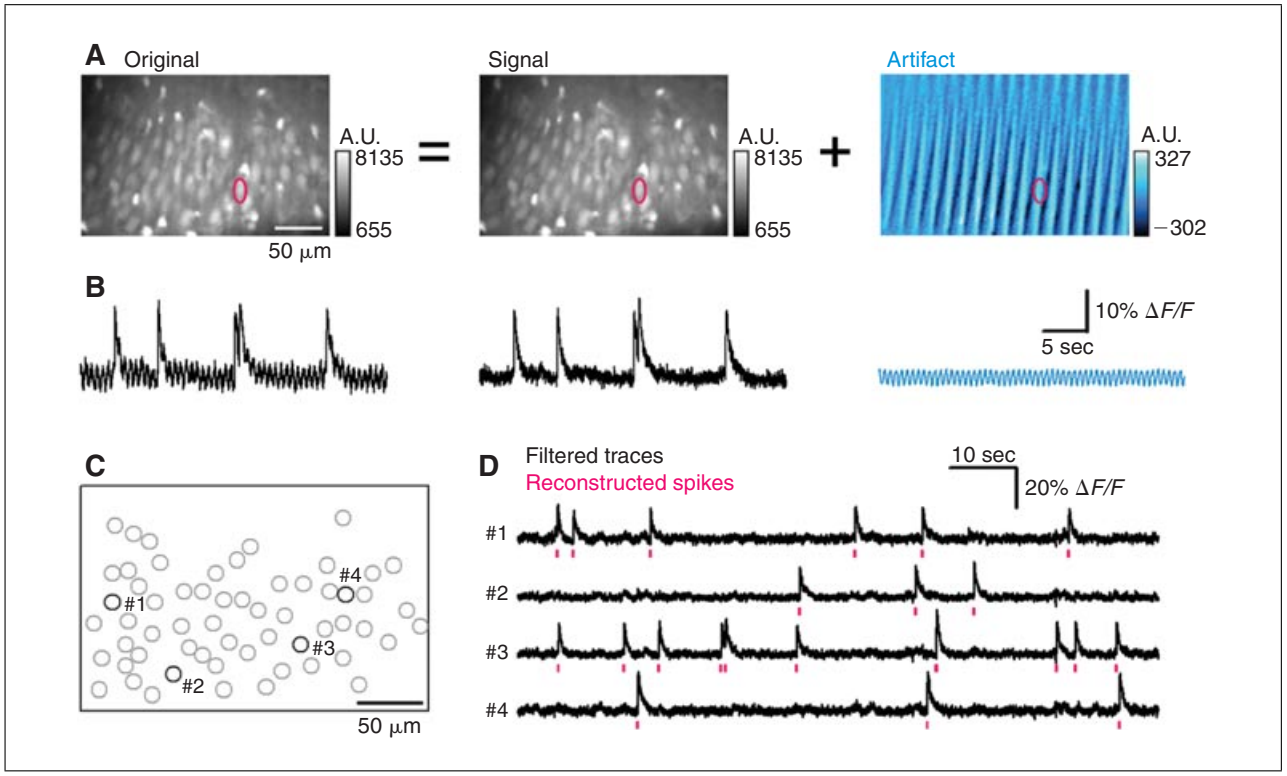

Figure 2.14.2 Post-hoc data processing of fMCI movies. (**A**) Example fMCI from CA3 neurons in hippocampal slice cultures. OGB-1AM was applied using the spot-loading method. The original movie (left, 100 fps) was image-processed post-hoc with a custom-made denoising program and separated into signals (middle) and periodic noise artifact (right); see Supplementary Movie 1. (**B**) Representative raw fluorescence trace (left), denoised signal trace (middle), and noise artifact (right) of the neuron indicated by the red ellipse in A. (**C**) Spatial locations of all neurons identified in A. (**D**) Calcium-signal traces for the four neurons labeled in C. Reconstructed spike times are indicated at the bottom of each trace. To access the supplemental movie (NS0214_denoise example.mp4), see the supplemental file at *http://www.currentprotocols.com/protocol/ns0214*.

effect, see Supplementary Movie 1. To access the supplemental movie (NS0214_denoise example.mp4), see the supplemental file at http://www.currentprotocols.com/protocol/ns0214.

12. If noise remains, retry the MATLAB routine on the output file "signal.tif."

    Under mathematically ideal conditions, repetitive filtering by this algorithm will completely eliminate any form of periodic noise.

13. Determine the regions of interest (ROIs) that encompass the somata of imaged neurons.

    ROIs should be within the soma contours; otherwise, the fluorescent signal may be contaminated by that of neighboring cells or neuropiles.

14. Average the fluorescence intensities of all pixels within the ROIs for each frame and generate a time series of the mean fluorescence in each cell.

15. Calculate the ratio of the fluorescence change from the baseline value.

    We usually define the baseline value as the averaged value of the lowest 30% of fluorescence intensities occurring 0 to 10 sec before the selected time point.

    The timing of spikes are manually or automatically determined based on the onsets of the calcium transients (Sasaki et al., 2008).

SPOT LOADING OF BRAIN TISSUE WITH DYES

ALTERNATE PROTOCOL

Spot loading is useful for late-DiV slice cultures or acute slices prepared from adult animals because they are poorly loaded with the immersion-loading method.

***Additional Materials** (also see Basic Protocol)*

Brain slices, such as late-DiV organotypic slices and acute slices from adult animals
Oregon Green 488 BAPTA-1 (OGB-1) acetoxymethyl ester (AM) (Invitrogen)
Pluronic F-127
Extracellular artificial cerebrospinal fluid (aCSF; see recipe), oxygenated with carbogen gas (95% O_2/5% CO_2)

45-μm-pore-diameter filter
Patch pipet (2–5 MΩ)
Micromanipulator

1. Transfer an acute or organotypic slice into an imaging chamber.
2. Dissolve OGB-1 AM in 10% Pluronic F-127/DMSO to yield a dye concentration of 2 mM. Dilute this solution 1:9 with aCSF and filter it through a 45-μm-pore-diameter filter before use.

   Undiluted dye can be stored for >1 month at −20°C.

3. Fill a glass micropipet with the dye solution. Insert the pipet tip into the cell layer 10- to 20-μm below the slice surface.
4. Apply a constant pressure of 10 to 20 kPa for 1 to 3 min to eject the dye solution.
5. Remove the pipet and wait for >30 min to allow the removal of unabsorbed calcium indicator and obtain a stable fluorescence level.

   This protocol yields a stained area with a diameter of 200 to 500 μm.

6. Set the parameters and acquire images.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Extracellular artificial cerebrospinal fluid (aCSF)

125 mM NaCl
3.5 mM KCl
2 mM $CaCl_2$
1 mM $MgCl_2$
25 mM $NaHCO_3$
1.25 mM NaH_2PO_4
10 mM D-glucose
Adjust pH to 7.4 by bubbling with carbogen (95% O_2/5% CO_2)

Other versions of aCSF may also be suitable; the exact composition will depend on the experiment.

COMMENTARY

Background Information

The information basis of neural networks relies on neuronal circuitry, in which neurons communicate with each other through complicated spiking behaviors. Sequential activities of neurons reflecting circuit connectivity compose neural representations. Information embedded in each representation relies on precise timing of neuronal activity, and such strict timing of activity within the order of milliseconds plays critical role in neuronal plasticity and in transferring messages to other neurons. Conventional physiological recording techniques (e.g., patch clamp, EEG, or fMRI) cannot fully tell us the information contents coded by the fine spatio-temporal sequence of neuronal population; thereby our knowledge about the relationship between neuronal circuitry and neuronal representation is fairly limited. Understanding the emergent properties of spike sequences in neuronal networks requires new-generation techniques for large-scale parallel recordings of the activity of neuron populations. At present, functional multineuron calcium imaging (fMCI) is the only technique that can record spikes simultaneously from hundreds of neurons or more at a single-cell resolution with identifiable locations of the monitored cells (Smetters et al., 1999; Takahashi et al., 2007). fMCI takes advantage of the fact that an action potential is followed by a transient calcium increase in the cell body of the neuron. The amplitude of this calcium increase is large enough that a single action potential is reliably detectable as a somatic fluorescence change when the neuron is loaded with calcium-sensitive indicators. Therefore, the timing of spikes occurring in multiple neurons can be reconstructed by simultaneously monitoring individual neurons.

Confocal microscopy and two-photon microscopy have been used for large-scale fMCI in vitro and in vivo. These types of microscopy usually use a single beam of an excitation laser to illuminate one point on the specimen. This focal point is raster-scanned over the microscopic field using a mechanical device, such as a galvanomirror system. The mechanical scanning process constrains the speed of image acquisition, typically ranging from 0.5 to 30 frames/sec (fps), which may be too slow to capture precise timings of neuronal spikes. Nipkow-type spinning-disk confocal microscopy resolves this problem by splitting one laser source into ~1000 light beams through multiple pinholes on a rotating circular plate called the Nipkow disk, named after Paul Nipkow, a 19th-century German engineer (Fig. 2.14.1A,B). In the latest version of the most widely used confocal scanner (e.g., CSU-X1, Yokogawa Electric), 20,000 pinholes (each 50 μm in diameter) are arranged spirally on the disk at an interval of 250 μm so that they can uniformly cover the entire microscopy field. The disk-rotation velocity is adjustable from 1500 to 10,000 rpm, and every 30° of rotation covers the full field. Therefore, this confocal scanner enables remarkably rapid image acquisitions at up to 2000 fps when combined with a high-speed photodetector, such as an electron-multiplying charge-coupled device (CCD) camera (Takahashi et al., 2010). This speed barely meets the minimal criterion required for the evaluation of the spatio-temporal patterns of neural spikes with millisecond precision. Another important

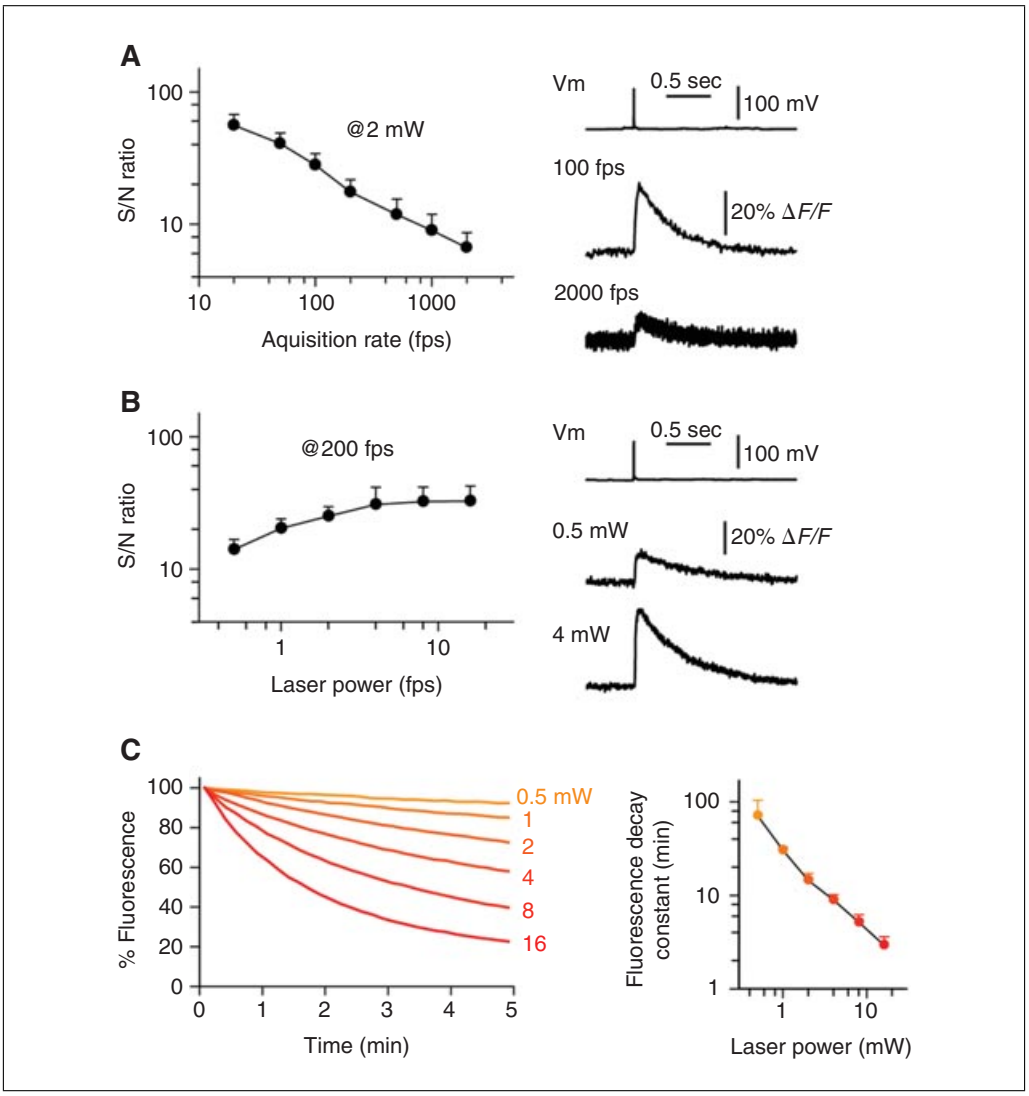

Figure 2.14.3 Post-hoc processing of calcium-imaging data. (**A**) S/N ratios of action-potential-induced calcium transients decrease with the image-acquisition rate (20 to 2000 fps). Preparations were illuminated at a laser power of 2 mW. Neurons were fired with a brief current injection in the current-clamp mode (top right). Representative fluorescent signals observed at 100 and 2000 fps are shown in the traces on the right. The S/N ratio was defined as the ratio of the calcium-transient amplitude to the standard deviation (SD) of its baseline fluctuation. Data are reported as the means ± SDs of four neurons. (**B**) S/N ratios increase with laser power (0.5 to 16 mW). Movies were taken at 200 fps. Neurons were fired with a short current injection with a patch-clamp electrode (top right). Representative fluorescence signals observed at 0.5 and 4 mW are shown in the right traces. Data are reported as the means ± SDs of four neurons. (**C**) Baseline fluorescence intensity bleaches as a function of time under continuous laser illumination. Left traces represent the average photobleaching functions at various laser intensities ranging from 0.5 to 16 mW (four slices each). The right plot shows the relationship between the laser power and the photobleaching decay constant. Data are reported as the means ± SDs of four neurons. Abbreviations: S/N, signal-to-noise.

advantage of this microscopy technique is reduced photobleaching (and thus low phototoxicity; Sasaki et al., 2007). The latter comes from the fact that the photobleaching rate is supralinearly proportional to the intensity of laser excitation (see Fig. 2.14.3C). Note that the intensity of a single laser line split through a Nipkow disk is substantially lower than in ordinary confocal microscopy, typically 1–5 μW. Therefore, Nipkow-type microscopy provides a unique opportunity for long-lasting, high-speed fMCI, which is thus applicable even for online experimental manipulations including visually targeted patch-clamp recordings (Ishikawa et al., 2010) and optical mapping of the synaptic connectivity

in a neuronal network of interest (Sasaki et al., 2009). Additionally, a Nipkow disk enables light permeation into deeper tissues than the conventional scanning system (Takahara et al., 2011). Although still inferior to two-photon microscopy, a Nipkow disk allows imaging of up to 150-μm depth.

Critical Parameters and Troubleshooting

Loading brain tissue with calcium indicator dye

In this unit, we introduce two methods to load brain slices with calcium indicator dyes. The immersion-loading method is more convenient and useful for loading all cells on the slice surface at a time. However, application of this method is usually limited to the younger tissues, such as early DiV of organotypic cultures and acute slices from young animals. This is probably due to the lack of connectivity of these tissues, which allows penetration of the dye into the tissue. In contrast, the spot-loading method is helpful to stain a small cell population in a region of interest and applicable even to older brain tissue including slices of late DiV and from adult animals.

Both methods also require high-quality brain slices. This is especially critical for the immersion-loading method, in which only healthy neurons on the clear surface of the tissue can be loaded with dyes and show calcium transients. Dead cells can also capture the dye at their somata and emit undesired excessively bright signals, which severely reduce the imaging quality.

Imaging

The light through the spinning disk can penetrate into the surface layer of the tissue up to tens of micrometers. Because strongly bright cells on the surface of acute slices are presumably damaged or dead due to the cutting procedure, the imaging plane should be set to slightly deeper layers where cells are alive and healthy. For deeper imaging, the spot-loading method is more practical to label cell population deeper (up to 150 μm) with dyes compared with immersion method by which only the surface layers are labeled.

Setting the parameters for image acquisition largely depends on the experimental setup and its software. Below we describe some essential points to improve imaging with Nipkow-type microscopy. The main advantage of the Nipkow system is its high-scanning speed. The latest model of the Nipkow disk can rotate at up to 10,000 rpm and scan the full field at 2000 fps. Thus, actual rate of image acquisition is limited by readout speed of the CCD camera. The readout speed of a CCD camera depends on the total number of the vertical (but not horizontal) lines in an image area. Therefore, vertical cropping of the imaged area is employed for faster data acquisition. In addition to image cropping, the image size can also be reduced by binning the pixels in an image, although the binning impairs spatial resolution. This may cause contaminations of calcium signals among adjacent cells. Moreover, movies acquired at a high speed almost inevitably contain wave-like periodic noise; i.e., interference fringes due to differential interaction between the Nipkow-disk rotation speed and the camera readout rate. Increasing (or decreasing) the rotational speed of the disk may reduce the interference. If the noise remains, offline noise removal is also available (see below).

Gain should be adjusted so that the fluorescent signal remains within the dynamic range of the camera. Bursting spikes may produce a several-fold increase in fluorescence above the baseline value.

Post-hoc image processing

Fast Nipkow-disk scanning movies often include periodic artifacts, such as moving striped waves. Our written MATLAB routine “denoise.m” extracts any form of periodic artifacts by determining the periodicity, amplitude, and phase distribution of the artifact (Fig. 2.14.2A and Supplementary Movie 1). This algorithm determines the time-series of artifacts to minimize the normalized mean squared error (NMSE). When the exact period is unknown, but the user specifies a range in which the true period of the artifact exists, our routine automatically searches for the artifact period that maximizes the signal-to-noise ratio within the specified range. Because the artifact period varies with the Nipkow-disk rotational speed and camera scanning rate, users are required to input a possible range to the routine. If the range is unknown, the user can set it by trial and error based on the resulting “signal.tif” and “artifact.tif” files. A periodic artifact may remain even after the application of our procedure. This occurs when there are multiple artifact sources with different periods or when the form of the artifact is very different from a sine curve. In this case, repetitive application of this procedure can eliminate the remaining artifact.

Table 2.14.1 Troubleshooting[a]

Problem	Possible cause	Solution
Cells are poorly stained by immersion loading	Unhealthy cells and tissues	Work with another slice
	Older tissue	Use slice cultures on earlier DiV or acute slices from younger animals. Younger tissues have more extracellular space and less myelination, which helps dyes defuse into the tissues and enhances dye loading into neurons. Try the spot-loading method.
Dyes are not ejected via patch pipets	Pipet tip clogged	Use a new pipet. If blockage repeatedly occurs, refilter dye solution or make fresh.
	Loss of pressure inside pipets	Increase the intrapipet pressure or decrease the pipet resistance To optically confirm dye ejection and diffusion, load 200 μM Alexa Fluor 488 hydrazide (Invitrogen), as well as OGB-1AM into the pipet
Cells are poorly stained by spot loading	Insufficient amount of dye ejected	Increase the intrapipet pressure or the duration of ejection Wait until cells are loaded with dye after stopping the dye ejection. It usually takes more than 30 min to reach the maximal fluorescence intensity and the full dye diffusion.
	Unhealthy cells and tissues	Work with another slice
Scanning speed is limited to a low rate	Large imaging area (many vertical lines)	Increase the binning or crop the image size to reduce the number of vertical lines acquired. The acquisition rate largely depends on the readout and vertical shift speed of the CCD camera.
Wave-like periodic noise is observed	Interference with the rotational rate of the Nipkow disk and the frame speed of the CCD camera	Increase (or decrease) the rotational speed of the Nipkow disk and minimize the periodic noise. If the noise remains, apply our custom-made MATLAB routine designed for denoising (see "Post-hoc image processing").

[a] Additional troubleshooting for topics not covered in manuscript body.

Additional troubleshooting

Please refer to Table 2.14.1 for common problems that might be encountered and their solutions.

Anticipated Results

With this technique, spiking activities can be monitored simultaneously from tens to hundreds of neurons at up to 2000 fps in acute or cultured hippocampal slices. A single action potential elicits a calcium influx into the soma, which causes a 5% to 30% increase in the fluorescence intensity above baseline. After the post-hoc denoising process (Fig. 2.14.2A,B), the spike-induced change in fluorescence has a sufficiently high signal-to-noise ratio to enable the detection of each spike (Fig. 2.14.2C,D). The signal-to-noise ratio decreases at higher image-acquisition rates, but it is still nearly 10:1 even at 2000 fps (Fig. 2.14.3A). Higher laser powers can increase the signal-to-noise ratio (Fig. 2.14.3B) and the photobleaching rates (Fig. 2.14.3C). The photobleaching curve can be fit with an exponential decay, and the decay constant decreases nonlinearly with the laser power. Therefore, the practical laser power is in the range of 0.5 to 4 mW.

Time Considerations

The loading procedure typically requires 45 to 90 min. The warm-up and operation of the confocal imaging system depend on the equipment and model types, although it usually takes 5 to 10 min. The duration of the experiment depends on the question being addressed. Post-hoc processing and analysis of

an obtained movie will require 1 to 5 hr, which depends on the data size.

Acknowledgements

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