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Abstract. We report a novel fluorescence imaging approach to imaging nonfluorescence-labeled biological tissue samples. The method was demonstrated by imaging neurons in Golgi-Cox-stained and epoxy-resin-embedded samples through the excitation of the background fluorescence of the specimens. The dark neurons stood out clearly against background fluorescence in the images, enabling the tracing of a single dendritic spine using both confocal and wide-field fluorescence microscopy. The results suggest that the reported fluorescence imaging method would provide an effective alternative solution to image nonfluorescence-labeled samples, and it allows tracing the dendritic spine structure of neurons.

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Golgi-Cox staining has been recognized as one of the most elegant and effective procedures for studying the morphology of neurons.¹ The neurons stained using the Golgi-Cox method appear as black deposits, enabling the visualization of the dendritic branching pattern and dendritic spines using light microscopy and a tracing algorithm.^{2,3} With the optical absorption characterization of black deposits, one can image the neuron morphology via reflection or transmission wide-field imaging techniques.⁴⁻⁶ Generally, nonfluorescence-labeled biological tissues are sliced, and Golgi-Cox-stained biological samples are imaged through the optical absorption approach. A slice imaging depth of less than several tens of micrometers is difficult

to achieve. During sample preparation, as the embedded Spurr resin has strong fluorescence, we can utilize the background fluorescence to image the black stained samples. In this study, to demonstrate the fluorescence imaging method, we show the fluorescence data of mouse brain slices stained using the Golgi-Cox method. By using this method, the Golgi-Cox-stained tissues could be imaged using currently available fluorescence imaging techniques. To our knowledge, this is the first report of imaging Golgi-Cox-stained neurons using the contrast between bright-background fluorescence and dark-labeled neurons.

The epoxy resin Spurr, a classical embedding reagent, is commonly used for achieving ultrathin or half-ultrathin sections in a brain tissue block.^{6,7} The broad fluorescence spectrum of Spurr is shown in Fig. 1(a). The spectral measurement shown in the figure was performed using a fluorescence spectrometer (FP-6500 Spectrofluorometer, Jasco). With excitation of different wavelengths, polymerized Spurr emits fluorescence with a dominant characteristic band [Fig. 1(a)]. When polymerized Spurr is excited by wavelengths of 400, 430, 460, and 490 nm, the emission band exhibits a redshift to wavelengths of ~470, 500, 530, and 550 nm, respectively [Fig. 1(a)]. As is well known regarding the Golgi-Cox method, the stained neurons appear as black deposits.⁸ Black deposits have the characteristic of absorbing the energy and always display a black pattern when imaged. Polymer Spurr emits strong fluorescence when excited with light of a certain wavelength. Therefore, it is a good choice to image stained neurons by utilizing light-matter interaction phenomena because we can embed a mouse brain with Spurr after Golgi-Cox staining and polymerize the brain tissue. When the coronal brain section is excited, the unstained background emits bright fluorescence because of Spurr permeation and polymerization. We can image the dark nerve cells using fluorescence illumination, as shown in Fig. 1(b). From the cartoon picture, the background seldom emits fluorescence without excitation. Once excited, however, black neurons stand out clearly against the bright-background fluorescence. Comparison of images (data not shown) with 405-, 458-, and 488-nm lasers shows no significant difference in the neuron structure. The ratio of signal to noise of the image captured with a 488-nm laser is best for some reasons, so we choose a 488-nm laser for the later experiment.

Golgi-Cox staining is based on the principle of heavy metallic impregnation of neurons, which allows the visualization of the fine structure of a neuron.¹ Dendritic spines are tiny protrusions of neurons first described by Santiago Ramón y Cajal using Golgi staining.^{9,10} Changes in the morphology and density of dendritic spines are usually regarded as a sign of the dynamics of synaptic function.^{10,11} We demonstrate the imaging of dendritic spines of the Golgi-Cox-stained Spurr-embedded pyramidal neurons in a mouse hippocampus by using confocal fluorescence microscopy. A brain slice of a seven-week-old male Kunming (KM) mouse was prepared using the modified Golgi-Cox method,¹² in which the darkening solution is LiOH instead of ammonium hydroxide. The mouse was deeply anesthetized, and the brain was carefully removed and then placed in a Golgi-Cox solution in the dark at room temperature for fixation and impregnation. The brain was used for more than three-month impregnation and then cut into 100- μ m-thick slices in a microtome cryostat. After 1% LiOH alkali treatment for 30 s, the brain slices were gradually dehydrated, embedded with Spurr (SPI, USA), sealed with cover glass, and then

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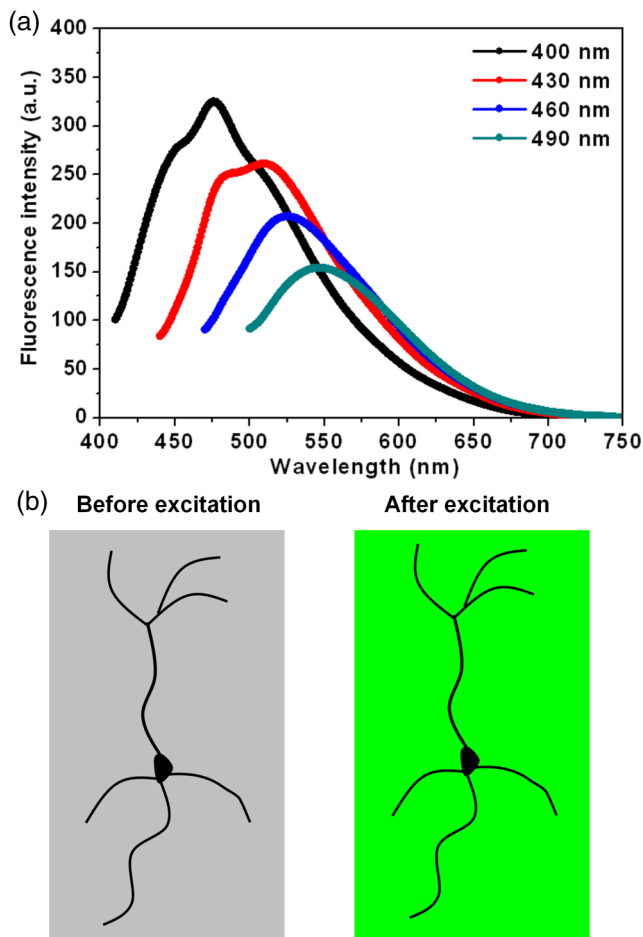


Fig. 1 Fluorescence imaging diagram of Golgi-Cox-stained neurons. (a) Fluorescence spectrum of polymerized Spurr with excitation from 400 to 490 nm. When polymerized Spurr interacts with light of wavelengths 400, 430, 460, and 490 nm, the emission peak appears at ~ 470 , 500, 530, and 550 nm. (b) The polymerized Spurr-embedded specimen before excitation (left). When the surface of the specimen is excited, the section absorbs incident light and emits bright fluorescence, while dark neurons absorb incident light and appear as black patterns (right). The pseudo color of polymerized Spurr fluorescence is green.

kept in an oven at 60°C for 36 h. After the polymerized brain was cooled to room temperature, it was kept dry in the dark until data acquisition. Neuron morphology in the subcortex was imaged using a 488-nm laser (5-mW output power) and $20\times$ objective (dry, N.A. 0.8); the laser power at the specimen will be lowered to 20 to 30% of the output power. Images were acquired using inverted confocal fluorescence microscopy (LSM710, Zeiss). With Spurr fluorescence illumination, we can observe clear soma and dendrite structures in the entire scanning area [Fig. 2(a)]. An apical dendrite and its spines were imaged using a 488-nm laser (15-mW output power) and a $63\times$ objective (water immersion, N.A. 1.20), and are shown at $3\times$ magnification in Fig. 2(b) [corresponding to the red box in Fig. 2(a)]. The output power of the 488-nm laser in the confocal microscopy is 5 mW when imaging with a $20\times$ objective and 15 mW when imaging with a $63\times$ objective. Depending on the morphology, spines can be classified as follows: stubby (short without neck), thin (thin with a small head and a long neck), mushroom (bulbous head with a narrow neck), and cup-shaped or branching

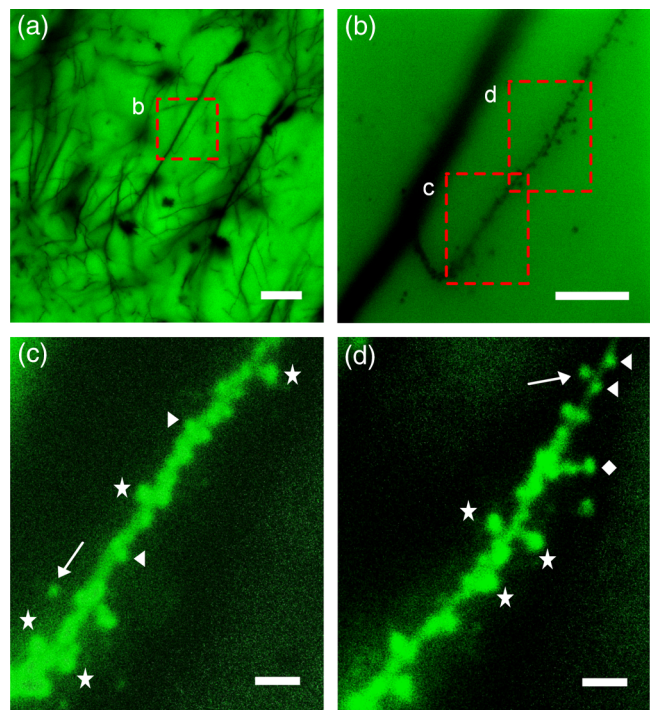


Fig. 2 Confocal fluorescence imaging of pyramidal neurons of Golgi-Cox-stained Spurr-embedded slices of a mouse brain hippocampus. (a) Neuron morphology in the subcortex. Scale bar: $50\ \mu\text{m}$. (b) Spines along partial neuron dendrites at $3\times$ magnification. Scale bar: $10\ \mu\text{m}$. (c) and (d) Dendritic spines in the boxes in (b) with inverted color at $4\times$ magnification. The arrow head, arrow, star, and diamond indicate dendritic spines of stained neurons. Scale bar: $2\ \mu\text{m}$.

(one neck protruding from dendritic shaft and splitting into two subnecks, and one small head for each subneck).¹⁰ In order to show the clear spine morphology of the apical dendrite, in Figs. 2(c) and 2(d), we acquired images of the part shown in the boxes in Fig. 2(b) at $\sim 4\times$ magnification by using a $63\times$ water objective with color the inverted using Photoshop software. We can clearly observe the stubby (arrow head), thin (arrow), mushroom (star), and branched (diamond) spines in Figs. 2(c) and 2(d). The results of confocal fluorescence imaging of the stained neurons show that this method is suitable for tracing fine spine structures of pyramidal neurons in the hippocampus. Through the comparison of the fluorescence imaging approach with a conventional Golgi-staining imaging method, the image from the fluorescence imaging approach demonstrates a better, at least comparative, ability to reveal the fine structure of Golgi-stained neurons.

We can also record many dendritic spines of Golgi-Cox-stained cortical pyramidal neurons of a mouse-brain coronal section with wide-field fluorescence imaging. For this purpose, a brain of a seven-week-old male KM mouse was prepared using the modified Golgi-Cox method described above.¹² A home-made bright-field line-scan imaging system with ultramicrotome sectioning of Spurr-embedded tissue was used for wide-field fluorescence imaging.^{6,13} All the images were obtained using laser illumination (488 nm, continuous-wave mode, Sapphire) with ~ 30 -mW output through an objective lens (LUMPlanFLN, $40\times$, N.A. 0.8, water immersion, Olympus) and using the strategy of imaging first and cutting-off later. The axial serial imaging was performed using a diamond

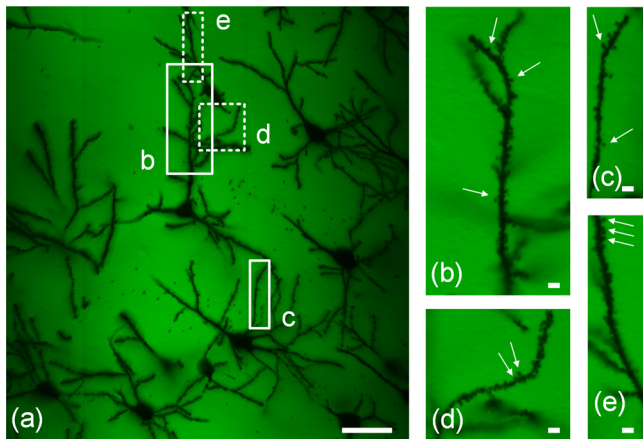


Fig. 3 Wide-field fluorescence imaging of cortical pyramidal neurons of Golgi-Cox-stained Spurr-embedded mouse-brain coronal section. (a) Projection of 75 frames of wide-field fluorescence imaging. Scale bar: 50 μm . (b), (c), (d), and (e) Four three-frame-overlay areas of interest from the insets in (a) (areas in boxes shown in b, c, and d; the area in the dotted box shown in e) from a, respectively. The arrows indicate the dendritic spines of stained neurons. Scale bar: 3 μm .

knife and three-dimensional stage movement with a voxel resolution of $0.3 \mu\text{m} \times 0.3 \mu\text{m} \times 1 \mu\text{m}$. A 75-frame projection view of wide-field fluorescence imaging is shown in Fig. 3(a). The staining of entire dendritic trees of cortical neurons was confirmed.¹⁴ For exploring the spines, adjacent three-frame images were overlapped [Figs. 3(b), 3(c), 3(d), and 3(e)]. Apical and lateral dendritic spines from two different somas [Figs. 3(b) and 3(c)] or the same stained neuron [Figs. 3(b) and 3(d)] can be clearly observed, similar to the previous result of imaging.² Although it has been argued in a previous report that the Golgi-Cox method is not optimal for the impregnation of dendritic spines,¹⁵ the report also suggests that it is one of the best methods to calculate the spine density in a rat brain.¹ In particular, the wide-field fluorescence imaging of stained neurons shows that this method is convenient for distinguishing the spines of the same area, regardless of whether the dendrites are crossed. In contrast to confocal imaging without fluorescence illumination,^{2,3,16} we adopt the strategy of imaging first and cutting-off later, which will be fully discussed in another paper under submission. This fluorescence imaging mode removes much defocusing interference and yields clear micron-level structures [Figs. 3(b), 3(d), and 3(c)]. We can obtain the fine spine structure from crossed complex regions in the neocortex at micron/submicron levels [Figs. 3(b) and 3(e)].

In summary, we developed a fluorescence imaging method for Golgi-Cox-stained epoxy-resin-embedded neurons. The fluorescence of polymerized Spurr allowed us to brighten the background around the black neurons. Dendritic spines along one dendrite could be recorded according to their type, distinguished on multiple intersecting dendrites, and traced along the axial direction. By using this method, one may image a specimen without fluorescent markers through commercial fluorescence microscopy. To our knowledge, this is the first report of imaging Golgi-Cox-stained neurons by using the contrast between bright-background fluorescence and dark-labeled neurons. Still, due to the absorption of Golgi-stained black deposit in confocal fluorescence imaging, the

imaging of a thick brain section of Golgi staining sample is still challenging. By combining this image approach with the strategy of imaging first and cutting-off later,^{13,17} one may achieve three-dimensional, high-resolution imaging of the Golgi-Cox-stained neurons throughout the whole mouse brain from superficial to deep layers.

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