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## Automatic Identification of Fluorescently Labeled Brain Cells for Rapid Functional Imaging

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<sup>1</sup>Department of Physics, <sup>2</sup>Division of Neurobiology, <sup>3</sup>Graduate Program in Neurosciences, <sup>4</sup>Department of Computer Science and Engineering, and <sup>5</sup>Center for Neural Circuits and Behavior, University of California at San Diego, La Jolla, California

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Valmianski I, Shih AY, Driscoll JD, Matthews DW, Freund Y, Kleinfeld D. Automatic identification of fluorescently labeled brain cells for rapid functional imaging. J Neurophysiol 104: 1803-1811, 2010. First published July 14, 2010; doi:10.1152/jn.00484.2010. The on-line identification of labeled cells and vessels is a rate-limiting step in scanning microscopy. We use supervised learning to formulate an algorithm that rapidly and automatically tags fluorescently labeled somata in full-field images of cortex and constructs an optimized scan path through these cells. A single classifier works across multiple subjects, regions of the cortex of similar depth, and different magnification and contrast levels without the need to retrain the algorithm. Retraining only has to be performed when the morphological properties of the cells change significantly. In conjunction with two-photon laser scanning microscopy and bulk-labeling of cells in layers 2/3 of rat parietal cortex with a calcium indicator, we can automatically identify  $\sim$ 50 cells within 1 min and sample them at  $\sim$ 100 Hz with a signal-to-noise ratio of  $\sim 10$ .

#### INTRODUCTION

In vivo two-photon laser scanning microscopy (TPLSM) of brain cells labeled with a functional indicator is a powerful and increasingly popular method to probe neural function. This approach, for example, enables the simultaneous detection of intracellular  $Ca^{2+}$  changes in populations of neurons and astrocytes within the middle to upper layers of the rodent cerebral cortex (Kerr et al. 2005; Ohki et al. 2005). However, it has been a challenge to achieve high temporal resolution across regions of cortex because only one voxel is measured at a time. The limit on temporal resolution for functional imaging arises not from an inability to scan the laser beam more rapidly, but rather from the efficiency of two-photon excitation of the dye and the need to avoid damage to tissue from high laser powers.

When cells of interest occupy only a small fraction of the field of view, one means to increase the sampling rate is to scan along an arbitrary path that passes cyclically through the cells of interest (Göbel and Helmchen 2007; Göbel et al. 2007; Lillis et al. 2008; Lörincz et al. 2007; Rothschild et al. 2010) rather than scanning the entire field with a raster pattern. The rate-limiting step to determine the scan path through a large number of cells is the need to manually annotate the location of all somata. Automatic identification of somata in TPLSM images is a challenge due to low signal-to-noise ratios associated with images deep within the cortex and differences in the fluorescence intensity of cells caused by uneven uptake of dye. Traditional spatial segmentation approaches that use handmade morphometric filters do not generalize across preparations. In particular, because of the need to differentiate between cell somata, blood vessels, and unusually bright areas of neuropil, approaches that segment based solely on time-averaged intensity or predetermined masks do not perform well. Furthermore, techniques that focus on temporal variation are effective for disentangling cells in populations of asynchronously active neurons and astrocytes but fail to detect cells that do not spike or to differentiate cells that spike synchronously (Mukamel et al. 2009; Ozden et al. 2008; Sasaki et al. 2008).

Here we address the issue of rapid scanning with a cell segmentation algorithm that uses machine learning to automatically identify the location of somata and an optimized scan algorithm to compute a scan path that preferentially passes through labeled somata while minimizing the time spent scanning neuropil and unidentified tissue. This approach can incorporate scans across and along cerebral blood vessels (Schaffer et al. 2006) to permit simultaneous measurements of neuronal activity, astrocytic activity, and blood flow. Although arbitrary scanning has been previously implemented for functional imaging by TPLSM (Göbel et al. 2007; Lillis et al. 2008; Rothschild et al. 2010), our approach further optimizes the scan pattern and integrates it with automated segmentation.

#### METHODS

#### Experimental methods

ANIMAL PREPARATION. Our subjects were Sprague-Dawley rats from Charles River, ranging in mass from 270 to 310 g. Initial surgeries were performed under isoflurane (Baxter Healthcare) anesthesia, with 4% (vol/vol) in 30% oxygen and 70% nitrous oxide for induction and 1-2% (vol/vol) for maintenance. Craniotomies were placed over the hindlimb representation of the somatosensory cortex, with a window size of  $\sim 4 \times 4$  mm centered at 2.5 mm medial-lateral and -1.0 mm anterior-posterior, as described (Kleinfeld et al. 1998; Shih et al. 2009) A metal frame that supports a window made from a no. 1 coverslip was mounted above the craniotomy and filled with 1.5% (wt/vol) agarose in an artificial cerebral spinal saline (Kleinfeld and Delaney 1996). Catheters were placed in the femoral artery for continuous measurement of blood pressure (BP-1, World Precision Instruments) and withdrawal of arterial blood for blood gas analysis (RapidLab 248, Bayer). The femoral vein was separately catheterized for drug and anesthetic delivery. Isoflurane was discontinued before imaging, and anesthesia was transitioned to  $\alpha$ -chloralose with an intravenous bolus injection of 50 mg/kg for induction and a steady flow of 40 mg/kg/h for maintenance (Devor et al. 2008). Body temperature was maintained at 37°C with a feedback-regulated heat pad (50-7053-F, Harvard Apparatus). Heart rate and blood oxygen levels were continuously monitored using a pulse oximeter (8600V,

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Nonin). Intraperitoneal injections of 5% (wt/vol) glucose in 1 ml saline were given every 2 h to prevent dehydration. The care and experimental manipulation of our mice and rats have been reviewed and approved by the Institutional Animal Care and Use Committee at the University of California at San Diego.

SOMATOSENSORY CORTEX MAPPING. We mapped the hindlimb region of the somatosensory cortex using intrinsic optical imaging of blood oxygenation, as described (Drew and Feldman 2009; Frostig et al. 1993). The contralateral hindlimb was electrically stimulated with 1 mA, 10 ms wide pulses delivered at 3 Hz for 3 s (Devor et al. 2008). Images were acquired with a 12-bit CCD camera (1M60, Dalsa) with a macroscope composed of camera lenses (Ratzlaff and Grinvald 1991). An initial image of the cortical vasculature was taken using 430 nm illumination to provide a map for dye injections. The cortical surface was illuminated at 630 nm and images of a 3 × 3 mm field, at 1,024 × 1,024 pixel resolution, were obtained at 58 frames/s and binned into 256 × 256 pixel images at 2 frames/s for analysis.

CALCIUM DYE INJECTION AND IMAGING. In vivo two-photon imaging was performed using the membrane-permeant Ca<sup>2+</sup> indicator Oregon green BAPTA1 (OGB1)-AM (Invitrogen) as described (Stosiek et al. 2003). Briefly, OGB1-AM was dissolved in 20% (wt/vol) Pluronic F-127 in DMSO to a concentration of 10 mM. This solution was diluted 1:10 with a buffered saline, 150 mM NaCl, 2.5 mM KCl, and 10 mM HEPES at pH 7.4, to yield a final dye concentration of 1 mM for loading into a micropipette tip. The coverslip over the cranial window was removed, the electrode was lowered into the appropriate region of cortex, and the dye was pressure injected, at 0.07 bar for 1 to 5 s, into the hindlimb somatosensory cortex at a depth 250–300  $\mu$ m below the pial surface. The pipette was left in place for 5 min to allow equilibration of the dye with the tissue and then removed. The exposed cortical surface was incubated with 50  $\mu$ M sulforhodamine101 (SR101, Sigma) in buffered saline for 10 min to label cerebral astrocytes (Nimmerjahn et al. 2004). Finally, the cranial window was resealed. All imaging was performed with a two-photon microscope of local design (Tsai and Kleinfeld 2009) using a  $40 \times$  dipping objective and galvanometric scan mirrors (6210H scanners with MicroMax 673xx dual-axis servo driver, Cambridge Technology) and MPScope (Nguyen et al. 2006, 2009) for acquisition and control. The optimized scan algorithm is readily integrated with this software. The excitation wavelength was 800 nm, and the collection band of OGB1 fluorescence was 350-570 nm and that of SR101 was 570-680 nm. Images were  $256 \times 256$ pixels or  $400 \times 256$  pixels in size, and a time series consisted of 200 frames collected at 5 or 10 Hz. Two sensory stimulation protocols were used in conjunction with the imaging. A single 10 ms stimulus was applied to the hindlimb to induce neuronal responses, whereas thirty 10 ms pulses readily induced changes in both neuronal activity and blood flow.

#### Computational procedures

IMPLEMENTATION OF THE CELL SEGMENTATION ALGORITHM. The algorithm was implemented in MATLAB code and C++ code compiled into MEX, i.e., MATLAB accessible, libraries. Robust-Boost, which is an improved version of the Adaboost algorithm, was used as realized in JBoost version 2.0r1, freely available at jboost. sourceforge.net. The output classifiers generated by JBoost take the form of MATLAB ".m" files. Cross-validation and other classification metrics were evaluated using Python and Perl scripts distributed with JBoost; we note that nfold.py, VisualizeScore.py, and atree2graphs.pl are particularly useful. All calculations made use of a workstation with a Intel Pentium D Processor with 4 MB of cache memory and a 3.2 GHz clock speed.

Our realization of the algorithm is organized into five principle parts: I) training code to generate the first step classifier; 2) annotation and training code to help annotate and then generate the second step

classifier; 3) segmentation code whose input is full-field TPLSM images and whose output is the result of the second classifier; 4) PathGUI code that interacts with the segmentation code to find cells, construct an optimized path through them, and interact with the TPLSM control software; and 5) PathAnalyzeGUI code that is used as a quick analysis tool to check the accuracy of the path. Additional analysis code was developed to segment raw scan data, identify onset times, and automatically differentiate astrocytes from neurons.

The training code used to generate the first step classifier takes full-field TPLSM data as input. Adobe Photoshop was used to perform annotations. Not all pixels that are parts of cells need to be annotated, but modes care must be taken to avoid labeling pixels inaccurately. The annotation and training algorithm that is used to generate the second step classifier uses the output of the first step classifier after it is thresholded at multiple levels. A graphical user interface was developed to assist annotation. For each candidate cell produced, the annotator is presented with its outline and can choose whether the outline segments a cell, not a cell, or an ambiguous region is ambiguous.

Both classifier training codes interact with JBoost using command line calls from MATLAB. The RobustBoost algorithm requires three parameters to be chosen. The first parameter, i.e., rb\_epsilon, characterizes the expected amount of error in the annotations of the training set; the default value is 0.1. The second parameter, i.e., rb\_theta, characterizes how much separation is desired between two classes; the default value is 0. The third parameter, i.e., rb\_sigma, characterizes how the potential function changes with time; the default value is 0.1. To construct the first classifier, rb\_epsilon was set to 0.15, rb\_theta was set to 0.2, and rb\_sigma was set to 0.06, rb\_theta was set to 0.1, and rb\_sigma to 0.1. The final decision tree contains hundreds of nodes, each with a tunable threshold on a particular feature.

The final classifier is relatively insensitive to the exact values of the parameters. Two parameters, i.e., rb\_theta and rb\_sigma, can be changed by a factor of two to three with negligible effect. The most critical parameter is rb\_epsilon, which corresponds to the fraction of expected errors in the annotation. This parameter should be set to the lowest number for which the algorithm converges, We used the training error found after 300 rounds of training with LogitBoost (Friedman et al. 2000) to estimate this number. LogitBoost is a common boosting procedure with no adjustable parameters apart from the number of training rounds and produces slightly smaller training error than RobustBoost but has a greater test error. In practice, the value of rb\_epsilon may be changed by a factor of 1.1 to 1.2 with little effect on the test error. If the parameter rb\_epsilon is set too low, RobustBoost does not converge.

OPTIMIZED SCAN ALGORITHM. The cell detection algorithm is integrated with a scan algorithm to generate a near optimal path between the segmented cells. The location and spatial extent of all cells are tabulated in terms of regions of interest (ROIs) formed by rectangular bounding boxes around each cell. This scan algorithm seeks to 1) maintain a constant scan speed over regions of interest, such as segmented cells; 2) scan each cell with a single straight line that, for computational simplicity, is restricted to cross the cell through the corners of a bounding box; 3) maximize the speed of the scan when the laser is not passing though a region of interest; and 4) minimize the total time along the path by optimizing the order in which cells are scanned. Mathematical details of the algorithm are given in the APPENDIX.

The scan path is further optimized by rearranging the order in which the ROIs are scanned, as well as by selecting among one of four vectors that pass along the diagonals of each ROI, through the use of the ANT System algorithm (Di Caro and Dorigo 1998). The ANT System algorithm is used iteratively. Initially a large set of paths is generated through a search among nearest neighboring cells to min-

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imizes the time it takes to go from one ROI to a specified second ROI, cycling among all ROIs. Once all of the possible paths are generated, they are weighed by the total distance of each path. This weighting determines the interaction energy between any two ROIs; the interaction energy is set to zero if no path exists between a given pair of ROIs. In the next iteration, a modified nearest neighbor search is performed; this time, the nearest neighbor is determined by a weighted function of the time it takes to move between a pair of ROIs and the interaction energy between the two sites. This process iterates, with the energy growing the more a path between two sites is used, until the ANT System algorithm converges on a final, optimized pathway among all sites. A lucid discussion is found in Wikipedia (en.wikipedia.org/wiki/Ant\_colony\_optimization).

ON-LINE DOWNLOAD. All of the software presented in this paper will be available for download at physics.ucsd.edu/neurophysics/links.html.

#### RESULTS

Cell segmentation consists of two classification steps. In the first step, we classify individual pixels as to whether they are part of a cell. The pixels identified as being part of a cell are divided into connected elements that form candidate cells. In the second step, we classify these connected elements as to whether they are indeed cell somata as opposed to other features. The first step yields a significant number of false positives. The second step removes most of these false positives and generates the final decision as to the locations of the cells. Both steps use classifiers generated by the RobustBoost algorithm (Freund 2009), which is relatively insensitive both to explicit errors in human annotations and inconsistencies in labeling of ambiguous regions (Schapire et

al. 1998). RobustBoost is part of a family of machine learning algorithms called Boosting, which have been used in several biological image segmentation problems (Giannone et al. 2007; Liu et al. 2008). The classifiers consist of a nonbinary decision tree whose nodes correspond to thresholds on selected features and whose output is a score that corresponds to whether a given pixel is part of a cell. RobustBoost iteratively adds nodes and adjusts the thresholds to optimize the prediction given by the decision tree relative to the manually annotated images.

The first step classifier determines whether a pixel belongs to a cell. The inputs to the classifier are feature maps that highlight the objects of interest in the TPLSM data. To identify cells, we chose eight heuristics that evaluate temporal and spatial differences, including mean values, variances, covariances, correlations, and normalized versions of these quantities (Table 1). RobustBoost is used to generate the classifier, using as training data the full-field images of cortical regions in which pixels in the images are annotated as to whether they are part of a cell, not part of a cell, or if the determination is ambiguous. Once trained, the output from this classifier corresponds to a map of the score of a pixel being part of a cell. The output is median filtered to remove isolated pixels and thresholded at multiple levels to form connected elements that are candidate cells.

The second classifier scores whether a candidate cell is indeed a cell. This classifier takes as input a second set of feature maps computed from the output of the first stage, this time using six morphological properties (Table 2). We again use RobustBoost with training data in which we

TABLE 1. Feature map of time-series image data

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Index	Feature Map
1	$I^{Mean}_{x,y} = \langle  ilde{I}_{x,y}(t)  angle^{st} \dagger$
2	$I_{x,y}^{Var} = \langle [\tilde{I}_{x,y}(t) - I_{x,y}^{Mean}]^2 \rangle$
3	$I_{x,y}^{Cov} = \sqrt{\langle [\tilde{I}_{x,y}(t) - I_{x,y}^{Mean}] [\tilde{I}_{x+1,y}(t) - I_{x+1,y}^{Mean}] \rangle^2 + \langle [\tilde{I}_{x,y}(t) - I_{x,y}^{Mean}] [\tilde{I}_{x,y+1}(t) - I_{x,y+1}^{Mean}] \rangle^2}$
4	$I_{x,y}^{Corr} = \sqrt{\frac{\langle [\tilde{I}_{x,y}(t) - I_{x,y}^{Mean}] [\tilde{I}_{x+I,y}(t) - I_{x+1,y}^{Mean}] \rangle^2}{I_{x,y}^{Var} I_{x+1,y}^{Var}}} + \frac{\langle [\tilde{I}_{x,y}(t) - I_{x,y}^{Mean}] [\tilde{I}_{x,y+1}(t) - I_{x,y+1}^{Mean}] \rangle^2}{I_{x,y}^{Var} I_{x,y+1}^{Var}}$
5	$I_{x,y}^{NormMean} = I_{x,y}^{Mean} - \hat{I}_{x,y}^{Mean} / \hat{\sigma}_{x,y}^{Mean} \ddagger$
6	$I_{x,y}^{NormVar} = rac{I_{x,y}^{Var} - \hat{I}_{x,y}^{Var}}{\widehat{\sigma}_{x,y}^{Var}}$
7	$I_{x,y}^{NormCov} = \frac{I_{x,y}^{Cov} - \hat{I}_{x,y}^{Cov}}{\widehat{\sigma}_{x,y}^{Cov}}$
8	$I_{x,y}^{NormCorr} = rac{I_{x,y}^{Corr} - \hat{I}_{x,y}^{Corr}}{\widehat{\sigma}_{x,y}^{Corr}}$

 $\begin{aligned} *\langle I(t)\rangle &= 1 / N \sum_{t=1}^{N} I(t), \dagger \tilde{I}(t) = I(t) \otimes W_5, \text{ where } W_n \text{ is a uniform filter of } n \text{ pixels.} \\ \ddagger \tilde{I}(t) &= I(t) \otimes W_{21} \text{ and } \hat{\sigma}_{x,y} = \sqrt{\sum_{x=x-10}^{x+10} \sum_{y'=y-10}^{y+10} [I_{x,y}(t) - \hat{I}_{x,y}]^2} / \sqrt{(21)^2 - 1}. \end{aligned}$ 

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table 2.	Feature	тар	of	intermediate	cluster	data
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Index	Feature Map
1	Threshold level at which the candidate is generated
2	The area of the candidate, in pixels
3	The Euler number, defined as the number of objects in the candidate minus the number of holes in those objects.
4	The extent, defined as the area of the candidate divided by the area of the bounding box.
5	The eccentricity of an ellipse that has the same second-moments as the candidate.
6	The solidity of the candidate, determined as the ratio of the area of the candidate to that of the associated convex hull.

annotate the output of the first classifier to identify candidate cells as cells, not cells, or ambiguous objects. The final likelihood map generated by the trained classifier is thresholded at or near zero and contains only connected elements that are likely to be cells.

We realized the cell segmentation algorithm by training the first and second classifiers using 64 different datasets, i.e., 16 different regions imaged with TPLSM over four trials each in four animals (see examples in Fig. 1). Each data set consisted of 200 consecutive frames at a resolution of  $256 \times 256$  pixels or higher. Once the training was completed, we applied the cell segmentation algorithm to segment a test set. In all of our tests,

we used a single classifier that was trained only once with annotated data from different regions of the cortex, different magnification and contrast levels, and different animals.

The application of our method to test cases is shown in the example of Fig. 2, which shows the eight feature maps generated from the data (Fig. 2A) and the output from the first classifier (Fig. 2B); the thresholded version of this output, at multiple levels, is used as input to the second classifier. The output of the second classifier (Fig. 2C) is thresholded at zero to yield the segmented cells (Fig. 2D). In practice, it takes several hours of computer processing time generate the classifiers for a particular preparation but only









FIG. 1. Examples of annotations used to generate the 2 classifiers. A: example, shown raw and annotated, for the 1st classifier. This image is 1 of 16 full field images annotated in Adobe Photoshop. Because 4 trials were performed for each annotated region, these annotations were used in learning on 64 stimulation trials. Green indicates that the pixel is part of cell somata, whereas blue indicates that it is uncertain whether a pixel is part of cell somata. All uncolored pixels are taken as examples of pixels that are not parts of cells. Notice that a very rough annotation was sufficient to produce good results. B: example to generate second classifier. A screenshot of a graphical user interface used to annotate whether a particular cluster of pixels is a cell, not a cell, or ambiguous region. Left: a large mean image is shown with a current candidate cell outlined. Outlines that have not yet been evaluated are colored blue, those that were selected as not cells are colored red, those that have been selected as cells are colored green, and those that were selected as ambiguous regions remain colored blue. Top right: a normalized mean image of the region. Bottom right: a mean image with all of the previously made selections out-

lined with appropriate colors.

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#### INTELLIGENT SCANNING



FIG. 2. Example of segmentation of a test data set. A: the 4 unnormalized filtered version of the raw data (Table 1, formulas 1–4). The color corresponds to amplitude of the filtered output. The normalized versions of filtered images from A (Table 1, formulas 5–8). B: the output of the 1st classifier. The color corresponds to the likelihood that a given pixel is a cell. C: the output of the 2nd step classifier, with isolated pixels, i.e., speckle noise, removed with a 5  $\times$  5 pixel median filter, along with the output values thresholded to form clusters of pixels that are candidate cells; we chose 6 levels, which correspond to pixels lying in the top 5, 10, 15, 20, 25, and 30% of the maximum amplitude. D: final classification made by thresholding the output shown in C.

a few minutes to apply the algorithm and segment all cells in a sequence of images.

To evaluate the cell segmentation algorithm, a *k*-fold cross-validation was performed for both the first and second step classifiers by lumping training sets from the 16 different brain regions and partitioning the data into five equal sets (k = 5). Five different estimates for test error were obtained by training on four of the five sets and using the remaining set to test. We observed an average estimated error of 0.07 (combined false positives and false negatives) for the test data. The area under the receiver operating characteristic (ROC) curve, a combined measure of method specificity and sensitivity, is 0.97 and is dominated by false positives (Fig. 3). An examination of incorrectly classified cells shows that they predominantly occur in areas where it is difficult for a human expert to consistently label the cells.

#### Implementation

As a proof-of-principle implementation of our algorithms, we performed >200 trials of fast scanning measurements across 23 fields in primary somatosensory cortex that responded to stimulation of the hindpaw (n = 4 rats). The scan-mirror speed was adjusted so that the imaging time for each cell was ~200  $\mu$ s per scan cycle, which yielded a signal-to-noise ratio sufficient to detect the nominal 10% fluorescence changes associated with calcium action potentials in neurons labeled with the indicator OGB1 (Dombeck et al. 2007; Kerr et al. 2005; 2007; Komiyama et al. 2010; Ohki et al. 2005; Rothschild et al. 2010). Recall that the calcium spikes are not necessarily associated with single

sodium spikes, since previous work has shown that many sodium spikes can contribute to a single calcium spike (Greenberg et al. 2008). We chose to study the upper layers of cortex for technical convenience and because different somata are well separated. Objects that overlap will be rejected, so that areas with extremely dense cells may be problematic and were thus avoided.

We present two typical examples of fast scanning in cortical layers 2/3 of rat somatosensory cortex with our approach. The first is a region with 68 identified cells, 64 neurons, and 4 astrocytes that was scanned at 70 Hz (Fig. 4, A-D). Data were acquired for 10 min with only minimal photobleaching. The second is a region with 20 identified cells, 19 neurons, and 1 astrocyte, along with three blood vessels, that was scanned at 110 Hz (Fig. 4, E-H). Data were acquired for 4 min, again with only minimal bleaching. In both examples, the cell segmentation algorithm was used in conjunction with full-field images from the OGB1 emission channel to determine all possible cell bodies (Fig. 4, A and E; see Supplemental Fig. S1<sup>1</sup> for an example of segmentation of 12 trials across 4 animals using the same classifier). Segmentation requires about 1 min of computation, and determination of the optimized pathway requires an additional 2 min. Cells that were co-labeled with the astrocytic marker sulforhodamine 101 (SR101) were automatically labeled as astrocytes; the coordinates of selected blood vessels were also marked. The optimized scan algorithm was used to find the shortest cycle time through all cells (Fig. 4, A and E), with  $\sim$ 70% of the scan time spent over ROIs, and a series of scan measurements was performed that encompassed periodic sensory stimulation (Fig. 4, B and F). The typical signal-

<sup>&</sup>lt;sup>1</sup> The online version of this article contains supplemental data.



FIG. 3. Validation statistics for the classifiers. A: a histogram of cross-validated examples binned by the scores they have received from the first classifier. Black are examples of pixels that are parts of cells, whereas gray are examples of pixels that are not parts of cells. B: receiver operating characteristic curve of the 1st classifier; the 2 dotted lines indicate the point on the ROC curve for which the score threshold is zero. Note that because ground truth is poorly defined, the ROC curve is only approximately representative of the real classifier errors. C: a histogram of cross-validated examples binned by the score they have received from the 2nd classifier. Black are examples of candidate cells that are actually cells, whereas gray are candidate cells that are not cells. D: an ROC curve of the 2nd classifier; the 2 dotted lines indicate the point on the ROC for which the score threshold is zero, which is the nominal final threshold for our algorithm. Note that because ground truth is poorly defined, the ROC curve is only approximately representative of the real classifier errors.

to-RMS-noise ratio, which we define as the ratio for a change in intracellular calcium induced by a single sensory stimulus, which we define as the ratio of the peak value of the response to the value of the RMS noise during baseline activity, is  $\sim 10$  (Fig. 4, *C* and *G*).

Our rapid segmentation process also facilitates on-line data analysis. For example, the average trial-by-trial activity of all cells as a function of time after stimulation is readily calculated (Fig. 4*D*). As a second example, changes in astrocytic calcium levels together with changes in the speed of red blood cells in a nearby microvessel are readily compared with the composite neuronal activity (Fig. 4*H*).

#### DISCUSSION

Our cell detection method can identify and find borders of  $\sim$ 70 cells in a 512 × 512 pixel image in 1 min, which appears to be at least 10-fold faster than human annotation. This is crucial for studies that involve longitudinal measurements of somatic activation, such as developmental plasticity (Golshani et al. 2009; Rochefort et al. 2009), or swelling of the brain, such as experimental stroke (Sigler et al. 2009), where recalculation of the scan path compensates for shifts in the position of cells. As a practical matter, multiple cells and blood vessels may be monitored typically at rates that are 10-fold greater than those achieved with full-field images.

Optimization of the scan algorithm insures that the majority of time is spent over somata and blood vessels of interest. We chose to optimize with use of the ANT system algorithm (Di Caro and Dorigo 1998). This approach was chosen over gradient descent algorithms, genetic algorithms (Potvin 1996), and convex hull algorithms (Nikolenko et al. 2007) because, for regions with >100 ROIs, the ANT system is relatively insensitive to internal parameters when computing the shortest pathway. Although there is no strong upper bound on the time for convergence of the ANT algorithm, the time increases slowly with an increase in the number of ROIs. The relatively high efficiency of this process may, in some instances, obviate the need to replace galvometric scanners with acousto-optical deflectors (AODs). Arbitrary path scanning can also be combined with AODs for three-dimensional scanning applications (Duemani-Reddy et al. 2008; Vucinić and Sejnowski 2007).

One potential limitation of optimized path scanning is that it is not compatible with schemes for correcting for motion artifacts (Dombeck et al. 2007). This implies that our method should be primarily used on anesthetized animals. Nonetheless, the segmentation part of our approach can be used to do postexperiment analysis of full-frame images collected from behaving animals. This allows one to analyze in hours what could take weeks to do manually.

The insensitivity of our algorithm to correlated activity implies that it may be superior to correlation-based algorithms (Mukamel et al. 2009; Sasaki et al. 2008). The natural capability of learning-based approaches, such as ours, to generate very complex morphological classifiers makes it superior to hand-tuned approaches, albeit at the

### Innovative Methodology

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FIG. 4. Two examples of cell segmentation and fast scanning for functional imaging of neurons and astrocytes in rat parietal cortex. A: a full-field image of a region with 68 cells, obtained at 4 frames/s, with a scan path superimposed on it in which all cells are sampled at 70 Hz. The green channel shows the fluorescence from Oregon Green Bapta-1, whereas the red channel shows fluorescence from Sulforhodamine 101. White shows the outlines of cells as determined by our algorithm. *B*: part of the raw data output from consecutive scans, including a hindlimb stimulation. *C*: activity of 10 cells, 9 neurons, and 1 astrocyte as indicated in *A* and *B*, during the same time interval as shown in *B*. The traces shown in the order of the cells that were scanned and represent typical results. *D*: distribution of onset times for changes in intracellular  $[Ca^{2+}]$  in all 68 cells after stimulation across 9 trials. *E*: a full-field image of 19 neurons, 1 astrocyte, and 3 blood vessels scanned at 110 Hz with a scan path superimposed on it. *F*: part of raw data output that includes a hindlimb stimulation event. *G*: activity of cells, neurons, and an astrocyte indicated in *E* and *F* during the same time interval as shown in *F*. *H*: the calcium response of the astrocyte (A1), the average neuronal response (N1–N19), and the speed of red blood cells in one capillary (V1).

cost of obtaining and annotating training data. At the same time, our approach can make use of specialized filters, such as automatic spike train deconvolution (Vogelstein et al. 2010), to provide a fuller analysis of TPLSM data. Last, the use of compiled languages or specialized hardware may greatly decrease the computational time to segment the image data and compute an optimized scan path.

#### APPENDIX

The portions of the scan path that pass through the ROIs are created as straight lines, given by

$$\mathbf{P} = \mathbf{P}_0 + \mathbf{V}_{\text{linear}} \cdot t$$

where P is a two-dimensional vector of voltages that specifies the deflection of the scan mirrors that in turn directs the beam. The

parameter  $\mathbf{P_o}$  is the initial voltage and the parameter  $\mathbf{V_{linear}}$  is the slew (in V/ms), whose magnitude determines the time spent crossing the cell and whose direction is set by the diagonal of the bounding box. The paths through each ROI are connected by third-order polynomial splines that are constructed so that the scan path is continuous in both voltage and slew. This creates a physically realizable path that is followed by the scan mirrors with a constant delay, typically 80  $\mu$ s for our scanners. The connecting paths between the ROIs are described by

$$\mathbf{P}_{\text{spline}} = \mathbf{P}_{i} + \mathbf{V}_{i} \cdot \boldsymbol{t} + \mathbf{C} \cdot \boldsymbol{t}^{2} + \mathbf{D} \cdot \boldsymbol{t}^{3}$$

where for computational convenience, the spline is taken to start at t = 0 and end at  $t = \tau$ , the initial voltage  $P_i$  and slew  $V_i$  are set to match the position and velocity of the end of the ROI preceding the spline, and the parameters **C** and **D** are found from

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$$C = \frac{3P_f}{\tau^2} - \frac{3P_i}{\tau^2} - \frac{2V_i}{\tau} - \frac{V_f}{\tau}$$

and

$$C = \frac{V_f}{3\tau^2} - \frac{V_i}{3\tau^2} - \frac{2C}{3\tau}$$

The value of  $\tau$  is the smallest positive real value that does not subject the mirrors to an acceleration larger than a hardware limit, denoted **m**, where typically **m** = 100 V/ms<sup>2</sup>. Candidate values for the shortest possible spline length are found by setting the acceleration to  $\pm$ **m** at the beginning and end of each spline, and finding all positive real values for  $\tau$ , i.e.

$$0 = \pm m\tau^2 + (4V_f + 2V_i)\tau + (6P_i - 6P_t)$$

for acceleration at the start of a spline and

$$0 = \pm m\tau^2 + (4V_i + 2V_t)\tau + (6P_i - 6P_t)$$

for acceleration at the end of a spline. This leads to multiple values for  $\tau$ ; we choose the smallest value that bounds the acceleration at the beginning and end of the spline but allows the mirrors to make positional errors on other parts of the spline (Supplemental Fig. S2). Thus  $|2C_x| < m$ ,  $|2C_y| < m$ ,  $|2C_x + 6D_x\tau| < m$ , and  $|2C_y + 6D_y\tau| < m$ .

The total time spent scanning across the regions between ROIs was minimized by estimating the optimum order in which to scan the ROIs. This is a "traveling salesman" problem in terms of minimizing the time between ROIs, for which the ANT System algorithm (Di Caro and Dorigo 1998) provides a robust and easily implemented approximate solution. Finally, the vector along the diagonals through each ROI is iteratively adjusted to further minimize the total time spent scanning across connecting sections.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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