33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

Modeling Two-Photon Calcium Fluorescence of Episodic V1 Recordings Using Multifrequency Analysis

Henry W. Zheng, Wasim Q. Malik, Caroline A. Runyan, Mriganka Sur, Emery N. Brown

Abstract – **The use of two-photon microscopy allows for imaging of deep neural tissue *in vivo*. This paper examines frequency-based analysis to two-photon calcium fluorescence images with the goal of deriving smooth tuning curves. We present a multifrequency analysis approach for improved extraction of calcium responses in episodic stimulation experiments, that is, when the stimulus is applied for a number of frames, then turned off for the next few frames, and so on. Episodic orientation stimulus was applied while recording from the primary visual cortex of an anesthetized mouse. The multifrequency model demonstrated improved tuning curve descriptions of the neurons. It also offers perspective regarding the characteristics of calcium fluorescence imaging of the brain.**

Keywords: Two-photon microscopy, calcium imaging, multifrequency analysis, visual cortex, tuning curve.

I. Introduction

Recently there has been increasing interest in the application of two-photon and multiphoton excitation microscopy in neural imaging. Two-photon microscopy as a technology offers several technical advantages over traditional confocal microscopy [1]. The use of confocal microscopy *in vivo* has been hindered by issues such as phototoxicity and photobleaching, limited depth imaging due to light scattering, and focal size. Fundamentally, these issues are related to the inefficient use of photons when imaging tissue – light is shined all over the sample, but only a small fraction is selected, focused, and collected. Because of the need to reduce the pinhole size to filter out stray photons, confocal microscopy suffers from a trade-off between resolution and image size. Furthermore, confocal microscopy, which is based on single-photon excitation, induces excessive photon absorption by the tissue, making phototoxicity and photobleaching a significant issue. Since greater intensity is needed to penetrate into deep tissue, confocal microscopes need to balance depth with phototoxicity.

The mechanism of two-photon excitation microscopy circumvents these difficulties by taking advantage of two low-energy photons to collide at the focal point, exciting the quantum state of the sample to a point equivalent to what a high-energy photon from a confocal microscope would excite to. The use of two low-energy photons to achieve the function of a single high-energy photon reduces phototoxicity, allowing imaging of far deeper tissue. Two-photon microscopy circumvents or mitigates the issues faced by confocal microscopy and is ideal for imaging large and deep swaths of tissue *in vivo*, making it a prime technology for use in neuroscience.

Calcium fluorescence is an effective analogue to voltage measurements, as $[Ca^{2+}]$ is a direct function of neural spike activity [2], [3]. The ability to observe calcium fluorescence over extended periods of time gives a novel way to monitor population activity. Furthermore, imaging calcium fluorescence using two-photon microscopy offers an unprecedented range and depth of viewing *in vivo*. Although the technology has recently been used, two-photon microscopy has yet to be characterized in an environment with episodic stimulation. Thus, the primary purpose of the paper is to develop a model tailored to episodic visual stimulation that characterizes calcium fluorescence as observed using two-photon microscopy.

Current techniques use simple approaches such as bin averaging of relative fluorescence to construct tuning curves [4]. In fluorescence microscopy, it is particularly important to establish a structured model because of three reasons. The first reason is due to the high amount of error inherent in noninvasive imaging – due to the presence of tissue obstructing the path of photons reflecting from deep in the brain, there is high variability in the effective florescence detected by the camera. Two, the relatively slow sampling rate of scanning the layers of tissue leads to a larger proportion of the data being noise and hinders attempts at interpolation. Third, the concentrations of calcium inside neurons fluctuate nonlinearly with voltage and interact in complex ways with many other cellular processes [5]. Because of the low sampling rate combined with large noise,

Manuscript received April 15, 2011. This work was supported in part by NIH Grants DP1 OD003646, R01 EB006385, EY07023 and EY017098.

H. W. Zheng is with Yale University, New Haven, CT 06520 (e-mail: henry.zheng@yale.edu).

W. Q. Malik is with Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114. He is also with the Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139 (e-mail: wqm@mit.edu).

C. Runyan is with the Picower Institute for Learning and Memory, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139 (e-mail: crunyan@mit.edu).

M. Sur is with the Picower Institute for Learning and Memory, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139 (e-mail: msur@mit.edu).

E. N. Brown is with the Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114. He is also with the Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139 (e-mail: enb@neurostat.mit.edu).

it becomes important to apply a model that incorporates the structure of the data so as to more effectively distinguish between useful data and noise.

The rest of the paper is organized as follows: Section 2 describes the materials and experimental setup used. Section 3 discusses the details of constructing a model based on the data, such as the algorithms used for parameter estimation. Section 4 examines the modeling effort, characterizes patterns observed, and evaluates the efficacy of the model. Section 5 offers concluding remarks on the characterization of the signal and gives direction for future work.

II. Materials and Methods

All experimental procedures followed have been approved by the Massachusetts Institute of Technology Committee on Animal Care, and adhere to NIH guidelines for the Care and Use of Laboratory Animals.

A. Animal and Microscope Setup

Multiphoton imaging of the fluorescent calcium indicator Oregon Green Bapta (OGB) was performed in the visual cortex of anesthetized mice *in vivo*. Neurons were bulk-loaded with OGB by intracortical injection of the AM-ester conjugated form of OGB using standard techniques. Imaging was performed with a custom-made two-photon laser scanning microscope [6]. Time series traces of images with a field-of-view of approximately 250×250 μm were collected at 1 Hz. The images were taken from cortical layer 2/3, which was readily distinguished from layer 1 on the basis of the relative density of astrocytes and neurons. Per set, the data were collected from 46 neurons in the V1 of a single mouse. Seven sets were collected in total.

B. Stimulus Protocol

Visual stimuli were delivered via a 17" LCD display placed 0.15 m away from the eyes of the animal. The stimuli were generated with the Matlab software package using the PsychoPhysics Toolbox [7]. The stimulation protocol consisted of square-wave gratings with 100% contrast which drifted at 3 Hz. The gratings alternated between appearing and disappearing, each occurring for 4 seconds. Data frames were recorded at 1 Hz. Between each appearance, the grating rotated 20°, from 0° to 340°. Thus, the stimulus rotates 340° in 144 seconds and the time series of the response of a neuron to this stimulus approximates a full orientation tuning curve. Prior to recording these responses, 10 image frames (10 seconds) were acquired in the absence of any visual stimulus to establish the baseline response level (Figure 1).

Figure 1: Diagram of Experimental Setup. White boxes indicate no stimulus, black boxes indicate presence of stimulus. Each box represents 4 seconds, except for the first box which is 10 sec. The grid above visually represents the stimulus seen by mouse at each moment in time. 0° to 100° is shown – experimentally range extends to 360°.

Image files collected by two-photon microscopy were imported into Matlab and analyzed with custom routines. The cell bodies were identified by inspection and outlined manually. Only cells with fluorescence distinguishable from the neuropil were chosen for subsequent analysis. The fluorescence pixels were averaged over the area of the cell. Using an episodic stimulus scheme forms the foundation for the multifrequency model, which presupposes the periodic presence and absence of stimulus. This allows for a more informed analysis and modeling of the calcium flux in mouse visual cortex.

III. Statistical Model and Analysis

Given that the goal is to construct tuning curves for the cells, it is important to find a mathematically relevant approach to model the fluorescence curve. Periodogram analysis of the time series shows that many of the cells exhibit an activity profile that suggests a sinusoidal structure composed of a few main frequencies (Figure 3). Thus, we used the following multi-frequency sinusoid model,

$$F = \sum_{i=1}^{N} A_i \sin(\omega_i t + \varphi_i) + \kappa(t), \qquad (1)$$

where the overlapping sinusoids form, due to constructive and destructive interference, a carrier-wave component representing the fundamental frequency (i=1) that the fluorescence signal oscillates with. Secondary (i=2,3) frequencies modulate the carrier frequency, creating the envelope that will be used to extract the tuning curve. Based on spectral analysis (Figure 3a), calculated by finding the power using mean-squared Fourier transform, we observed that most of the cellular signals were composed of two or three frequencies (N = 2, 3). These components can be seen as high-power peaks near the low-frequency section of the periodogram. $\kappa(t)$ denotes the residuals that form the noise component, and will be used to examine the applicability of the multifrequency model to the data.

The algorithm used for parameter estimation is a nonlinear least squares approach implemented using the Matlab curve fitting toolbox [8]. F is the measured fluorescence levels in (1), composed of the model-based predicted fluorescence with parameter values $\boldsymbol{\theta} = \{A_i, \omega_i, \varphi_i\}$. The system was solved for $\boldsymbol{\theta}$ using an iterative nonlinear regression technique, where $\hat{\boldsymbol{\theta}}$ was stepwise approached and each step was calculated by:

$$\Delta\hat{\boldsymbol{\theta}} = (\mathbf{J}^{\mathrm{T}}\mathbf{J})^{-1}\mathbf{J}^{\mathrm{T}}\Delta F. \qquad (2)$$

$\mathbf{J}$ is the Jacobian of the residual and ΔF the step size to re-calculate a more accurate iteration of $\hat{\boldsymbol{\theta}}$. A trust-region estimation technique was used to calculate the step size of ΔF and iteratively approach the optimal $\hat{\boldsymbol{\theta}}$. The covariance of $\hat{\boldsymbol{\theta}}$ can be found by:

$$\mathrm{Cov}(\hat{\boldsymbol{\theta}}) = \sigma^2(\mathbf{J}^{\mathrm{T}}\mathbf{J})^{-1} \qquad (3)$$

where $\sigma^2 = SS_E/(n\text{-}1)$. SS_E is the sum of the squares of the residuals, n is the number of data points, and values of the main diagonal in the covariance matrix $\mathrm{Cov}(\hat{\boldsymbol{\theta}})$ form the standard error of the parameters.

IV. Results and Discussion

A. Fit Analysis

The sinusoid model described well the overarching profile of the neuron responses, in particular the slowly oscillating envelope that is the response to the changing stimulus. The Pearson correlation coefficient was used to evaluate the coherence of the model to the data and was greater than 0.6 for all cells, with some as high as 0.85. This shows that the model captured the majority of the stimulus-evoked response by the neurons. From Figure 2, the presence of many fluorescence measurements that measure below baseline fluorescence indicate that the information from multiphoton microscopy is littered with noise. Thus, it is important to use a more structured model to discern the stimulus-evoked component from the noise.

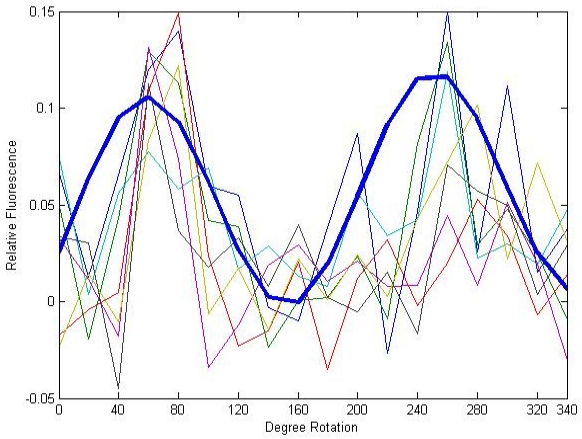

Fig. 2. Comparison of tuning curves based on multifrequency versus standard approach. Bold curve is the tuning curve predicted by the multifrequency model. Standard approach calculated by directly averaging fluorescence of cell body over different angle stimuli.

Comparing the tuning curve predicted by the multifrequency approach against simple averaging of fluorescence at different angles is revealing about the character of the response of the neuron to episodic stimulus. As can be seen above, V1 neurons respond more consistently when gratings at specific angles are first seen (namely those from 0° to 180°), but this consistency deteriorates when the grating direction is seen again. This suggests an interesting hidden mechanism that affects the activity of these neurons, which should be investigated.

It was found that, for most neurons, two or three sinusoids were adequate for an appropriate description of the data. As seen in Figure 3a, the periodogram of the original signal

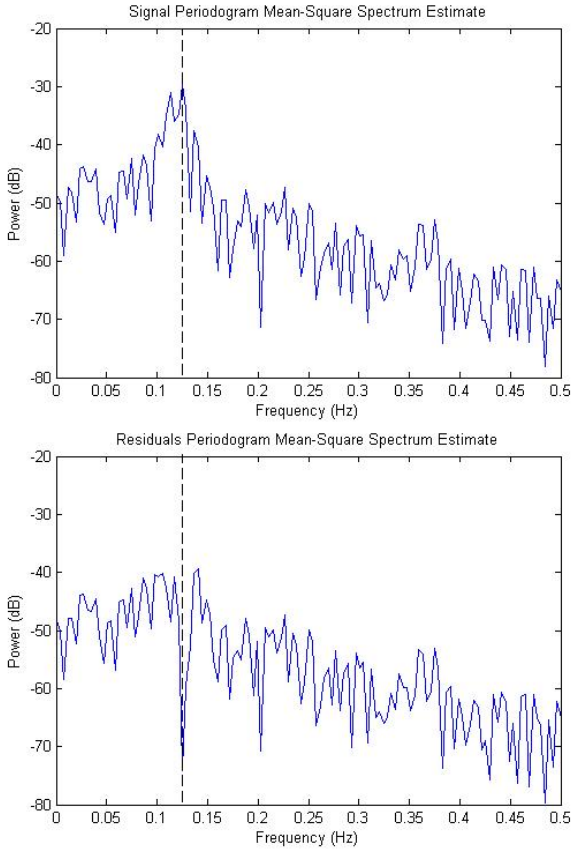

Fig. 3. (a) Top, periodogram of fluorescence signal; (b) Bottom, periodogram of residuals. The vertical line indicates $f = 0.125$ Hz, the frequency of the stimulus.

show two main stimulus-evoked frequencies at around $f = 0.125$ Hz that were captured by the multifrequency model. The frequencies captured in the sinusoids were all low-frequency components of the data equal to the low frequency of the stimulus change, the low sampling frequency, and the relatively slow speed of calcium flux in the neuron. Since one on-off cycle of the stimulus corresponded to an experimental period of 8 seconds, that the data reveals a strong frequency component at 0.125 Hz confirms that the main frequency was captured by the multifrequency model.

B. Residual Analysis

Based on characteristics of residuals, the multifrequency model seems appropriate. This implies that the model captured the major low-frequency components of the data and the noise captured the remaining, high-frequency stimulus-independent component of the signal. A periodogram of the raw fluorescence data show two high power frequencies at approximately f=0.125 Hz, corresponding to two major sinusoids that comprise the signal, while the residual has a far less pronounced profile of frequency peaks than the original signal and form a band of low-power noise captured in the residual.

C. Tuning Curve

As seen in Figure 2, reconstructing the tuning curve from the multifreqiency model presented a much less noisy tuning curve than from the unmodeled data. The first half-cycle of stimulus (angle rotations between 0° and 180°) exhibited a much more consistent and distinct rise in calcium levels compared to the second half-cycle (from 200° to 340°). This is most likely because the neuron is preferentially tuned to an orientation around 70°, but due to the symmetry possessed by a rotating grating, show sensitivity to an orientation of 250°.

The asymmetry of the tuning curve illustrates an important principle that must be considered when constructing models for calcium fluorescence. Despite the apparent symmetry of a grating oriented at 70° or at 250°, these neurons do not respond symmetrically. Thus, any model designed to capture the response must be flexible enough to accommodate an asymmetrical neuron response to a symmetrical signal input.

This poses difficulties because of the effect of noise upon the derivation of a tuning curve by any algorithm. Due to the observation made above, any model of the neurons in the visual cortex must be flexible enough to accommodate asymmetry. However, the problem arises when such asymmetry is not stimulus-derived, but due to a large noise component. The primary logic behind assuming symmetry when using a multifrequency model is to reduce the effect of large noise factors. A more flexible model that can incorporate more variability might erroneously capture noise as information. This is an issue that must be addressed when considering any model in a high-noise environment, such as in two-photon calcium fluorescence imaging. Thus, future models of multiphoton calcium fluorescence imaging will incorporate and quantify the complex modulations that calcium undergoes in the neuron.

A comparison between the predicted modeled fluorescence against traditional processing techniques (Figure 2) show significant qualitative differences that point to the usefulness of the model to extract pertinent information. Traditional methods would not have detected significant difference between baseline the midpoint orientation between baseline and highest activity. However, it is known that V1 cells possess partial sensitivity to off-target orientations and have a range of tuning widths, which is captured in the model [9]. Thus, qualitatively the model captures the intended activity of the neuron and agrees with the results derived from voltage-based models.

V. Conclusion

The paper examined the issue of understanding and modeling multiphoton microscopy and presented a model for intelligent smoothing of two-photon calcium fluorescence imaging data. Using our multifrequency model, stimulus-evoked responses from neurons in the visual cortex were accurately captured in the presence of large noise. The characteristics of the model and noise were examined, leading to greater insight regarding the character of calcium fluorescence imaging and of VI neurons and offering insight into future models of multiphoton fluorescence microscopy. The most promising direction to take this approach is to elaborate on more sophisticated models that take advantage of situational factors such as orientation maps or cortical thickness. With a more sophisticated model that incorporates noise due to structure of the cortex into account, a more powerful method could be constructed that would further make two-photon calcium fluorescence microscopy a viable neuroimaging method.

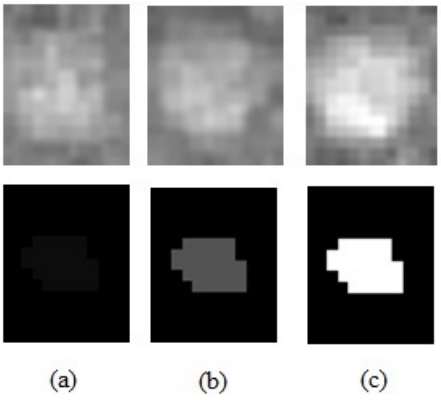

Fig. 3. Unprocessed (top) and modeled (bottom) fluorescence responses at (a) baseline, (b) 40° orientation, and (c) 80° orientation.

Acknowledgment

The authors would like to thank Nathan Wilson for his help during the experimental process.

References

[1] W. Denk, K. Svoboda, "Photon Upmanship: Why Multiphoton Imaging is More Than a Gimmick," *Neuron*, vol 18, pp. 351–357, Mar. 1997.

[2] R. Yuste, W. Denk, "Dendritic spines as basic functional units of neuronal integration," *Nature*, vol. 375, pp. 682–684, Jun. 1995.

[3] C. Stosiek, O. Garaschuk, K. Holthoff, A. Konnerth, "In vivo two-photon calcium imaging of neuronal networks," *Proc. Natl. Acad. Sci. USA*, vol. 100, pp. 7319–7324, Apr. 2003.

[4] W.Q. Malik, J. Schummers, M. Sur, E. N. Brown, "Denoising Two-Photon Calcium Imaging Data," *PLoS ONE* 6(6): e20490, Jun. 2011.

[5] K. Svoboda, R. Yasuda, "Principles of Two-Photon Excitation Microscopy and Its Applications to Neuroscience," *Neuron*, vol. 50, pp. 823–839, Jun. 2006.

[6] A. Majewska, G. Yiu, R. Yuste, "A Custom-Made Two-Photon Microscope and Deconvolution System," *Eur. J. Physiol*, vol. 44, pp. 398-408, Dec. 2000.

[7] D. H. Brainard, "The Psychophysics Toolbox," *Spatial Vision*, vol. 10, pp. 433-436, Jan. 1997.

[8] D. C. Montgomery, C. L. Jennings, M. Kulahci, *Introduction to Time Series Analysis and Forecasting*. Wiley: 2008.

[9] J. Schummers, J. Mariño, M. Sur, "Synaptic integration by V1 neurons depends on location within the orientation map," *Neuron*, vol. 36, pp. 969–978, Dec. 2002.