



REVIEW

## **REVISED** Calcium imaging analysis – how far have we come?

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### Abstract

Techniques for calcium imaging were first demonstrated in the mid-1970s, whilst tools to analyse these markers of cellular activity are still being developed and improved today. For image analysis, custom tools were developed within labs and until relatively recently, software packages were not widely available between researchers. We will discuss some of the most popular methods for calcium imaging analysis that are now widely available and describe why these protocols are so effective. We will also describe some of the newest innovations in the field that are likely to benefit researchers, particularly as calcium imaging is often an inherently low signal-to-noise method. Although calcium imaging analysis has seen recent advances, particularly following the rise of machine learning, we will end by highlighting the outstanding requirements and questions that hinder further progress and pose the question of how far we have come in the past sixty years and what can be expected for future development in the field.

### Keywords

Calcium Imaging, Denoising, Motion Correction, Classification, Quantification, Machine Learning, Neural Networks



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### Open Peer Review

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**REVISED Amendments from Version 1**

This latest version of the review article "Calcium imaging analysis – how far have we come?" contains a clearer breakdown of the possible image analysis options for different experimental set-ups of calcium imaging experiments such as *in vitro* versus *in vivo* methods. We also expand on the 'Quantification' section as this is an important section that was previously very brief. In this section we discuss identification of action potentials from calcium signals as this can be a controversial topic.

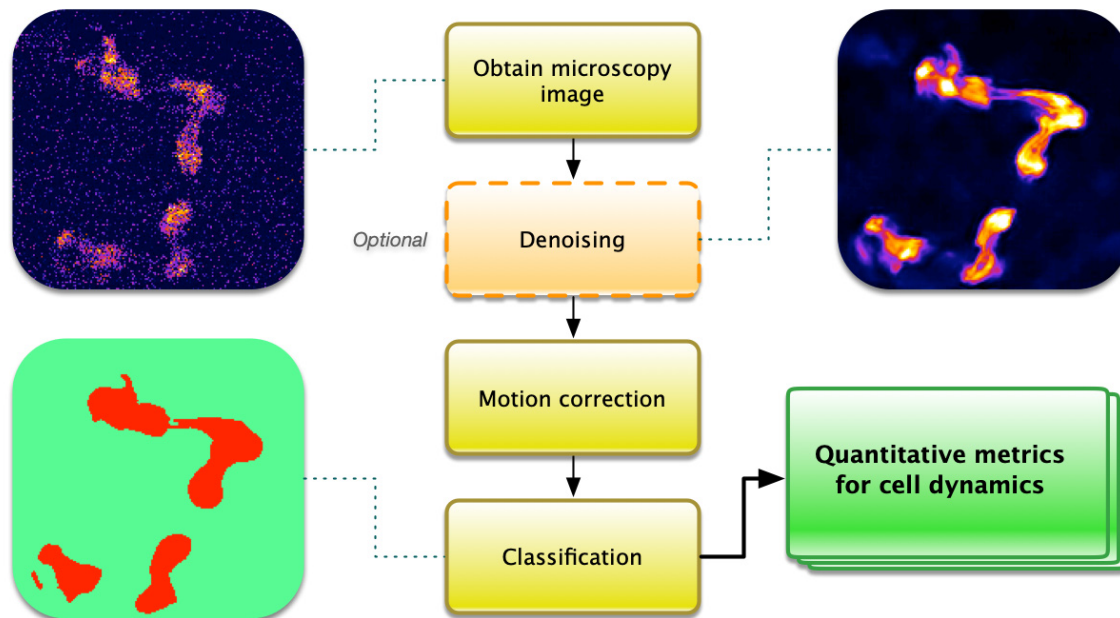
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**Introduction**

The ability to image calcium ion ( $\text{Ca}^{2+}$ ) dynamics in cells has long been of interest, particularly in the neurosciences, where it can be used as a marker for neuronal excitability. The origins of calcium imaging began in the mid-1970s (Blinks *et al.*, 1976; Moisescu *et al.*, 1975), however the most  $\text{Ca}^{2+}$  specific BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid)-based dye was developed in 1980 by Roger Tsien, and its derivatives are still used today (Tsien, 1980). In the past forty years, the methods available for measuring  $\text{Ca}^{2+}$  fluxes in cells have expanded to include ratiometric, fluorescence lifetime, or fluorescence intensity-based dyes, and genetically-encoded calcium indicators (GECIs) (Miyawaki *et al.*, 1997; Ohkura *et al.*, 2005). The use of microscopy modalities has also advanced to include light-sheet

microscopy (LSM; Huisken *et al.*, 2004) for long-term imaging, and 2-photon microscopy (2PM; Denk *et al.*, 1990) for deep tissue and cell specific uncaging techniques. The combination of  $\text{Ca}^{2+}$  indicator and imaging modality used will reflect the properties of the sample and the scientific question, as well as the methodologies available to the researcher. For example, *in vitro* imaging, or *in vivo* invertebrate imaging, may use exogenous or GECIs, imaged using LSM, epifluorescence, 2PM or other fluorescence microscopes depending on the temporal and spatial resolution, timescale of imaging, and thickness of the sample being taken into consideration. Other specialist options for *in vivo* imaging of GECIs are available for imaging in awake and behaving animals including miniaturized forms of 1- or 2- photon endoscopic fluorescence microscopes (miniscopes) for single-cell *in vivo* recordings (Cai *et al.*, 2016; Chen *et al.*, 2013; Silva, 2017).

Calcium imaging is an inherently noisy method due to the high spatiotemporal information desired from a sample often showing low signal-to-noise alongside drift or cell movement, particularly for living organisms. In recent years, a number of software packages have been written for individual aspects of the commonly used pipeline in calcium imaging analysis (Figure 1). This processing pipeline includes image denoising, motion correction, classification for cell identification, and quantification of calcium signals. As calcium imaging is used across a broad range of samples, from sub-cellular, cellular,



**Figure 1.** The steps of a common pipeline for calcium imaging analysis can be subdivided into three areas before quantitative analysis is performed. Denoising is an optional step that can help to improve signal-to-noise and enhance features. Motion correction may be necessary in cases of drift or movement. Classification can select regions of interest for which quantitative analysis is performed.

networks, bulk tissue dynamics to whole organisms and behaving animals, aspects of this pipeline can vary substantially with our ‘one size fits all’ approach.

## Denoising

Live-cell imaging generally requires short exposure times and low excitation power to limit the effects of photo-toxicity and photo-bleaching. This leads to image degradation in the form of noise. In fluorescence microscopy the two prevalent noise sources are Poisson noise and Gaussian noise. Poisson noise is caused by the stochastic and discrete nature of photon emission and tends to be dominant at low light levels, whereas Gaussian noise describes the intrinsic thermal and electronic fluctuation in the image sensor (Luisier *et al.*, 2011)

Although denoising is not a required step in the pipeline, effective denoising can improve the subsequent steps by artificially enhancing signal-to-noise. Traditionally, image denoising has been based on local averaging approaches, such as the application of a Gaussian smoothing filter (Buades *et al.*, 2005; Lindenbaum *et al.*, 1994). Alternative methods include a local filter method such as anisotropic filters (Broser *et al.*, 2004; Kitamura & Häusser, 2011) (Perona & Malik, 1990), or in the frequency domain, Wiener filters (Wiener, 1950) and wavelet thresholding methods (Donoho, 1995) (Besbeas *et al.*, 2004; Wegner *et al.*, 2006).

Local methods are computationally light but have clear limitations. First, the averaging often involved in local methods introduces blur, causing features to appear less well defined. Second, they do not perform well for high noise levels, since the correlations between neighbouring pixels deteriorate (Shao *et al.*, 2014). In the context of calcium imaging, local methods have been shown to perform well (Malik *et al.*, 2011).

Non-local filters solve some of the problems by using self-similarity of natural images beyond neighbouring pixels, thus exploiting global information (Shao *et al.*, 2014). The first method to propose this is the non-local means method (Buades *et al.*, 2005), in which subregions of an image referred to as patches are restored by weighted averaging of all other patches in an image. Since then, there have been a number of improvements such as invariance to patches that are rotated or mirrored with respect to each other (Grewenig *et al.*, 2011), improved computational efficiency, and automated parameter tuning and extension to 3D image stacks (Coupé *et al.*, 2008). Although non-local filters are better at high noise levels, they will typically lead to artefacts like over-smoothing (Shao *et al.*, 2014). A modern, well-balanced and state-of-the-art non-local method is ND-SAFIR, which is specifically geared towards application in fluorescence microscopy imaging (Boulanger *et al.*, 2010). ND-SAFIR is a powerful method for removing Poisson-Gaussian noise. It is based on non-local means denoising using a variance stabilisation step, followed by calculating the spatial and temporal patch-based weighted averages for intensity values. The method is widely applicable between experimental samples and can be used directly for 2D+t and 3D+t datasets (Buades *et al.*, 2011).

In recent years, deep learning methods have become state-of-the-art for denoising. Methods such as DnCNN (Zhang *et al.*, 2017), FFDNet (Zhang *et al.*, 2018) and CARE (Weigert *et al.*, 2018) rely on convolutional neural networks that are trained in a supervised learning approach. However, this requires ground truths to be available for model training, which may be difficult to obtain in practice. A different approach was developed in noise2noise (Lehtinen *et al.*, 2018), where instead of learning the mapping from noisy images to clean targets, the model is trained with other noisy images as targets. The images must be corresponding pairs displaying the same objects but with independent noise. Assuming the noise sources underlying the images have zero-mean distributions, the weights of the network will then converge during training to the same values as a network trained with clean targets because the noise that manifests in the weights cancels out. A more recent method, noise2void (Krull *et al.*, 2019), aims to resolve this issue of needing ground truths, by using self-supervised learning. Here, the network is optimised to predict the value of each pixel from the values of neighbouring pixels in an image, thus requiring no separate ground truths. In another recent method, DeepInterpolation (Lecoq *et al.*, 2020), the need for ground truth training data is avoided by treating the denoising task as a nonlinear interpolation problem. This assumes that the data have a sequential component, such that spatiotemporally overlapping features can be exploited. DeepCAD is a new deep self-supervised denoising method that reduces detection noise and thereby improve the signal-to-noise more than tenfold, which it claims can improve the accuracy of neuron extraction and spike inference (Li *et al.*, 2021).

## Motion correction

Motion correction is often required to ensure consistent image processing across a time stack. We distinguish between two types of motion: (a) drift occurring in the imaging system itself caused by thermal gradients in the microscope, vibrations and mechanical instability (Kreft *et al.*, 2005); (b) subject motion such as fluctuations in the immersion media or the movement of organisms (Jenkinson *et al.*, 2002). Drift will typically play a significant role when imaging the same field of view over multiple days, which can be rectified by using standard registration methods (Dubbs *et al.*, 2016) (Thévenaz *et al.*, 1998).

More complex motion such as organism movement can be harder to correct as it is often non-uniform, over a large area, and causes movement in-and-out of the focal plane. These require non-rigid registration methods or motion tracking. A commonly used example available in Python and MATLAB is Non-Rigid Motion Correction, NoRMcorre (Pnevmatikakis & Giovannucci, 2017), which uses patch-based field of view registration whereby separate images are then merged by smooth interpolation. The popularity of NoRMcorre may in part be due to its general applicability.

Two correction methods have been produced for *in vivo* imaging in awake rodents, one based on the rigid-transform-based Lucas–Kanade (gradient descent) (Lucas & Kanade, 1981)

image registration algorithm using MathWorks® MATLAB platform (Greenberg & Kerr, 2009), the other using a Hidden Markov Model (Dombeck *et al.*, 2007). Although effective, these methods have not been packaged for easy implementation and are reliant on cells remaining in the x- and y- dimensions as it cannot track following movement between z-axes. In cases with z-axis movement, tracking-based methods may be more reliable, and specialist options exist using control theory and machine learning approaches for post-processing (Nguyen *et al.*, 2017), or applied to a motorised stage (Cong *et al.*, 2017; Kim *et al.*, 2017). A MathWorks® MATLAB toolbox, miniscope 1-photon imaging pipeline (MINIPIPE), has been developed to include denoising, motion correction and signal extraction (Lu *et al.*, 2018). MINIPIPE motion correction includes several steps including the Lucas-Kanade and Kanade-Lucas-Tomasi (Lucas & Kanade, 1981; Shi & Tomasi, 1994) trackers, and Log-Demons registration (Vercauteren *et al.*, 2009), and outperforms the Lucas-Kanade, Kanade-Lucas-Tomasi, and NoRMCorr for using sample 2-photon videos (Lu *et al.*, 2018).

Tracking methods specifically designed to be more basic to implement and widely available include plug-ins for image processing packages (Abramoff *et al.*, 2004) such as Trackmate (Tinevez *et al.*, 2017), or Time Series Analyzer (Balaji, 2014).

## Classification

Classification is required to ensure that the quantification can be performed over specific regions of interest, such as for subcellular area, specific cells, or tissue regions. Classification can be achieved through pixel- or object-based segmentation. Pixel-based methods map each pixel to a class according to the spectral similarities. Popular pixel-based methods for calcium image analysis include Maximum Likelihood Classification (MLC) (Malik *et al.*, 2011) or Otsu thresholding to separate ‘light’ and ‘dark’ clustered pixels (Otsu, 1979) as used as part of the SIMA Python package ROI pipeline (Kaifosh *et al.*, 2014).

Object-based segmentation is a two-step process using both spectral and spatial/contextual information to group pixels into objects which are then classified. CaImAn is an open-source package with modules for classification, motion correction, source extraction, and spike deconvolution. The classification method is based on convolutional neural networks (Giovannucci *et al.*, 2019). It was packaged into EZcalcium in an effort to improve usability by providing a GUI in MathWorks® MATLAB (Cantu *et al.*, 2020). However, using limited CaImAn function in EZcalcium does not easily allow for segmentation of more complex structures or large organelles or clusters of cells and is better for somas or smaller, less complex areas. Cellpose is another generalist, deep learning-based segmentation method that uses entirely open source packages in Python with a GUI to aid implementation. There is also a web-based option for testing Cellpose, which makes it very easy to use (Stringer *et al.*, 2020), though it too can be limited at detecting more complex cell shapes such as dendrites and axons.

DenoiSeg is an extension of Noise2Void that offers an end-to-end neural network, which is jointly optimised to

denoise and segment images. The denoising capability is learnt by the self-supervised learning principle that noise2void introduced (Krull *et al.*, 2019). By combining this with a supervised learning approach using a few annotated ground truths of segmentation maps, the final segmentation performance ends up performing better than without co-learning, i.e. having two separate networks perform the respective tasks (Buchholz *et al.*, 2020).

Cell classification methods have been discussed with the conclusion that ‘learning-based methods score among the best-performing methods, but well-optimized traditional methods can even surpass these approaches in a fraction of the time’ (Vicar *et al.*, 2019).

## Quantification

The aim of each step is for signal extraction to allow a quantitative output from the images of calcium signals. The most commonly used measure is the relative fluorescence variation ( $\Delta F/F_0$ ) for classified cells. Packages will therefore either provide this data of the baseline fluorescence ( $F_0$ ) and deviations from baseline ( $\Delta F$ ), for further analysis, or provide a direct plot. Background subtraction may need to be considered as not all packages will take this into account. Multiple methods can be used, including subtracting the intensity values from a region of the image that does not contain  $Ca^{2+}$  indicator from the intensity values in regions of interest. However care should be taken using background subtraction with ratiometric indicators (Shkryl, 2020).  $F_0$  baseline values can be calculated by averaging the values before the onset of stimulation in the same region (Galizia *et al.*, 1999), or by low-pass filtering the signal (Balkenius *et al.*, 2009) (For review (Balkenius *et al.*, 2015).

Signal extraction from single cells can be particularly difficult for *in vivo* recordings due to large background fluxes and high spatial overlaps of cells outside of the focus plane which is further increased in 1-photon compared to 2-photon imaging. Semi-automated ROI analysis (Barbera *et al.*, 2016; Klaus *et al.*, 2017; Pinto & Dan, 2015), principal component analysis independent components analysis (PCA/ICA) (Mukamel *et al.*, 2009), clustering based approaches (like Suite2P; Pachitariu *et al.*, 2017), and constrained nonnegative matrix factorization (CNMF) (Pnevmatikakis *et al.*, 2016) approaches are techniques that have been explored with different strengths for detecting background and spatial overlap. An ‘extended’ CNMF method (CNMF-E) has been developed with an adjusted spatiotemporal background model that outperformed PCA/ICA for the simulated and experimental datasets that were tested (Zhou *et al.*, 2018). For a package method, the toolbox MINIPIPE combines a CNMF (Pnevmatikakis *et al.*, 2016) with additional steps to remove false positives (Lu *et al.*, 2018). CaImAn also builds upon the CNMF algorithm (Pnevmatikakis *et al.*, 2016) to allow it to be fully automated, and CNMF-E for 1-photon endoscopic data (Zhou *et al.*, 2018).

Another feature commonly needed by researchers is timing of neuronal action potentials (APs) or ‘spike detection’ through deconvolution of the extracted signal. A wide range of algorithms can be used as discussed in the results to the

*Spikefinder* challenge (Berens *et al.*, 2018) as there are multiple methods of varying complexity that can be used. EZcalcium directly shows the raw fluorescence, inferred activity and deconvolved neural ‘spiking’, whereby the data can then be exported into file formats for proprietary (.mat, .xlsx) or open (.csv) software programmes for further analysis (Cantu *et al.*, 2020; Giovannucci *et al.*, 2019). The ability to accurately detect spikes requires knowledge of ground truth, usually from electrophysiological recordings. Calcium imaging can be susceptible to variation between neuron type, calcium indicator and its concentration used, the optical resolution, the sampling rate and the noise level. Therefore, it is fundamental to understand how specific indicators react under the given imaging conditions, which cannot be readily generalized across protocols. To try and improve the accuracy of spike detection, a toolkit using a supervised algorithm of spike inference has been developed using a ‘ground truth database’ from a large number of sets of calcium imaging with corresponding electrophysiological measurements (Rupprecht *et al.*, 2021).

## Conclusion

A great number of analysis advancements have been made since calcium imaging was first developed. Popular packages for various steps of the pipeline (Figure 1) include CaImAn, SIMA, Suite2P, and EZcalcium (Cantu *et al.*, 2020; Giovannucci *et al.*, 2019; Kaifosh *et al.*, 2014; Pachitariu *et al.*, 2017). Although these packages are great starting tools for the community, many require programming knowledge in Python or commercial packages such as MathWorks® MATLAB. Many of the available options are only semi-automated and the limited automated options available are often designed for a very limited experimental context and are not actively supported when problems are experienced, e.g. other than for cells of a specific size and shape imaged *in vitro*. EZcalcium is one of the most intuitive options, which has improved the usability of CaImAn, NoRMCorre, but again seems best suited to analyse cell bodies. Suite2P and EZcalcium both attempt to offer an automated pipeline from raw images to spike extraction with no prior programming knowledge required by the user (Cantu *et al.*, 2020; Pachitariu *et al.*, 2017). As both packages are suited to similar experimental data, the choice may be based upon personal preference.

It therefore seems that perhaps some of the biggest advances could be made by designing packages for detecting neuritic structures or organelles and improving the spatial resolution of the analysis to be intracellular, such as has been used for calcium sparks (Berens *et al.*, 2018). Longitudinal tracking of specific cells across imaging sessions also remains a challenge so that individual cells can be identified between multiple imaging sessions. A MathWorks® MATLAB toolkit has been made with reported error rates of < 5 % (Sheintuch *et al.*, 2017); an alternative approach is also available using CaImAn (Giovannucci *et al.*, 2019) though direct comparisons between these methods is difficult without knowing ground truths. Calcium imaging for population activity has also been highlighted as an area that requires further research, particularly when imaging over larger fields of view. Using models specific for neuron types imaged may improve detection of APs, which are commonly under-represented in population activity measurements (Huang *et al.*, 2021). Recent toolboxes with large datasets containing ground-truths may reduce false negatives during analysis (Rupprecht *et al.*, 2021). On the other end of the scale, pipelines for functional imaging in organisms such as zebrafish, *C. elegans* and *Drosophila*, where motion correction is often required and improved analysis for connectomics purposes are much needed.

As the application of machine learning in calcium imaging analysis matures, a higher level of automation and throughput for analysis tasks can be expected to follow. This will be enabled by more generalised and robust machine learning models. The barrier to training and deploying these methods will also reduce as more research is made into few-shot learning (using small training datasets) in addition to training approaches such as self-supervised and unsupervised learning.

## Data availability

No data are associated with this article.

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## References

- Abramoff MD, Magalhaes PJ, Ram SJ: **Image Processing with ImageJ**. *Biophotonics International*. 2004; **11**(7): 36–42.  
[Reference Source](#)
- Balaji J: **Time series analyzer**. 2014; (Accessed: 7 June 2021).  
[Reference Source](#)
- Balkenius A, Bisch-Knaden S, Hansson B: **Interaction of visual and odour cues in the mushroom body of the hawkmoth *Manduca sexta***. *J Exp Biol*. 2009; **212**(Pt 4): 535–541.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Balkenius A, Johansson AJ, Balkenius C: **Comparing Analysis Methods in Functional Calcium Imaging of the Insect Brain**. *PLoS One*. Public Library of Science, 2015; **10**(6): e0129614.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Barbera G, Liang B, Zhang L, *et al.*: **Spatially Compact Neural Clusters in the Dorsal Striatum Encode Locomotion Relevant Information**. *Neuron*. Cell Press, 2016; **92**(1): 202–213.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Berens P, Freeman J, Deneux T, *et al.*: **Community-based benchmarking improves spike rate inference from two-photon calcium imaging data**. *bioRxiv*. 2018; 177956.  
[Publisher Full Text](#)
- Besbeas P, De Feis I, Sapatinas T: **A Comparative Simulation Study of Wavelet**

- Shrinkage Estimators for Poisson Counts.** *Int Stat Rev.* John Wiley & Sons, Ltd. 2004; **72**(2): 209–237.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Blinks JR, Prendergast FG, Allen DG: **Photoproteins as biological calcium indicators.** *Pharmacol Rev.* 1976; **28**(1): 1–93.  
[PubMed Abstract](#)
- Boulanger J, Kervrann C, Bouthemy P, et al.: **Patch-based nonlocal functional for denoising fluorescence microscopy image sequences.** *IEEE Trans Med Imaging.* 2010; **29**(2): 442–454.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Broser PJ, Schulte R, Lang S, et al.: **Nonlinear anisotropic diffusion filtering of three-dimensional image data from two-photon microscopy.** *J Biomed Opt.* International Society for Optics and Photonics, 2004; **9**(6): 1253–1264.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Buades A, Coll B, Morel JM: **A non-local algorithm for image denoising.** *2005 IEEE Computer Society Conference on Computer Vision and Pattern Recognition (CVPR'05).* 2005; **2**(0): 60–65.  
[Publisher Full Text](#)
- Buades A, Coll B, Morel JM: **Non-Local Means Denoising.** *Image Processing On Line.* 2011; **1**: 208–212.  
[Publisher Full Text](#)
- Buchholz TO, Prakash M, Schmidt D: **DenoiSeg: joint denoising and segmentation.** *European Conference on Computer Vision.* 2020; 324–337.  
[Publisher Full Text](#)
- Cai DJ, Aharoni D, Shuman T, et al.: **A shared neural ensemble links distinct contextual memories encoded close in time.** *Nature.* Nature Publishing Group, 2016; **534**(7605): 115–118.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Cantu DA, Wang B, Gongwer MW, et al.: **EZcalcium: Open-Source Toolbox for Analysis of Calcium Imaging Data.** *Front Neural Circuits.* Frontiers Media S.A. 2020; **14**: 25.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Chen TW, Wardill TJ, Sun Y, et al.: **Ultrasensitive fluorescent proteins for imaging neuronal activity.** *Nature.* Nature, 2013; **499**(7458): 295–300.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Cong L, Wang Z, Chai Y, et al.: **Rapid whole brain imaging of neural activity in freely behaving larval zebrafish (*Danio rerio*).** *eLife.* 2017; **6**: e28158.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Coupé P, Yger P, Prima S, et al.: **An optimized blockwise nonlocal means denoising filter for 3-D magnetic resonance images.** *IEEE Trans Med Imaging.* 2008; **27**(4): 425–441.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Denk W, Strickler JH, Webb WW: **Two-photon laser scanning fluorescence microscopy.** *Science.* 1990; **248**(4951): 73–76.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Dombeck DA, Khabbazi AN, Collman F, et al.: **Imaging Large-Scale Neural Activity with Cellular Resolution in Awake, Mobile Mice.** *Neuron.* Elsevier. 2007; **56**(1): 43–57.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Donoho DL: **De-noising by soft-thresholding.** *IEEE Trans Inf Theory.* 1995; **41**(3): 613–627.  
[Publisher Full Text](#)
- Dubbs A, Guevara J, Yuste R: **moco: Fast Motion Correction for Calcium Imaging.** *Front Neuroinform.* Frontiers, 2016; **10**: 6.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Galizia CG, Menzel R, Hollodobler B: **Optical imaging of odor-evoked glomerular activity patterns in the antennal lobes of the ant conopnotus rufipes.** *Naturwissenschaften.* Naturwissenschaften, 1999; **86**(11): 533–537.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Giovannucci A, Friedrich J, Gunn P, et al.: **CaImAn an open source tool for scalable calcium imaging data analysis.** *eLife.* NLM (Medline), 2019; **8**: e38173.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Greenberg DS, Kerr JN: **Automated correction of fast motion artifacts for two-photon imaging of awake animals.** *J Neurosci Methods.* Elsevier, 2009; **176**(1): 1–15.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Grewnig S, Zimmer S, Weickert J: **Rotationally invariant similarity measures for nonlocal image denoising.** *J Vis Commun Image Represent.* 2011; **22**(2): 117–130.  
[Publisher Full Text](#)
- Haykin S, Widrow B: **Least-Mean-Square Adaptive Filters.** *Wiley Online Library.* 2003; 31.  
[Publisher Full Text](#)
- Huang L, Ledochowitsch P, Knoblich U, et al.: **Relationship between simultaneously recorded spiking activity and fluorescence signal in gcamp6 transgenic mice.** *eLife.* eLife Sciences Publications Ltd, 2021; **10**: e51675.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Huisken J, Swoger J, Del Bene F, et al.: **Optical sectioning deep inside live embryos by selective plane illumination microscopy.** *Science.* 2004; **305**(5686): 1007–1009.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Jenkinson M, Bannister P, Brady M, et al.: **Improved optimization for the robust and accurate linear registration and motion correction of brain images.** *NeuroImage.* 2002; **17**(2): 825–841.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kaifosh P, Zaremba JD, Danielson NB, et al.: **SIMA: Python software for analysis of dynamic fluorescence imaging data.** *Front Neuroinform.* Frontiers Research Foundation, 2014; **8**: 80.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Kim DH, Kim J, Marques JC, et al.: **Pan-neuronal calcium imaging with cellular resolution in freely swimming zebrafish.** *Nat Methods.* Nature Publishing Group, 2017; **14**(11): 1107–1114.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kitamura K, Häusser M: **Dendritic Calcium Signaling Triggered by Spontaneous and Sensory-Evoked Climbing Fiber Input to Cerebellar Purkinje Cells *In Vivo*.** *J Neurosci.* Society for Neuroscience, 2011; **31**(30): 10847–10858.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Klaus A, Martins GJ, Paixao VB, et al.: **The Spatiotemporal Organization of the Striatum Encodes Action Space.** *Neuron.* Cell Press, 2017; **95**(5): 1171–1180.e7.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Kreft M, Stenovec M, Zorec R: **Focus-drift correction in time-lapse confocal imaging.** *Ann N Y Acad Sci.* Ann N Y Acad Sci, 2005; **1048**: 321–330.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Krull A, Buchholz TO, Jug F: **Noise2void - learning denoising from single noisy images.** *Proceedings of the IEEE/CVF Conference on Computer Vision and Pattern Recognition.* 2019; 2129–2137.  
[Publisher Full Text](#)
- Lecoq J, Oliver M, Siegle JH, et al.: **Removing independent noise in systems neuroscience data using DeepInterpolation.** *bioRxiv.* Cold Spring Harbor Laboratory, 2020; 2020.10.15.341602.  
[Publisher Full Text](#)
- Lehtinen J, Munkberg J, Hasselgren J, et al.: **Noise2Noise: Learning Image Restoration without Clean Data.** *arXiv:1803.04189v3.* 2018.  
[Reference Source](#)
- Li X, Zhang G, Wu J, et al.: **Reinforcing neuron extraction and spike inference in calcium imaging using deep self-supervised denoising.** *Nat Methods.* 2021.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Lindenbaum M, Fischer M, Bruckstein A: **On Gabor's contribution to image enhancement.** *Pattern Recogn.* 1994; **27**(1): 1–8.  
[Publisher Full Text](#)
- Lu J, Li C, Singh-Alvarado J, et al.: **MIN1PIPE: A Miniscope 1-Photon-Based Calcium Imaging Signal Extraction Pipeline.** *Cell Rep.* 2018; **23**(12): 3673–3684.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Lucas BD, Kanade T: **Iterative image registration technique with an application to stereo vision.** 1981; **2**: 674–679.  
[Reference Source](#)
- Luisier F, Blu T, Unser M: **Image denoising in mixed poisson-gaussian noise.** *IEEE Trans Image Process.* 2011; **20**(3): 696–708.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Malik WQ, Schummers J, Sur M, et al.: **Denoising two-photon calcium imaging data.** *PLoS One.* PLoS One, 2011; **6**(6): e20490.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Miyawaki A, Llopis J, Heim R, et al.: **Fluorescent indicators for Ca<sup>2+</sup> based on green fluorescent proteins and calmodulin.** *Nature.* Nature Publishing Group; 1997; **388**(6645): 882–887.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Moiescu DG, Ashley CC, Campbell AK: **Comparative aspects of the calcium-sensitive photoproteins aequorin and obelin.** *Biochim Biophys Acta.* 1975; **396**(1): 133–140.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Mukamel EA, Nimmerjahn A, Schnitzer MJ: **Automated Analysis of Cellular Signals from Large-Scale Calcium Imaging Data.** *Neuron.* NIH Public Access, 2009; **63**(6): 747–760.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Nguyen JP, Linder AN, Plummer GS, et al.: **Automatically tracking neurons in a moving and deforming brain.** *PLoS Comput Biol.* Edited by E. Dyer. Public Library of Science, 2017; **13**(5): e1005517.  
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Ohkura M, Matsuzaki M, Kasai H, et al.: **Genetically encoded bright Ca<sup>2+</sup> probe applicable for dynamic Ca<sup>2+</sup> imaging of dendritic spines.** *Anal Chem.* American Chemical Society, 2005; **77**(18): 5861–5869.  
[PubMed Abstract](#) | [Publisher Full Text](#)
- Otsu N: **Threshold Selection Method From Gray-Level Histograms.** *IEEE Trans Syst Man Cybern.* 1979; **9**(1): 62–66.  
[Publisher Full Text](#)
- Pachitariu M, Stringer C, Dipoppa M, et al.: **Suite2p: beyond 10,000 neurons with standard two-photon microscopy.** *bioRxiv.* Cold Spring Harbor

Laboratory. 2017; 061507.

[Publisher Full Text](#)

Perona P, Malik J: **Scale-space and edge detection using anisotropic diffusion.** *IEEE Trans Pattern Anal Mach Intell.* 1990; **12**(7): 629–639.

[Publisher Full Text](#)

Pinto L, Dan Y: **Cell-Type-Specific Activity in Prefrontal Cortex during Goal-Directed Behavior.** *Neuron.* Cell Press, 2015; **87**(2): 437–450.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Pnevmatikakis EA, Giovannucci A: **NoRMCorre: An online algorithm for piecewise rigid motion correction of calcium imaging data.** *J Neurosci Methods.* Elsevier B.V. 2017; **291**: 83–94.

[PubMed Abstract](#) | [Publisher Full Text](#)

Pnevmatikakis EA, Soudry D, Gao Y, *et al.*: **Simultaneous Denoising, Deconvolution, and Demixing of Calcium Imaging Data.** *Neuron.* Cell Press, 2016; **89**(2): 285–99.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Rupprecht P, Carta S, Hoffmann A, *et al.*: **A Database and deep learning toolbox for noise-optimized, generalized spike inference from calcium imaging.** *bioRxiv.* 2021.

[Publisher Full Text](#)

Shao L, Yan R, Li X, *et al.*: **From Heuristic Optimization to Dictionary Learning: A Review and Comprehensive Comparison of Image Denoising Algorithms.** *IEEE Trans Cybern.* 2014; **44**(7): 1001–1013.

[PubMed Abstract](#) | [Publisher Full Text](#)

Sheintuch L, Rubin A, Brande-Eilat N, *et al.*: **Tracking the Same Neurons across Multiple Days in Ca<sup>2+</sup> Imaging Data.** *Cell Rep.* Elsevier B.V., 2017; **21**(4): 1102–1115.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Shi J, Tomasi C: **Good features to track.** In *1994 Proceedings of IEEE Conference on Computer Vision and Pattern Recognition.* Publ by IEEE, 1994; 593–600.

[Publisher Full Text](#)

Shkryl VM: **Error correction due to background subtraction in ratiometric calcium measurements with CCD camera.** *Heliyon.* Elsevier Ltd, 2020; **6**(6): e04180.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Silva AJ: **Miniaturized two-photon microscope: Seeing clearer and deeper into the brain.** *Light Sci Appl.* Nature Publishing Group, 2017; **6**(8): e17104.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Stringer C, Wang T, Michaelos M: **Cellpose: A generalist algorithm for cellular segmentation.** *bioRxiv.* 2020; 2020.02.02.931238.

[Publisher Full Text](#)

Thévenaz P, Ruttimann UE, Unser M: **A pyramid approach to subpixel registration based on intensity.** *IEEE Trans Image Process.* 1998; **7**(1): 27–41.

[PubMed Abstract](#) | [Publisher Full Text](#)

Tinevez JY, Perry N, Schindelin J, *et al.*: **TrackMate: An open and extensible platform for single-particle tracking.** *Methods.* Academic Press Inc. 2017; **115**: 80–90.

[PubMed Abstract](#) | [Publisher Full Text](#)

Tsien RY: **New Calcium Indicators and Buffers with High Selectivity Against Magnesium and Protons: Design, Synthesis, and Properties of Prototype Structures.** *Biochemistry.* 1980; **19**(11): 2396–2404.

[PubMed Abstract](#) | [Publisher Full Text](#)

Vercauteren T, Pennec X, Perchant A, *et al.*: **Diffeomorphic demons: efficient non-parametric image registration.** *NeuroImage.* Academic Press, 2009; **45**(1 Suppl): S61–S72.

[PubMed Abstract](#) | [Publisher Full Text](#)

Vicar T, Balvan J, Jaros J, *et al.*: **Cell segmentation methods for label-free contrast microscopy: Review and comprehensive comparison.** *BMC Bioinformatics.* BioMed Central Ltd. 2019; **20**(1): 360.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Wegner FV, Both M, Fink RHA: **Automated Detection of Elementary Calcium Release Events Using the à Trous Wavelet Transform.** *Biophys J.* The Biophysical Society, 2006; **90**(6): 2151–63.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Weigert M, Schmidt U, Boothe T, *et al.*: **Content-aware image restoration: pushing the limits of fluorescence microscopy.** *Nat Methods.* 2018; **15**(12): 1090–1097.

[PubMed Abstract](#) | [Publisher Full Text](#)

Wiener N: **Extrapolation, interpolation, and smoothing of stationary time series: with engineering applications.** Technology Press. 1950.

[Reference Source](#)

Zhang K, Zuo W, Chen Y, *et al.*: **Beyond a Gaussian denoiser: Residual learning of deep CNN for image denoising.** *IEEE Trans Image Process.* 2017; **26**(7): 3142–3155.

[PubMed Abstract](#) | [Publisher Full Text](#)

Zhang K, Zuo W, Zhang L: **FFDNet: Toward a fast and flexible solution for CNN Based image denoising.** *IEEE Trans Image Process.* 2018; **27**(9): 4608–4622.

[PubMed Abstract](#) | [Publisher Full Text](#)

Zhou P, Resendez SL, Rodriguez-Romaguera J, *et al.*: **Efficient and accurate extraction of in vivo calcium signals from microendoscopic video data.** *eLife.* NLM (Medline), 2018; **7**: e28728.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)



# Open Peer Review

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## Version 2

Reviewer Report 16 September 2021

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**Anubhuti Goel** 

Department of Psychology, University of California, Riverside (UCR), Riverside, CA, USA

The authors have addressed my concerns.

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Systems neuroscience, Calcium imaging, mouse behavior, electrophysiology.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 02 September 2021

<https://doi.org/10.5256/f1000research.76662.r92939>

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**Christopher Rowlands** 

Department of Bioengineering, Imperial College London, London, UK

**Isabell Whiteley** 

Department of Bioengineering, Imperial College London, London, UK

We would like to thank the authors for responding to all of our points stated in the first review. While there are still some parts where improvements could still be made, these issues are comparatively minor, and thus we feel this paper now has a more complete review of the steps required to detect calcium signals in neurons.

Overall, this review provides important information for researchers using calcium imaging, and can be used as a resource for finding analysis tools that fit their needs. The new definitions provided give further clarity to the concepts and tools discussed in the paper, and the up to date citations will direct the reader to papers that have applied these tools to their research. After all these changes and additions to the manuscript, we have no further corrections and feel that the paper is ready for indexing.

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Microscopy, instrument development, automation and data analysis, neurophotonics, biophotonics

**We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 27 August 2021

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**William Zeiger** 

Department of Neurology, University of California, Los Angeles (UCLA), Los Angeles, CA, USA

The authors have adequately addressed all suggested critiques. I have no further comments.

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Stroke, neural circuits, two-photon calcium imaging

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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Version 1

Reviewer Report 07 June 2021

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**Anubhuti Goel** 

Department of Psychology, University of California, Riverside (UCR), Riverside, CA, USA

The review by Miranda et al provides an overview of the calcium imaging pipeline and the open source resources available to the scientific community. Overall the review is a helpful resource to anyone aiming to analyze their calcium imaging data. However a clearer understanding of which resource is better suited to which model system or type of analysis will be a helpful addition (maybe some examples will be particularly insightful). The authors mention Suite2P and EZCalcium as the top analysis tools available however it is not clear which one the reader should pick. Furthermore both Suite2P and EZCalcium come with a user interface which means other than downloading either python or MATLAB no programming knowledge is needed. This fact is not clear in the review.

One reference is mentioned as "Balaji, UCLA". The authors should specify if indeed that is a personal communication or perhaps an unpublished tool.

**Is the topic of the review discussed comprehensively in the context of the current literature?**

Yes

**Are all factual statements correct and adequately supported by citations?**

Partly

**Is the review written in accessible language?**

Yes

**Are the conclusions drawn appropriate in the context of the current research literature?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Systems neuroscience, Calcium imaging, mouse behavior, electrophysiology.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 16 Aug 2021

**Miranda Robbins**, MRC Laboratory of Molecular Biology, Cambridge, UK

The review by Miranda et al provides an overview of the calcium imaging pipeline and the open source resources available to the scientific community. Overall the review is a helpful resource to anyone aiming to analyze their calcium imaging data.

*We thank the reviewer for their helpful suggestions for our article.*

However a clearer understanding of which resource is better suited to which model system or type of analysis will be a helpful addition (maybe some examples will be particularly insightful).

*We have included in the introduction information about the methods that may better suit in vitro, versus in vivo imaging methods:*

**"The combination of Ca<sup>2+</sup> indicator and imaging modality used will reflect the properties of the sample and the scientific question, as well as the methodologies available to the researcher. For example, *in vitro* imaging, or *in vivo* invertebrate imaging, may use exogenous or GECIs, imaged using LSM, epifluorescence, 2PM or other fluorescence microscopes depending on the temporal-resolution, timescale of imaging, and thickness of the sample being taken into consideration. Other specialist options for *in vivo* imaging of GECIs are available for imaging in awake and behaving animals including miniaturized forms of 1- or 2- photon endoscopic fluorescence microscopes (miniscopes) for single-cell *in vivo* recordings (Cai et al., 2016; Chen et al., 2013; Silva, 2017)."**

*We have added more information for 1 photon in vivo imaging paragraph in 'motion correction' section.*

*We have added more information to the in vivo quantification section:*

**"Signal extraction from single cells can be particularly difficult for in vivo recordings due to large background fluxes and high spatial overlaps of cells outside of the focus plane. Semi-automated ROI analysis (Pinto and Dan, 2015; Barbera et al., 2016; Klaus et al., 2017), principal component analysis independent components analysis (PCA/ICA) (Mukamel, Nimmerjahn and Schnitzer, 2009), and constrained nonnegative matrix factorization (CNMF) (Pnevmatikakis et al., 2016; Zhou et al., 2018) approaches are techniques that have been explored with different strengths for detecting background and spatial overlap. An 'extended' CNMF method has been developed with an adjusted spatiotemporal background model that outperformed PCA/ICA for the simulated and experimental datasets that were tested (Zhou et al., 2018). For a package method, the toolbox MIN1PIPE combines a CNMF (Pnevmatikakis et al., 2016) with additional steps to remove false positives (Lu et al., 2018)."**

The authors mention Suite2P and EZCalcium as the top analysis tools available however it is not clear which one the reader should pick. Furthermore both Suite2P and EZCalcium come with a user interface which means other than downloading either python or MATLAB no programming knowledge is needed. This fact is not clear in the review.

*We have added the following in the conclusion:*

**"Suite2P and EZcalcium both attempt to offer an automated pipeline from raw images to spike extraction with no prior programming knowledge required by the user (Cantu et al., 2020; Pachitariu et al., 2016). As both packages are suited to similar experimental data, the choice may be based upon personal preference."**

One reference is mentioned as "Balaji, UCLA". The authors should specify if indeed that is a personal communication or perhaps an unpublished tool.

*We understand that this may cause confusion; as this plug-in is not published and we have now*

*added the ImageJ webpage as a reference.*

**Competing Interests:** No competing interests were disclosed.

Reviewer Report 24 May 2021

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**William Zeiger** 

Department of Neurology, University of California, Los Angeles (UCLA), Los Angeles, CA, USA

In their review "Calcium imaging analysis – how far have we come?", Robbins et al. present a concise overview of the basic steps involved in transforming raw microscopy images from calcium imaging experiments to quantifiable data. This is an extremely important topic with broad relevance to many fields. As such, the review should draw considerable interest. The review is well written. It is logically organized, with the analysis pipeline broken down into 4 distinct steps, each of which is discussed separately. The language is easy to follow for a broad audience of varying degrees of expertise. Many of the most popular software implementations are covered at least to some degree. Overall, I think this will be a useful and important review with significant impact.

However, I do have some suggestions that I believe could strengthen the manuscript. My main concern is that the target audience for the review is not clear. Calcium imaging is broadly used in many areas of the life sciences, including both in vitro and in vivo preparations, across multiple species, multiple tissues, and widely varying spatial scales (sub-cellular, cellular, bulk tissue signals). Given that much of the review is devoted to denoising and motion correction (problems which are relatively minor in in vitro preparations with no organismal movement and relatively high signal-to-noise), and that quantification focuses on spike detection, the review feels most well suited to in vivo two-photon imaging applications in the brain. If this was the intention, it would benefit the review to make this more explicit and provide more discussion tailored to this technique, particularly in the quantification section. If this assumption is not correct and the review is meant to be targeted at a more general audience, I would suggest the introduction be expanded to include at least a brief discussion of the types of calcium indicators available (ratiometric, FRET, fluo, GCaMP, etc) and imaging techniques in use (widefield fluorescence, confocal, 2P, miniscopes, etc). This is important for a beginner/generalist audience as the indicator and imaging technique used will strongly influence the subsequent processing pipeline needs.

In addition to this primary concern I have a few other suggestions that would improve the scope of the review:

- The quantification section should be significantly expanded. This step is the shortest in description, yet arguably can be fraught with the most pitfalls to which investigators fall prey.

- CaImAn is included under the “classification” section, but includes modules to do more than just classify, including motion correction and registration across imaging sessions
- Spike detection is mentioned, but it should be made explicit that spike detection is completely dependent on knowledge of ground truth about how specific indicators under specific imaging conditions relate and cannot be readily generalized across labs/indicators/preparations. See this recent preprint (Rupprecht et. al <https://www.biorxiv.org/content/10.1101/2020.08.31.272450v2><sup>1</sup>)
- On a related point about spike detection, the authors may want to include information about the relative sensitivity (or in some cases lack thereof) of calcium indicators for spike detection (see Hunag et. al <https://elifesciences.org/articles/516752>).
- It would be interesting for the authors to comment on 1P miniscope calcium imaging, as this technique is rapidly disseminating and poses specific challenges for imaging analysis. See Lu et. al ([https://www.cell.com/cell-reports/fulltext/S2211-1247\(18\)30826-X3](https://www.cell.com/cell-reports/fulltext/S2211-1247(18)30826-X3))
- Longitudinal tracking of cells across imaging sessions remains a challenge for the field – I am not aware of many algorithms that are widely available to tackle this problem. It could be included in the future directions.
- The Allen institute recently published a preprint on a new denoising technique which looks promising (See Lecoq et. al <https://www.biorxiv.org/content/10.1101/2020.10.15.341602v2><sup>4</sup>)

## References

1. Rupprecht P, Carta S, Hoffmann A, Echizen M, et al.: Database and deep learning toolbox for noise-optimized, generalized spike inference from calcium imaging. *bioRxiv*. 2020. [Publisher Full Text](#)
2. Huang L, Ledochowitsch P, Knoblich U, Lecoq J, et al.: Relationship between simultaneously recorded spiking activity and fluorescence signal in GCaMP6 transgenic mice. *Elife*. 2021; **10**. [PubMed Abstract](#) | [Publisher Full Text](#)
3. Lu J, Li C, Singh-Alvarado J, Zhou Z, et al.: MIN1PIPE: A Miniscope 1-Photon-Based Calcium Imaging Signal Extraction Pipeline. *Cell Reports*. 2018; **23** (12): 3673-3684 [Publisher Full Text](#)
4. Lecoq J, Oliver M, Siegle J, Orlova N, et al.: Removing independent noise in systems neuroscience data using DeepInterpolation. *bioRxiv*. 2020. [Publisher Full Text](#)

## Is the topic of the review discussed comprehensively in the context of the current literature?

Partly

## Are all factual statements correct and adequately supported by citations?

Yes

## Is the review written in accessible language?

Yes

**Are the conclusions drawn appropriate in the context of the current research literature?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Stroke, neural circuits, two-photon calcium imaging

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 16 Aug 2021

**Miranda Robbins**, MRC Laboratory of Molecular Biology, Cambridge, UK

In their review "Calcium imaging analysis – how far have we come?", Robbins et al. present a concise overview of the basic steps involved in transforming raw microscopy images from calcium imaging experiments to quantifiable data. This is an extremely important topic with broad relevance to many fields. As such, the review should draw considerable interest. The review is well written. It is logically organized, with the analysis pipeline broken down into 4 distinct steps, each of which is discussed separately. The language is easy to follow for a broad audience of varying degrees of expertise. Many of the most popular software implementations are covered at least to some degree. Overall, I think this will be a useful and important review with significant impact.

*We thank Dr. Zeiger for his positive comments about our review and interesting literature references that he has provided.*

However, I do have some suggestions that I believe could strengthen the manuscript. My main concern is that the target audience for the review is not clear. Calcium imaging is broadly used in many areas of the life sciences, including both in vitro and in vivo preparations, across multiple species, multiple tissues, and widely varying spatial scales (sub-cellular, cellular, bulk tissue signals).

Given that much of the review is devoted to denoising and motion correction (problems which are relatively minor in in vitro preparations with no organismal movement and relatively high signal-to-noise), and that quantification focuses on spike detection, the review feels most well suited to in vivo two-photon imaging applications in the brain. If this was the intention, it would benefit the review to make this more explicit and provide more discussion tailored to this technique, particularly in the quantification section. If this assumption is not correct and the review is meant to be targeted at a more general audience, I would suggest the introduction be expanded to include at least a brief discussion of the types of calcium indicators available (ratiometric, FRET, fluo, GCaMP, etc) and imaging techniques in use (widefield fluorescence, confocal, 2P, miniscopes, etc). This is important for a beginner/generalist audience as the indicator and imaging technique used will strongly influence the subsequent processing pipeline needs.

*We were indeed targeting the review at a more general reader. We have therefore expanded the regions of the introduction where we mention calcium indicators and imaging techniques as well as including a sentence to discuss differences between in vivo and in vitro samples as follows:*

**“The combination of Ca<sup>2+</sup> indicator and imaging modality used will reflect the properties of the sample and the scientific question, as well as the methodologies available to the researcher. For example, in vitro imaging, or in vivo invertebrate imaging, may use exogenous or GECIs, imaged using LSM, epifluorescence, 2PM or other fluorescence microscopes depending on the temporal-resolution, timescale of imaging, and thickness of the sample being taken into consideration. Other specialist options for in vivo imaging of GECIs are available for imaging in awake and behaving animals including miniaturized forms of 1- or 2- photon fluorescence microscopes (miniscopes) for single-cell in vivo recordings (Chen et al., 2013; Cai et al., 2016; Silva, 2017).**

**As calcium imaging is used across a broad range of samples, from sub-cellular, cellular, networks, bulk tissue dynamics to whole organisms and behaving animals, aspects of this pipeline can vary substantially with no ‘one size fits all’ approach.”**

In addition to this primary concern I have a few other suggestions that would improve the scope of the review:

- The quantification section should be significantly expanded. This step is the shortest in description, yet arguably can be fraught with the most pitfalls to which investigators fall prey.

*We have expanded the quantification section:*

**“Signal extraction from single cells can be particularly difficult for *in vivo* recordings due to large background fluxes and high spatial overlaps of cells outside of the focus plane. Semi-automated ROI analysis (Barbera et al., 2016; Klaus et al., 2017; Pinto & Dan, 2015), principal component analysis independent components analysis (PCA/ICA) (Mukamel, Nimmerjahn, & Schnitzer, 2009), and constrained nonnegative matrix factorization (CNMF) (Pnevmatikakis et al., 2016; Zhou et al., 2018) approaches are techniques that have been explored with different strengths for detecting background and spatial overlap. An ‘extended’ CNMF method has been developed with an adjusted spatiotemporal background model that outperformed PCA/ICA for the simulated and experimental datasets that were tested (Zhou et al., 2018). For a package method, the toolbox MIN1PIPE combines a CNMF (Pnevmatikakis et al., 2016) with additional steps to remove false positives (Lu et al., 2018).”**

- CaImAn is included under the “classification” section, but includes modules to do more than just classify, including motion correction and registration across imaging sessions

*We have clarified this by adding the sentence:*

**“CaImAn is an open-source package with modules for classification, motion correction, source extraction, and spike deconvolution. The classification method is based on convolutional neural networks( Giovannucci et al., 2019).”**



- Spike detection is mentioned, but it should be made explicit that spike detection is completely dependent on knowledge of ground truth about how specific indicators under specific imaging conditions relate and cannot be readily generalized across labs/indicators/preparations. See this recent preprint (Rupprecht et. al <https://www.biorxiv.org/content/10.1101/2020.08.31.272450v2><sup>1</sup>)

*Thank you for bringing this very interesting preprint to our attention. We appreciate this point and we have included the addition:*

**“The ability to accurately detect spikes requires knowledge of ground truth, usually from electrophysiological recordings. Calcium imaging can be susceptible to variation between neuron type, calcium indicator and its concentration used, the optical resolution, the sampling rate and the noise level. Therefore, it is fundamental to understand how specific indicators react under the given imaging conditions, which cannot be readily generalized across protocols. To try and improve the accuracy of spike detection, a toolkit using a supervised algorithm of spike inference has been developed using a ‘ground truth database’ from a large number of sets of calcium imaging with corresponding electrophysiological measurements (Rupprecht et al., 2021).”**

- On a related point about spike detection, the authors may want to include information about the relative sensitivity (or in some cases lack thereof) of calcium indicators for spike detection (see Hunag et. al <https://elifesciences.org/articles/51675><sup>2</sup>).

*We have included a discussion in the conclusion about the sensitivity of spike detection:*

**“Calcium imaging for population activity has also been highlighted as an area that requires further research, particularly when imaging over larger fields of view. Using models specific for neuron types imaged may improve detection of Aps, which are commonly under-represented in population activity measurements (Huang et al., 2021). Recent toolboxes with large datasets containing ground-truths may reduce false negatives during analysis (Rupprecht et al., 2021).”**

- It would be interesting for the authors to comment on 1P miniscope calcium imaging, as this technique is rapidly disseminating and poses specific challenges for imaging analysis. See Lu et. al ([https://www.cell.com/cell-reports/fulltext/S2211-1247\(18\)30826-X](https://www.cell.com/cell-reports/fulltext/S2211-1247(18)30826-X)<sup>3</sup>)

*We have added discussion of miniscopes to Introduction:*

**“Other specialist options for in vivo imaging of GECIs are available for imaging in awake and behaving animals including miniaturized forms of 1- or 2- photon fluorescence microscopes (miniscopes) for single-cell in vivo recordings (Chen et al., 2013; Cai et al., 2016; Silva, 2017).”**

*to the Motion correction section:*

**“A MathWorks® MATLAB toolbox, miniscope 1-photon imaging pipeline (MIN1PIPE), has been developed to include denoising, motion correction and signal extraction (Lu et al., 2018). MIN1PIPE motion correction includes several steps including the Lucas-Kanade and Kanade-Lucas-Tomasi (Lucas and Kanade, 1981; Shi and Tomasi, 1994)**

**trackers, and Log-Demons registration (Vercauteren et al., 2009), and outperforms the Lucas-Kanade, Kanade-Lucas-Tomasi, and NoRMcorre for using sample 2-photon videos (Lu et al., 2018)."**

to the *Quantification* section:

**"For a package method, the toolbox MIN1PIPE combines a CNMF (Pnevmatikakis et al., 2016) with additional steps to remove false positives (Lu et al., 2018)."**

- Longitudinal tracking of cells across imaging sessions remains a challenge for the field – I am not aware of many algorithms that are widely available to tackle this problem. It could be included in the future directions.

*We have added to the discussion on future challenges:*

**"Longitudinal tracking of specific cells across imaging sessions also remains a challenge so that individual cells can be identified between multiple imaging sessions. A MathWorks® MATLAB toolkit has been made with reported error rates of < 5 % (Sheintuch et al., 2017); an alternative approach is also available using CaImAn (Giovannucci et al., 2019) though direct comparisons between these methods is difficult without knowing ground truths."**

- The Allen institute recently published a preprint on a new denoising technique which looks promising (See Lecoq et. al <https://www.biorxiv.org/content/10.1101/2020.10.15.341602v2><sup>4</sup>)

*This is an interesting preprint that does indeed seem promising. It can be seen as an extension of the Noise2void method, which we already touch on. We have added the following sentence to the manuscript:*

**"In another recent method, DeepInterpolation (Lecoq et al., 2020), the need for ground truth training data is avoided by treating the denoising task as a nonlinear interpolation problem. This assumes the data have a sequential component, such that spatiotemporally overlapping features can be exploited."**

**Competing Interests:** No competing interests were disclosed.

Reviewer Report 05 May 2021

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**Christopher Rowlands**

Department of Bioengineering, Imperial College London, London, UK

**Isabell Whiteley**

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The authors present a brief review of processing and analysis methods for calcium imaging data in neuroscience. The article breaks down the processing steps typically required to view calcium signals from neurons, including denoising, motion correction, and classification, and then the quantification of the data. It appears that the primary contribution of the article is to summarize the most recent and up-and-coming methods for the processing of calcium activity in neurons. The abstract describes the intentions of the paper well, bringing the reader up to date on the last 60 years of calcium analysis tools, however several claims are made which are not followed up in the manuscript. For example, the authors do not highlight their preferred methods as they state they will in the abstract, much less provide justification for these choices. The abstract concludes by stating the authors will describe the future needs in the field, and this is successfully done in the conclusion, albeit briefly and without much justification – we would have liked to see some examples of the kinds of groups who might be making progress in these fields, or papers in different fields which might be applicable to calcium activity monitoring.

Overall we find the contents of this review to be beneficial to users applying calcium imaging to their research, however some additional information or clarification needs to be included or clarified. The following corrections should be made to the review before formal 'publication': The description of the purpose and benefits of denoising is well explained at the start of the section. In the following sections of motion correction and classification however, the authors proceed directly to the methods used to perform the task, and while this is comprehensive, they fail to justify why these steps are needed in the calcium processing and analysis pipeline. An 'introduction' sentence or paragraph for each section is needed.

The acronym BAPTA should be defined before it is used.

In the motion-correction section, more distinction should be made between 'camera-based' motion correction, where the whole image is captured simultaneously, and 'scanning-based' motion correction. There are distinct differences in capabilities and limitations for each approach (for example, scanning-based imaging can suffer from 'warping' which affects camera-based imaging much less, but can also sample regions of interest as opposed to the whole image, speeding up imaging to overcome motion artifacts).

The authors successfully reference the original papers for the different approaches to denoising, motion correction, and classification, but they should also include the references for when the techniques were first applied and used in calcium image analysis, as this is the focus of the review.

The standard of presentation is OK, but should really be improved before 'publication'. The first sentence of the abstract is difficult to parse and is not a complete statement. Furthermore, throughout the manuscript there are several difficult-to-parse sentences and some that are ungrammatical (e.g. "to include ratiometric, fluorescence lifetime, or fluorescence intensity, based reporters, and genetically-encoded options [...] alongside dyes", "rendering features to be less defined.", "ND-SAFIR is a powerful method for removing Poisson-Gaussian noise, which is based on non-local means denoising [...] to first use a variance stabilisation step, followed by spatial and temporal patch-based weighted averages of intensity values", "over multiple days. which can be well rectified using standard registration methods" and so on). Finally, the structure could be improved ("Motion correction can be split into two main categories" really should be followed by an explanation of what those two categories are, and background subtraction is alluded to in the "Quantification" section but not elaborated upon).

Figure captions should generally be self-contained and comprehensive – they should explain what the reader is expected to observe/pay attention to, and what conclusions should be drawn. Some more elaboration in the figure caption would therefore be welcome.

Other more minor adjustments that are recommended (but not required) include:  
It would be nice to see a definition of Poisson-Gaussian noise since this is unlikely to be understood by many readers. Similarly clear definition of a ‘patch’ would be helpful, as would explanation of the difference between local and non-local methods.

As stated previously, further expansion of the methods used for the analysis of calcium signals, such as calculation of  $dF/F$  and spike sorting, that are touched on in the quantification section, would be helpful. This section is very brief but would seem to be the section of most interest to the general reader.

The authors state the paper will describe the methods used for calcium imaging analysis however the focus of the paper is more on the pre-processing methods rather than the analysis. It would be nice to elaborate further on what kinds of analyses are performed on the data after pre-processing – how these calcium traces can be used to infer useful neurobiological information.

In summary, this is a brief but well-focussed review on a topic that is of interest to several researchers and thus should be formally published after the necessary revisions have been made.

**Is the topic of the review discussed comprehensively in the context of the current literature?**

Yes

**Are all factual statements correct and adequately supported by citations?**

Yes

**Is the review written in accessible language?**

Partly

**Are the conclusions drawn appropriate in the context of the current research literature?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Microscopy, instrument development, automation and data analysis, neurophotonics, biophotonics

**We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.**

Author Response 16 Aug 2021

**Miranda Robbins**, MRC Laboratory of Molecular Biology, Cambridge, UK

The authors present a brief review of processing and analysis methods for calcium imaging data in neuroscience. The article breaks down the processing steps typically required to view calcium signals from neurons, including denoising, motion correction, and classification, and then the quantification of the data. It appears that the primary contribution of the article is to summarize the most recent and up-and-coming methods for the processing of calcium activity in neurons.

*We thank the reviewers for highlighting areas of improvement in our review article.*

The abstract describes the intentions of the paper well, bringing the reader up to date on the last 60 years of calcium analysis tools, however several claims are made which are not followed up in the manuscript. For example, the authors do not highlight their preferred methods as they state they will in the abstract, much less provide justification for these choices.

*We acknowledge this inconsistency between the abstract and the content of the manuscript. On closer inspection, we have decided that we do not want to explicitly emphasise our personal preferences and justify them, but rather objectively discuss the strengths of the respective methods and leave it to the reader – particularly as the “best” methods will often be dependent upon the experimental methods used by individual researchers. We have therefore removed the mentioning of “our preferred [methods]” in the relevant sentence in the abstract from:*

**“We will discuss some of the most popular, alongside our preferred, methods for calcium imaging”**

The abstract concludes by stating the authors will describe the future needs in the field, and this is successfully done in the conclusion, albeit briefly and without much justification – we would have liked to see some examples of the kinds of groups who might be making progress in these fields, or papers in different fields which might be applicable to calcium activity monitoring.

*We have extended the discussion on future challenges to include:*

**“Longitudinal tracking of specific cells across imaging sessions also remains a challenge so that individual cells can be identified between multiple imaging sessions. A MathWorks® MATLAB toolkit has been made with reported error rates of < 5 % (Sheintuch et al., 2017); an alternative approach is also available using CaImAn (Giovannucci et al., 2019) though direct comparisons between these methods is difficult without knowing ground truths. Calcium imaging for population activity has also been highlighted as an area that requires further research, particularly when imaging over larger fields of view. Using models specific for neuron types imaged may improve detection of APs, which are commonly under-represented in population activity measurements (Huang et al., 2021). Recent toolboxes with large datasets containing ground-truths may reduce false negatives during analysis (Rupprecht et al., 2021).”**

Overall we find the contents of this review to be beneficial to users applying calcium imaging to their research, however some additional information or clarification needs to be included or clarified. The following corrections should be made to the review before formal 'publication':

The description of the purpose and benefits of denoising is well explained at the start of the section. In the following sections of motion correction and classification however, the authors proceed directly to the methods used to perform the task, and while this is comprehensive, they fail to justify why these steps are needed in the calcium processing and analysis pipeline. An 'introduction' sentence or paragraph for each section is needed.

*We have added introductory sentences as follows:*

**"Motion correction is often required for samples due to drift during imaging, or organism movement."**

**"Classification is required to ensure that the quantification can be performed over specific regions of interest, such as for subcellular area, specific cells, or tissue regions."**

The acronym BAPTA should be defined before it is used.

*We have now included this.*

In the motion-correction section, more distinction should be made between 'camera-based' motion correction, where the whole image is captured simultaneously, and 'scanning-based' motion correction. There are distinct differences in capabilities and limitations for each approach (for example, scanning-based imaging can suffer from 'warping' which affects camera-based imaging much less, but can also sample regions of interest as opposed to the whole image, speeding up imaging to overcome motion artifacts).

*We agree with this suggestion to more clearly outline the types of motion correction described. Most importantly, we want to distinguish between motion that occurs in the imaging system (such as the camera-based and scanning-based motion effects mentioned here) and the more complex motion effects that are due to specimen movement. We have added the following to the "Motion correction" section:*

**"Motion correction is often required to ensure consistent image processing across a time stack. We distinguish between two types of motion: (a) drift occurring in the imaging system itself caused by thermal gradients in the microscope, vibrations and mechanical instability (Kreft *et al.*, 2005); (b) subject motion such as fluctuations in the immersion media or the movement of organisms (Jenkinson *et al.*, 2002)."**

The authors successfully reference the original papers for the different approaches to denoising, motion correction, and classification, but they should also include the references for when the techniques were first applied and used in calcium image analysis, as this is the focus of the review.

*We thank the reviewer for this comment. We can appreciate the point that references describing applications of processing methods specific to calcium imaging should be more plentiful. These references are often difficult to find from the existing reviews on calcium imaging and we have therefore tried to add relevant references to this review where possible:*

For anisotropic filters we have added the references: (Broser et al., 2004; Kitamura and Häusser, 2011). For wavelet thresholding methods we have included: (Besbeas, Feis and Sapatinas, 2004; Wegner, Both and Fink, 2006). We have added: "In the context of calcium imaging, local methods have been shown to perform well (Malik et al., 2011).".

For motion correction using rigid methods we have added (Dubbs, Guevara and Yuste, 2016).

In 'Classification' we have added (Malik et al., 2011) as an example of Maximum Likelihood Classification.

While the calcium imaging literature does not have a lot of examples of non-trivial image processing applications, we have augmented the section with a new reference that indeed does: "Malik, Wasim Q., et al. "Denoising two-photon calcium imaging data." PloS one 6.6 (2011)".

The standard of presentation is OK, but should really be improved before 'publication'. The first sentence of the abstract is difficult to parse and is not a complete statement. Furthermore, throughout the manuscript there are several difficult-to-parse sentences and some that are ungrammatical (e.g. "to include ratiometric, fluorescence lifetime, or fluorescence intensity, based reporters, and genetically-encoded options [...] alongside dyes", "rendering features to be less defined.", "ND-SAFIR is a powerful method for removing Poisson-Gaussian noise, which is based on non-local means denoising [...] to first use a variance stabilisation step, followed by spatial and temporal patch-based weighted averages of intensity values", "over multiple days. which can be well rectified using standard registration methods" and so on). Finally, the structure could be improved ("Motion correction can be split into two main categories" really should be followed by an explanation of what those two categories are, and background subtraction is alluded to in the "Quantification" section but not elaborated upon).

*Thank you for highlighting these errors. We have edited the sentences as follows:*

**"Techniques for calcium imaging were first demonstrated in the mid-1970s, whilst tools to analyse these markers of cellular activity are still being developed and improved today"**

**"In the past forty years, the methods available for measuring Ca<sup>2+</sup> fluxes in cells have expanded to include ratiometric, fluorescence lifetime, or fluorescence intensity-based dyes, and genetically-encoded calcium indicators (GECIs) ( Miyawaki et al., 1997; Ohkura et al., 2005)."**

**"... causing features to appear less well defined."**

**"ND-SAFIR is a powerful method for removing Poisson-Gaussian noise. It is based on non-local means denoising using a variance stabilisation step, followed by calculating the spatial and temporal patch-based weighted averages for intensity values."**

**"...view over multiple days, which can be rectified by using standard registration methods."**

**We have deleted the sentence “Motion correction can be split into two main categories” which has been replaced with the introductory sentence on motion correction.**

**We have expanded the sentence on background subtraction to say:**

**“Multiple methods can be used, including subtracting the intensity values from a region of the image that does not contain Ca<sup>2+</sup> indicator from the intensity values in regions of interest. However, care should be taken using background subtraction with ratiometric indicators (Shkryl, 2020). F0 baseline values can be calculated by averaging the values before the onset of stimulation in the same region (Galizia, Menzel and Holldobler, 1999), or by low-pass filtering the signal (Balkenius, Bisch-Knaden and Hansson, 2009)(For review Balkenius, Johansson and Balkenius, 2015). “**

Figure captions should generally be self-contained and comprehensive – they should explain what the reader is expected to observe/pay attention to, and what conclusions should be drawn. Some more elaboration in the figure caption would therefore be welcome.

*We have adapted the Figure caption as follows:*

**“Figure 1. The steps of a common pipeline for calcium imaging analysis can be subdivided into three areas before quantitative analysis is performed. Denoising is an optional step that can help to improve signal-to-noise and enhance features. Motion correction may be necessary in cases of drift or movement. Classification can select regions of interest for which quantitative analysis is performed.**  
”

Other more minor adjustments that are recommended (but not required) include: It would be nice to see a definition of Poisson-Gaussian noise since this is unlikely to be understood by many readers. Similarly clear definition of a ‘patch’ would be helpful, as would explanation of the difference between local and non-local methods.

*Thank you for these suggestions. We agree that these things were not necessarily clear to the general reader, so we have incorporated various changes to address these points. First of all, definitions of the noise sources in fluorescence microscopy and a reference that deals more thoroughly with the subject:*

**“Live-cell imaging generally requires short exposure times and low excitation power to limit the effects of photo-toxicity and photo-bleaching. This leads to image degradation in the form of noise. In fluorescence microscopy the two prevalent noise sources are Poisson noise and Gaussian noise. Poisson noise is caused by the stochastic and discrete nature of photon emission and tends to be dominant at low light levels, whereas Gaussian noise describes the intrinsic thermal and electronic fluctuation in the image sensor (Luisier et al., 2011). “**

*Secondly, non-local methods are now described as using global information, a distinction to local methods that we believe should now make the difference clear. Lastly, we have included a definition of patch as a block of pixels representing a subregion of an image.*



As stated previously, further expansion of the methods used for the analysis of calcium signals, such as calculation of  $dF/F$  and spike sorting, that are touched on in the quantification section, would be helpful. This section is very brief but would seem to be the section of most interest to the general reader.

The authors state the paper will describe the methods used for calcium imaging analysis however the focus of the paper is more on the pre-processing methods rather than the analysis. It would be nice to elaborate further on what kinds of analyses are performed on the data after pre-processing – how these calcium traces can be used to infer useful neurobiological information.

*We have included the following in the 'quantification' section:*

**"Packages will therefore either provide this data of the baseline fluorescence ( $F_0$ ) and deviations from baseline ( $\Delta F$ ), for further analysis, or provide a direct plot."**

**"Signal extraction from single cells can be particularly difficult for in vivo recordings due to large background fluxes and high spatial overlaps of cells outside of the focus plane which is further increased in 1-photon compared to 2-photon imaging. Semi-automated ROI analysis (Barbera et al., 2016; Klaus et al., 2017; Pinto & Dan, 2015), principal component analysis independent components analysis (PCA/ICA) (Mukamel et al., 2009), clustering based approaches (like Suite2P; Pachitariu et al., 2016), and constrained nonnegative matrix factorization (CNMF) (Pnevmatikakis et al., 2016) approaches are techniques that have been explored with different strengths for detecting background and spatial overlap. An 'extended' CNMF method (CNMF-E) has been developed with an adjusted spatiotemporal background model that outperformed PCA/ICA for the simulated and experimental datasets that were tested (Zhou et al., 2018). For a package method, the toolbox MIN1PIPE combines a CNMF (Pnevmatikakis et al., 2016) with additional steps to remove false positives (Lu et al., 2018). CaImAn also builds upon the CNMF algorithm (Pnevmatikakis et al., 2016) to allow it to be fully automated, and CNMF-E for 1-photon endoscopic data (Zhou et al., 2018)."**

**"The ability to accurately detect spikes requires knowledge of ground truth, usually from electrophysiological recordings. Calcium imaging can be susceptible to variation between neuron type, calcium indicator and its concentration used, the optical resolution, the sampling rate and the noise level. Therefore, it is fundamental to understand how specific indicators react under the given imaging conditions, which cannot be readily generalized across protocols. To try and improve the accuracy of spike detection, a toolkit using a supervised algorithm of spike inference has been developed using a 'ground truth database' from a large number of sets of calcium imaging with corresponding electrophysiological measurements (Rupprecht et al., 2021)."**

In summary, this is a brief but well-focussed review on a topic that is of interest to several researchers and thus should be formally published after the necessary revisions have been made.

*We thank you for your positive comments and helpful additions to our article.*

**Competing Interests:** No competing interests were disclosed.

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