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Identification and quantification of neuronal ensembles in optical imaging experiments

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Abstract

Recent technical advances in molecular biology and optical imaging have made it possible to record from up to thousands of densely packed neurons in superficial and deep brain regions *in vivo*, with cellular subtype specificity and high spatiotemporal fidelity. Such optical neurotechnologies are enabling increasingly fine-scaled studies of neuronal circuits and reliably co-active groups of neurons, so called ensembles. Neuronal ensembles are thought to constitute the basic functional building blocks of brain systems, potentially exhibiting collective computational properties. While the technical framework of *in vivo* optical imaging and quantification of neuronal activity follows certain widely held standards, analytical methods for study of neuronal co-activity and ensembles lack consensus and are highly varied across the field. Here we provide a comprehensive step-by-step overview of theoretical, experimental, and analytical considerations for the identification and quantification of neuronal ensemble dynamics in high-resolution *in vivo* optical imaging studies.

Graphical abstract

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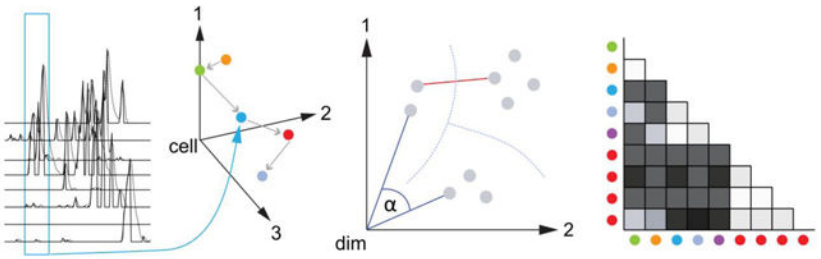
Author contributions

M.W. and J.PH. both conceptualized, wrote, discussed, and edited the manuscript. For figure 1G, data processing was carried out by J.P.H. using custom MATLAB® (MathWorks) code (see also Hamm et al., 2020). For figure 1 A, B, H (t-SNE density plots), and table 2 (depictions at section III, IV, V), data processing was carried out by M.W. using custom MATLAB® (MathWorks) code (see also Wenzel et al., 2019a). For figure visualizations, Adobe Illustrator® and Affinity Designer® were used. Both authors are independent principal investigators with individual funding resources.

Competing Interests Statement

The authors have no competing interests to declare. This statement is also included in the main manuscript, after the acknowledgments section.



Introduction

Neuronal ensembles are thought to represent basic building blocks of cortical function. Ever since Ramon y Cajal's disciple Lorente de Nó first hypothesized in the 1930s that instead of single neurons, the cooperative activity of afferent groups of neurons would trigger a response in effector neurons (Lorente de No, 1938), and ever since Donald J. Hebb cast these groups in the term "cell assemblies" (also commonly referred to as "ensembles"), and postulated that they would represent the basic units of perceptive integration (Hebb, 1949), their identification and characterization has become a main ambition of modern neuroscience.

In support of Nó's and Hebb's assembly hypothesis, influential theoretical considerations suggest that neural networks hold emergent collective computational abilities (Hopfield, 1982), and that population coding displays increased robustness in comparison to single cell information transmission (Kampa et al., 2011; Pouget et al., 2000). Inversely, a neural system relying on single cells rather than cooperative networks would be prone to transmission failure, or loss of information in the case of tissue damage (Buzsáki, 2010; Pouget et al., 2000).

While the body of theoretical literature on the function of neuronal assemblies experienced steady growth (Abeles, 1991; Braitenberg, 1978; Hopfield, 1982; Little, 1974; Palm et al., 2014), experimental investigations of neuronal ensembles in living tissue were however hindered by technical infeasibilities for most of the 20th century. Early studies of scalp level electrical brain activity with electroenchalography (EEG) first identified that brain networks exhibit emergent level rhythms or oscillations (Berger, 1929). Although brain rhythms recorded with EEG likely reflect the collective activity of spatially distributed ensembles (Buzsáki, 2010), they are defined in time (e.g. the 10Hz "alpha" rhythm) and lack neuronal resolution, representing a powerful but indirect means of ensemble measurement and quantification. An enormous change in experimental accessibility of local neural circuits was achieved with the advent of synaptic resolution biological imaging techniques, which allowed the simultaneous recording of densely packed local neuronal populations with unparalleled anatomical precision (Denk et al., 1990; Yuste and Denk, 1995). Since then, rapid progress in optical imaging (Duemani Reddy et al., 2008; Nguyen et al., 2001; Nikolenko et al., 2008; Yang and Yuste, 2017), molecular engineering of fluorescent indicators of neural activity (Mollinedo-Gajate et al., 2019), increasingly fine-scaled methods for manipulating brain activity (Carrillo-Reid et al., 2017; Deisseroth, 2011; Hess et al., 2014), and particularly computer technology and data science (Hu et al., 2018;

Paninski and Cunningham, 2018), have enabled the direct recording of spatially distributed neural activity patterns with single cell resolution, giving rise to fascinating new insights into the functional role of neuronal ensembles in basic brain function.

For example, initial *in vitro* calcium imaging studies in cortical sections (brain slices) identified that spontaneous activity is outlined by a limited set of spatiotemporal coactivity patterns, suggestive of cooperative activity in local neuronal ensembles (Cossart et al., 2003; Ikegaya et al., 2004). *In vivo* two-photon calcium imaging (2P-Ca^{2+}) studies have subsequently demonstrated similar spatiotemporal patterns, reactivating and explaining perception and behavior at greater than chance level in awake mouse visual cortex (Hamm et al., 2017; Miller et al., 2014), auditory cortex (Xin et al., 2019), parietal (Morcos and Harvey, 2016), and frontal cortices (Liang et al., 2018; Siniscalchi et al., 2019), together both highlighting robustness of the observation as well as suggesting a computational relevance of ensemble patterns. Elegant two-photon optogenetics experiments have demonstrated that activating even one or two neurons in a previously identified ensemble reactivates the rest of the group (suggestive of “pattern completion”) and influences behavior (Carrillo-Reid et al., 2019), confirming that ensemble activations could represent cortical attractors forming the building blocks of perception and thought (Carrillo-Reid and Yuste, 2020).

Recent evidence from cellular resolution imaging studies further points towards ensemble dysfunction (e.g. aberrant ensemble size or number by a given local volume, hyperactivity, unphysiological synchronization, diminished feature discrimination, temporal instability, dyscontextual coding such as erroneous novelty detection, or functional microcircuit break-down due to e.g. depolarization block) as a hallmark of various neurological disorders such as epilepsy (Jayant et al., 2019; Lillis et al., 2015; Liou et al., 2018; Muldoon et al., 2015; Wenzel et al., 2019a), neurodegenerative disease (Jáidar et al., 2010; Kuchibhotla et al., 2014; Marinković et al., 2019; Reznichenko et al., 2012), or psychiatric disorders (Hamm et al., 2020, 2017; Zaremba et al., 2017). In particular circumstances such as general anesthesia, reversible neuronal ensemble dysfunction may even be a desired effect supporting medically induced loss of consciousness (M. Wenzel et al., 2019). Collectively, these data under physiological conditions, and in the context of neuropsychiatric dysfunction, indicate that neuronal ensembles may comprise a practical target of study for developing a new generation of circuit-based neurotherapies.

Despite this proliferation of findings in multineuronal optical imaging studies, which point to the prevalence and importance of ensemble activity in brain circuits, the analytical methods of study of ensemble activity are highly varied. Indeed, nearly all of the above mentioned modern imaging studies have reported different algorithms and measures, although, importantly, there is a flow of considerations common to all such endeavors: preprocessing steps (going from images to a “raster plot”; from activity to a co-activity matrix), statistics for determining ensemble activity, algorithms for identifying reliable ensembles, statistics for discerning group-level phenomena from chance-level coincident coactivation, and, most importantly, a quantitative metrics for testing time, space, or conditional difference hypotheses. In this paper, we discuss each step in detail, providing not definitive presciptions but a range of potential options, couched in technical and theoretical

considerations. This is summarized in Table 1. We expect that these analysis steps and, importantly, metrics will become standardized as ensemble phenomena are better understood. Perhaps to the point that electrophysiology has “membrane resistance” and “rheobase”, optical imaging of ensemble activity will gain similar metrics.

What is meant by an ensemble?

There is no widely accepted formal definition of neuronal “ensemble”, and many laboratories have used different terms to describe the patterned activity they observe in neural population recordings. The result is that the field is flush with different operational definitions as well as different nomenclature, including reverberations, volleys, pools, assemblies, ensembles, groups, flashes, songs, synfire chains, phases, states, or attractors. For instance, the term “engram” refers a neuronal ensemble with a stable activation pattern (typically in the hippocampus), which is causally linked to a memory, activating during encoding and retrieval of the memory trace (Josselyn and Tonegawa, 2020). However, at the heart of all these different descriptions lies the assumption that the collective function of brain systems, which give rise to mental operations, consists of groups of neurons whose co-activity dynamics bear collective properties which are emergent: the effects and consequences, within brain systems and on behavior, of the co-activity of the whole is greater than the sum of its parts. The implications and bases of this assumption are philosophical in nature, and, depending on the goals of the study, the interested scientist may remain agnostic while still contributing to the neuroscientific enterprise. A thorough discussion of the nature, mechanisms, and functional consequences of ensemble activity is beyond the scope of this paper, and the reader is directed to more theoretically aimed reviews (Carillo-Reid et al., 2020). Here we focus more on practical considerations for the experimenter when recording neural populations.

General considerations

There are three common operational approaches for defining a neuronal ensemble: i) reader-centric, ii) input-centric, or iii) condition-centric (Buzsáki, 2010). According to each of these approaches, an ensemble might be identified experimentally by i) the activity of a set of neurons that can elicit an output (e.g. in an effector neuron, or behavior), ii) group-level responses to a defined stimulus (neural or external), or iii) group-level activity with respect to a specific experimental condition. The mentioned operational approaches are logically connected, yet depending on the framework of the respective experiment, their individual pursuit may be infeasible (e.g. the effector neuron of a recorded group of neurons may not be identifiable). Thus, usually one of the three approaches is chosen when the experimental framework is designed. For imaging experiments, this also implies that it is not generally necessary to know a priori, if the recorded ensemble members are synaptically connected. At first, one would need to decide whether multineuronal activtiy is non-random with respect to specific outputs, inputs, or circumstances, and thus, an ensemble.

Another general aspect of a neuronal ensemble is that it never comprises all, but rather few neurons of a neuronal system (Carrillo-Reid et al., 2019; Laughlin and Sejnowski, 2003; Miller et al., 2014; Prut et al., 1998). This fact, and the common assumption that neuronal

ensembles bear emergent functional properties, also imply that neuronal systems inherently comprise a set of ensembles (Palm, 1981). Importantly, the aggregate activity of ensembles enable higher accuracy of prediction due to smaller activity variance in comparison to single cells (Pouget et al., 2000). Inversely, individual neurons not only are stochastically active but also may be more promiscuous in terms of what they encode (Aronov et al., 2017; Mao et al., 2001; Miller et al., 2014), participating in more than one ensemble (Cai et al., 2016; Mao et al., 2001). By analogy, understanding a book by reading individual letters vs whole words; letters occur in many words, and are essential units, but the words are the functional building blocks. An important caveat to consider is whether ensemble activity is more reliable because it represents a truly emergent functional unit (like a *word*), or simply because the spontaneous uncorrelated activity of neurons, or "noise", cancels out when more cells are included in the "ensemble" or group-level predictor.

Temporal parameters

Rooted in de Nó's and Hebb's assembly hypothesis, a growing body of theoretical and experimental literature indicates that the activity of individual neurons that participate in a given ensemble must be orchestrated in time (Buzsáki, 2010). In this context, the terms coactivity, or synchrony, are often used. However, what may appear to be synchronous in imaging with e.g. Ca^{2+} indicator kinetics ranging in the tens to hundreds of milliseconds (Carrillo-Reid et al., 2017; Chen et al., 2013; Dana et al., 2019), might be asynchronous in techniques with millisecond temporal resolution (most prominently electrophysiology) (Diba et al., 2014). A practical approach to this issue would be to ask, which degree of co-activity allows for the effective integration of incoming afferent information (Buzsáki, 2010). While there is no definitive answer to this question, experimental studies have shown that the firing of an individual hippocampal neuron is most predictable from the firing of other neurons participating in the same ensemble within a 10–30 ms time window (Harris et al., 2003). Interestingly, this time window roughly corresponds to the membrane time constant of pyramidal neurons (Harris et al., 2003; Koch et al., 1996), as well as the cycle of beta-gamma band oscillations that are known to temporally organize feed-forward information processing in local cortical circuits (Buzsáki and Draguhn, 2004), especially as controlled by parvalbumin-positive interneurons (Cardin et al., 2009).

Further, with Ca^{2} imaging, it is possible that methodology will identify an ensemble that is "synchronous" at one scale (100 ms) which actually is a fast bout of reliable sequential activity, which is not strictly "synchronous" at fine scales (10 ms) but is nevertheless temporally structured (Luczak et al., 2009). This sequential activation of neurons in an ensemble might be outside of the scope for Ca^{2+} imaging, but the ability to identify the spatial aspects of this ensemble remains, allowing researchers to still characterize, identify, and quantify meaningful features of the ensemble (e.g. its spatiotemporal reliability within and across readers, inputs, or conditions).

Spatial parameters

Neuroanatomical, physiological, and theoretical evidence suggests that neuronal ensembles are mostly local entities (Scannell et al., 1995; Schüz et al., 2006). The vast majority of

neuronal connections in the neocortex have been shown to be local, there is evidence of local specialization in brain function, and theoretical considerations indicate that a cortical organization of parallel computing using a community structure increases the computational ability of the overall system (Sporns and Betzel, 2016). Indeed, a local ensemble organization of the cortex allows optimization of neuronal wiring, and thus space occupied by neuronal wiring (Chklovskii et al., 2002). To that same end, and the fact that neurons can participate in multiple ensembles (Cai et al., 2016; Mao et al., 2001; Miller et al., 2014), a recent study has also shown that ensembles can locally overlap in space even though encoding a different contextual memory (Cai et al., 2016). The spatial columnar organization of the cortex is a piece of luck for cellular resolution imaging studies, as (at least currently) the compromise of acquisition rate and sampled area still usually constrains neuronal imaging to comparably small imaged fields of view (typically not exceeding 800×800 microns, for a single focal imaging plane). Still, ensemble synchrony among distributed cortical networks, between columns and across brain regions, is undoubtedly significant as well (Engel et al., 1991), and some recent techniques involving two-photon random access mesoscopes (Sofroniew et al., 2016) have demonstrated success in measuring large (>4×4 mm), distributed brain networks (~3000 neurons) with adequate temporal resolution (>2 Hz).

Optical imaging as a powerful tool to study neuronal ensembles

While in comparison to electrical recordings, neural population imaging still heavily depends on indirect proxies for neural firing (most notably Ca^{2+} influx), and displays comparably poor temporal resolution, several key features make high resolution imaging an ideal tool to identify, and characterize neuronal ensembles in the alive brain. First, it allows recordings at near-real world density (Figure 1 A), which means that it enables the recording of many neurons packed together in dense local networks yet distributed over functionally relevant local volumes (see above). In comparison to single unit recordings, where the fidelity of assignment of extracellularly recorded action potentials to individual units strongly depends on the distance of individual neurons to the respective electrode and spike sorting algorithms (Pedreira et al., 2012), optical imaging allows for the unambiguous distinction of even immediately neighboring cells across the imaged field of view in two or three-dimensions (at the cost of recording speed), and simultaneous recordings of various molecularly labeled neuronal subtypes (e.g. parvalbumin-positive interneurons), which may carry distinct computational or disease related relevance (Baird-Daniel et al., 2017; Cammarota et al., 2013; He and Huang, 2018; Liou et al., 2018; Menendez de la Prida and Trevelyan, 2011; Sessolo et al., 2015; Trevelyan et al., 2006; Wenzel et al., 2019a). To this end, high resolution imaging enables a fine-scaled spatial quantification of neuronal ensemble dynamics within and across cortical layers (Andermann et al., 2013; Fang and Yuste, 2017; Fino and Yuste, 2011; Karnani et al., 2016; Wenzel et al., 2017), or their spontaneous, or stimulus-driven spatial overlap (Cai et al., 2016; Miller et al., 2014).

Another advantage of optical imaging in comparison to electrophysiological measurements is that an optically identified set of individual neurons, even synapses, can be repeatedly and reliably measured across weeks, even months (Holtmaat et al., 2009; Wenzel et al., 2019b; Yang et al., 2009), whereas in chronic *in vivo* electrode array recordings it remains

challenging to stably record from an initially identified set of single units across longer time spans (Niediek et al., 2016; Sharma et al., 2015). Finally, optical imaging also allows the reliable surveillance of neural inactivity, and activity dynamics under unphysiological circumstances such as epileptic seizures, where rapid changes in extracellular action potential shapes in the ictal core complicate reliable single unit activity tracking (Merricks et al., 2015). A comprehensive overview of commonalities and differences of high resolution optical imaging and electrophysiology in the context of ensemble studies is given in Table 1.

Choice of indicator and imaging technique

As imaging technology and molecular engineering of fluorescent markers of neuronal activity are advancing rapidly, a thorough review of the available indicators must be focused and timely. The interested reader is referred elsewhere (Bando et al., 2019a; Mollinedo-Gajate et al., 2019), as such a survey is outside the scope of this review. For the purposes of ensemble analysis, the choice of the fluorescent indicator, imaging technique, and experimental framework will always strongly depend on the scientific question asked and, likewise, will strongly influence the analytical approach adopted for addressing ensemble-level questions. Thus, a consideration of certain charachteristics of the chosen indicator, and its limitations, is a necessary step.

With respect to fluorescent indicators for measuring neuronal firing, green (GFP) or red-shifted (RFP) fluorophores coupled to genetically encoded calcium indicators have become the standard tools for neural population recoding *in vivo* today, owing to their superb signal to noise ratio, indicator kinetics allowing inference of spike rate, high photo-stability, and availability in transgenic mouse lines (Chen et al., 2013; Dana et al., 2019, 2018, 2014; Mollinedo-Gajate et al., 2019). On the flipside, Ca^{2+} indicator kinetics are slow in comparison to underlying neurobiological activity, as rapid neuronal action potentials (1ms) are monitored indirectly through measuring slower consequent Ca^{2+} influx across a neuron's membrane, and hyperpolarizing and sub-threshold depolarizing neural activity cannot be adequately captured by Ca^{2+} sensors (Knöpfel and Song, 2019). A highly sensitive indicator like GCaMP6s would enable the researcher to optimally detect *whether* a spiking event occured, but the ability to know how many spikes and the exact temporal pattern of said spikes outside of a ~200 ms window is absent. Faster indicators (e.g. GCaMP6f) can shorten this window but may come with losses in sensitivity when imaging larger populations *in vivo*, as signal-to-noise ratios are lower. More dense temporal sampling can patrially mitigate this potential loss in signal, yet may come at other costs, such as spatial scope or tissue damage, depending on the imaging technique (1- vs. 2-photon) and other factors (in vivo vs. in vitro, expression levels, etc.). This limitation does not necessarily affect whether and how coactive ensembles can be identified, but, as mentioned above, reliable rapid sequential activations within these ensembles within short time windows would be missed.

In the ideal case, membrane potential changes and neural firing would be imaged directly, and at millisecond precision. Developing such tools is an active field of intense ongoing research in current neuroscience (Bando et al., 2019a; Knöpfel and Song, 2019). However, even though recently progress has been made in the development of genetically encoded voltage indicators (Abdelfattah et al., 2019; Bando et al., 2019b; Kannan et al., 2018; Xu et

al., 2017), these tools still suffer from a critically low signal to noise ratio particularly in *in vivo* experiments, and unfavorable photo-stability properties limiting their use for experimental frameworks that require imaging sessions exceeding 10–15 minutes.

Whether 1-photon or 2-photon imaging should be used to study neuronal ensemble activity will again depend on the scientific question asked. Currently, in vivo two-photon imaging remains the gold standard for synaptic resolution imaging (Holtmaat et al., 2009; Lu et al., 2020; Silva, 2017; Turcotte et al., 2019), imaging of axonal projections for circuit analyses (Broussard et al., 2018; Glickfeld et al., 2013; Hamm et al., 2018), multi-color and volumetric imaging (Cheng et al., 2011; Han et al., 2019; Meng et al., 2019; Yang et al., 2016), and simultaneous cellular resolution imaging and optical manipulation (Carrillo-Reid et al., 2019, 2017; Nikolenko et al., 2008; Yang et al., 2018). On the other hand, one-photon wide-field imaging offers comparably higher temporal resolution per relative field of view. With the development of low-cost tools, and deconvolution and demixing algorithms (Pnevmatikakis et al., 2016), one-photon imaging of neuronal poplulations has become not only feasible, but has found widespread adoption in experiments in freely moving animals (Flusberg et al., 2008; Ghosh et al., 2011, Cai et al., 2016). Yet, separating signals from neighboring neurons is crucial for studying ensembles, and this still poses a challenge for one photon imaging. Recent advances in soma-restricted expression of fluorescent calcium indicators may however alleviate this challenge in the near future (Shemesh et al., 2020). For any automated scoring approach, the experimentor is strongly encouraged to manually verify the separation of neuronal signals, as well as confirm the veracity of extracted activity traces of individual neurons, prior to rigorous ensemble study.

Thus, for some experimental purposes, such as passive ensemble identification and quantification of single neuronal populations from local somata, one- and two-photon approaches may be roughly equivalent (ease of surgery and cost notwithstanding). If, for example, the experimental question requires ensembles to be manipulated optically, measured across multiple layers of tissue, or quantified at the level of axonal projections, an *in vivo* two-photon approach may be indicated. For an extensive comparison of current advanced imaging techniques, the reader may be referred to a recent in-depth review by Yang and Yuste (Yang and Yuste, 2017).

From images to activity

Extracting fluorescent signals (e.g. Ca^{2+} transients) from neuronal populations constitutes a critical step on the path from acquired images to ensemble analysis. Since it represents an early and seemingly straightforward part of data processing, the influence of deliberate decisions and quality control on the study of ensembles can be overlooked. An extra portion of care is necessary to make sure that the extracted signals correspond faithfully and reliably to the underlying neuronal activity, especially to the extent that it matches the goals of the end analysis (e.g. spikes, bursts, currents, etc). Prior to extracting neuronal signals, one first needs to ascertain that the imaged field of view (FOV) was spatially stable across the entire recording period, as e.g. locomotion-associated non-rigid distortions in the horizontal imaging plane, or motion of the field of view orthogonal to the imaging axis (e.g. FOV dipslacements) may lead to the loss of identified neuronal structures such as somata or

synapses resulting in spurious activity traces and co-activity estimates from distributed regions of interested (ROIs). Although motion correction algorithms have been developed that can rectify rigid (Dubbs et al., 2016; Thévenaz et al., 1998) and to some extent non-rigid (Pnevmatikakis and Giovannucci, 2017) pixel displacements (translation) somewhat parallel to the imaging plane, no algorithm can correct FOV displacements where ROIs leave or enter the FOV.

Once a stable ROI is identified, the method of scoring of neuronal activity from fluorescence traces has no clear standard in the literature. Removing the fluorescence of neighboring neuropil or cell-bodies, or from field-wide noise, using e.g. halo-subtraction or other deconvolution techniques is highly recommeneded, as these signals can induce spurious correlations among non-coactive neurons. Beyond this, we highlight a few points of consideration that can have a significant impact on ensemble analysis. Classically, the calculation of momentary change (delta; Δ) in fluorescence divided by baseline fluorescence (Δf/f) has been a standard in identifying cell activity (Figure 1 B). In calculating Δf/f, decisions must be made by the experimenter with regards to i) what constitutes the “baseline” and ii) in what time period change of fluorescence is calculated within (what is “momentary”). Regarding the first decision point, a mean fluorescence (f) from a period with no apparent calcium transients surrounding a given time point should be used, but, importantly, any division by baseline-f might be inappropriate when indicators (e.g. GCaMP6/7) or preparations (transgenic mice) are used where baseline fluorescence is very low (Chen et al., 2013). That is, in recordings with minimized background noise, short baseline periods with verly low average f-values could lead to denominators which, although seemingly equivalent across cells to the naked eye, could substantially affect estimates of neuronal activity (e.g. Δf/f differing 10-fold in cases of f=1.0 vs f=0.1). Alternatively, division by a whole-timecourse standard deviation, median, or maximum might prove more appropriate, especially in cases where spikes cannot be reasonably deconvolved from Δf/f.

Regarding the second decision point, basic data processing steps prior to ensemble analysis should involve some exclusion of signal decay periods (Figure 1 B) (Hamm et al., 2017; M. Wenzel et al., 2019), as indicator decay periods do not represent neuronal firing, but simple inactivation kinetics of the fluorescent sensor. As for example with Ca^{2+} indicators, such decay times may range in the hundreds of milliseconds (Chen et al., 2013; Dana et al., 2019). The potential for assessing spurious or unintended ensemble coactivity is therefore increased if signal decay periods are counted as “activity” in the analysis. Focusing on only the positive first-derivative of fluorescence traces mitigates this shortcoming, and allows one to focus on neurons which show f-increases (e.g. activity) in the same time window (Luis Carrillo-Reid et al., 2015; Hamm et al., 2020; Hamm and Yuste, 2016). However, doing so may require some smoothing of the fluorescene traces (e.g. using conservative filtering such as locally weighted regression smoothing; LOWESS) as well as the use of indicators with a high-saturation point and linearity within a large dynamic range (as increases in fluorescence during a decay should be registered equally as those appearing during baseline).

As with recent fluorescent sensors such as GCaMP6 or 7, duplets, or even single action potentials can be detected with high fidelity (Dana et al., 2019), basic amplitude thresholding of recorded transients represents a reasonable way to reduce noise in the

recorded population activity. The same applies to the exclusion of tissue pulsation artefacts and changes of fluorescence close to the baseline fluorescence that do not reflect neural firing. Somewhere along the way, processing population activity usually requires a decision about whether neuronal spiking is inferred from the continuous signal (Figure 1 B), or if the continuous signal itself is used for analysis. It is important to keep in mind that due to the comparably slow indicator kinetics, inferring spike times from calcium transients remains challenging, especially when the firing rate of a recorded neuron is high (Berens et al., 2018). Ground-truth determination of how Δf/f actually translates to spiking is highly indicated, ideally with electrophysiology and imaging of individual neurons. Yet again, even so, the translation of one neuron's ground truth firing rate in relationship to its calcium signal might not necessarily hold true for all other imaged neurons in the field of view, depending e.g. on neural subtype, or indicator load.

When possible, pure binarization of calcium signals is discouraged, as relative activation-level distributions across cells may be critical in assessing activity correlations among cells and in quantifying the pattern and/or degree of engagement of a given ensemble. Binarization may be a necessary measure, depending on the indicator and question of interest. For example, oregon green bapta (OGB) has a low dynamic range, but high sensitivity to individual spikes. As a dye not requiring transgenic animals or viral transduction, OGB with binarization may be better suited to some experimental preparations (Miller et al., 2014). Other reasons for binarization may be based in the use of algorithms that can exclusively handle binarized data structure (e.g. Lempel-Ziv-Welch compression), or if analysis is resource heavy due to the size of the dataset, or the analytical approach used (such as t-stochastic neighbor embedding, or t-SNE) (van der Maaten and Hinton, 2008), or both.

Importantly, entire analysis pipelines and processing routines have been developed and freely-distributed to automate all aspects of this process (Friedrich et al., 2017; Pnevmatikakis et al., 2016). In many cases, these approaches are becoming the standard in imaging neuroscience, involving the estimation of the background noise based on known characteristics of calcium and calcium indicator dynamics, calculation of activity from selected ROIs, and inference of neuronal spiking. In general, these alogorithms should be viewed as tools. Variations in experimental preparations, indicator expression or signal to noise ratio, sampling rate, animal age, and a number of other variables may influence the performance of these algorithms, so the experimenter is strongly encouraged to inspect and confirm the veracity of every ROI identified. Without proper calibration of algorithmic parameters, a spurious train of seemingly realistic calcium transients could be fit and extracted from an ROI timecourse of purely noise.

Eventually, data normalization should be part of an experimenter's considerations. That is, normalizing individual cell activation timecourses in order to integrate cells evenly and equitably in subsequent analyses of ensemble activations (e.g. frame-wise analyses, see below). For example, in the event of strongly varying indicator expression levels across examined neurons, a within-cell normalization (e.g. max norm) of activity may be useful, depending on how frames are compared to one another for assessing similarity (Hamm et al., 2020, 2017). Other normalization steps may be needed for theoretical considerations. Highly

active neurons may appear to “take part” in many ensembles in the same way that common letters like vowels may take part in many words; in which case, the presence of these neurons may be relatively less useful for identifying and differentiating separate ensemble patterns, and this inequality could be mitigated e.g. by term frequency inverse document frequency normalization (Carrillo-Reid et al., 2016).

From activity to co-activity

Once valid neuronal activity traces are extracted from the raw image series, a next step is to calculate neuronal co-activity in the dataset. Rapid recent advancements of optical (Han et al., 2019; Skocek et al., 2018) and electrophysiological (Jun et al., 2017; Khodagholy et al., 2015) population recording technology now allows the simultaneous measurement of the activity of up to thousands of neurons in the alive brain over months. The resulting multi-dimensional datasets typically comprise n neurons by x time points. A key aspect to be kept in mind is, again, that the term co-activity is closely associated with the respective recording technique (e.g. Ca^{2+} imaging versus single unit recording). A practical approach to this fundamental issue, as discussed above, is that co-activity could be understood as a window of concurrent neuronal activity that allows downstream signal integration and subsequent neuronal firing (e.g. “reader-centric”). It is reasonable to assume that this window of neuronal signal integration lies somewhere in the range between neuronal cell time constants (10–30 ms) and the length of a post-synaptic excitatory potential (up to 200 ms).

On the other hand, depending on the question, longer timescales may even be appropriate for some analyses (e.g. seconds to minutes); for example, quantifying correlations of neuronal activations across stimulus presentations (Agetsuma et al., 2017), contexts (Cai et al., 2016), or social interactions (Liang et al., 2018). It is not clear whether and how a group of neurons correlated in such longer time-scales deviates from a classical conceptualization of “ensemble”; indeed, such cells are coactive within a window of time and are likely connected in a synaptic matrix (directly or poly-synaptically, by virtue of their spatial proximity), and thus may be thought of an ensemble from a “condition-centric” viewpoint. Smaller, more temporally constrained ensembles could exist within these spatiotemporally broader neuronal groups, in the same way that, e.g., people from Manhattan and Hoboken can be said to be “neighbors”, sharing many experiences and influences, but not to the same degree that people from the same upper westside block are “neighbors”. Both scales of neuronal groupings are relevant for brain function (Cai et al., 2016; Ikegaya et al., 2004), and may correspond to different neurooscillatory frequency bands (Buzsaki, 2009). The time-scale of linking neurons into “ensembles” will depend on the scope of the experimental question.

Determining how exactly co-activity is quantified from two activity vectors has numerous options, including basic pearson correlations (Agetsuma et al., 2017), cosine-similarity (L. Carrillo-Reid et al., 2015), and euclidean distance (Hamm et al., 2018) (Figure 1D). Researchers are encouraged to investigate which may be most appropriate given the signal properties, experimental preparations, and questions of interest (e.g. with regard to time course). In many cases, neuronal activity distributions across time and space are not normally distributed, but are log-normal or heavily positively skewed; in such a case,

pearson correlation may be less appropriate than cosine-similarity (Hamm et al., 2017). Further, one must consider whether the absolute magnitude of a cell activation trace or population activity state should influence the calculated similarity, as, for example, Euclidean distance would depend on both relative and absolute activity levels, while cosine similarity would only depend on the angle between two population vectors.

It is important to relate recorded co-activity patterns to what would be expected in a null condition; e.g. an unconnected network of neurons receiving the same input. This may involve comparing the observed dataset against shuffled surrogates. Ideally, this should be carried out for each vector pair (e.g. cell-pair or frame-pair) using data recorded during the experiment or simulated based on the distributional properties of the data recorded during the experiment. The exact shuffling procedure will depend on the question asked, e.g. in one case, every individual neuron's firing rate needs to remain constant (within-cell shuffling), while in another case, population activity has to be kept constant for each time point (within-frame shuffling). For comparing two cells to see if their activity is truly "co-activity", a temporal shifting step is suggested (rather than a full random reallocation of timepoints) (Ikegaya et al., 2004), as this would retain autocorrelations (which could account from some of the co-activity estimate if e.g. two cells were randomly coactive at one time). Shuffling steps can be repeated thousands of times, and a null distribution can be generated. Comparing the original value of co-activation to this distribution can be used to generate p-values for statistical confirmation (Hamm et al., 2017; M. Wenzel et al., 2019). It is recommended that multiple shuffling regimes be applied to rigorously exclude spurious or uninteresting sources of the identified coativity, but also, that these shuffling procedures be applied separately. Too many simultaneously applied shuffles will overpower the analysis and lead to a type 1 error (e.g. create a null-distirbution that is too easily rejected).

Another important decision relates to the inclusion or exclusion of inactivity. On the one hand, the silence of a given neuron at a specific time point carries meaning in itself in comparison to other neurons that are co-active at the same time. Yet two signals with highly sparse activity, exhibiting only a few brief bursts across an experiment, could share a fair degree of correlation despite no true functional "co-activity", depending on how quiescent periods are dealt with analytically. Further, under certain conditions such as deep anesthesia, it might be of no use to include time points that include no neuronal activity whatsoever. Alternatively, one point to consider is that the target of interest in "ensemble" analysis is "ensemble activations" (Figure 1 A,B). Thus, one possible method is to first identify only timeperiods wherein the population of neurons is sufficiently active (so called "ensemble activations") based on some *a priori* (number of neurons; (Fang and Yuste, 2017)) or analytical threshold (via shuffling; (Hamm et al., 2017)). Then, ensemble analyses (including co-activity estimations) can proceed with only these timepoints.

From co-activity to ensembles

When a method for quantifying co-activity is chosen, the next step is to relate co-activation estimates across many signal pairs, arriving at some understanding of ensemble activity or functional network structure. A first point in the pipeline is to identify which points or signatures of co-activity represent an "ensemble." Practically speaking, neuronal coactivity

as it relates to ensembles can take at least two principal forms: cell-cell similarity, or how similar the activations of neuronal pairs are across time, and frame-frame similarity, or how similar the activation patterns of time points are across neurons. Simply put, starting from a rasterplot of neuronal activations (Figure 1B), is the co-activity matrix calculated with rows (cell-cell) or columns (frame-frame) as variables? In a cell-cell framework, the goal is to identify unique combinations of neurons which form ensembles; in a frame-frame framework, the goal is to identify unique patterns of active and non-active cells across the neuronal population which form the recurring activity states of the network.

In cell-cell and frame-frame co-activity pipelines alike, there are numerous options for moving from pair-wise to ensemble-wise analyses. A k-means approach can be used to identify functional cell combinations from activity traces (e.g. cell-cell (Ozden et al., 2008)) or commonly recurring populations states from cell activation patterns (Figure 1 C) (e.g. frame-frame; (Luis Carrillo-Reid et al., 2015a; Hamm et al., 2017). K-means clustering analysis is an algorthim which iteratively combines group observations (e.g. cells or frames) into a set of k-number of clusters to minimize some within-group distance metric (e.g. in frame or cell space, respectively). The distance metric used here is the similarity measure of co-activity (e.g cosine, Euclidean distance, or watershed segmentation; Figure 1D). The number of clusters can be estimated *a priori* by the researcher, or varied systematically through numerous k-means analyses (Hamm et al., 2020). By analysing cluster metrics (e.g. within vs between cluster similarity; distance between clusters) at various values of k (Figure 1G), the status of ensemble activity between multiple conditions can be compared in an unbiased manner (e.g. Hamm et al., 2020). Further, the relative peak of within cluster similarity for each experiment may suggest the true number of clusters in the imaged activity.

The “space” in which observations are clustered (e.g. frames or cells) can bias the results if particular spatial dimensions (e.g. frames or cells) are overrepresented or highly correlated (Alrabea et al., 2013; Lee et al., 2012); therefore, prior to k-means, a dimensional reduction of the activity space into a subset of principle components is helpful (e.g. PCA; Figure 1C middle, G left). While PCA comprises a linear combination of frames or cells, a t-distributed stochastic neighbor embedding (t-SNE) approach derives non-linear combinations of frames or cells to optimally separate clusters (of cells or frames) in the resulting 2 or 3 dimensional t-SNE space (Figure 1C right, H left; (Hamm et al., 2020; van der Maaten and Hinton, 2008; M. Wenzel et al., 2019)).

Further, a given promiscuous neuron can take part in multiple ensembles or activity states, as is a known feature e.g. of layer 2/3 neocortical populations (Miller et al., 2014), but the analytical implications for dealing with this feature differ depending on the framework (e.g. cell-cell vs frame-frame co-activity). Too many promiscuous cells, active in many ensembles, may alter the precision and performance of k-means clustering algorithms in frame-space (on neurons), and researchers should consider, in this case, other algorithms. Alternative to k-means, a watershed segmentation method can be applied to the t-SNE output to identify the clusters of activity patterns or neurons based on the shrinking and delineation of spatial boundaries around probability maxima (M. Wenzel et al., 2019) (Figure 1 D, H left). Another strong method for clustering complex datasets is affinity

propogation, which starts with cell-pair or ensemble activation (frame) similarity metrics and iteratively identifies ensemble/state exemplars (Frey and Dueck, 2007). Importantly, validation, by carrying out the intial analyses on a subset of the data and testing the solution (e.g. ensemble definitions) in the remaining data is highly encouraged.

From here, many potential properties or aspects can be estimated, depending on the experimental questions and nature of the datasets and tools. This could include: number or average size of distinct ensembles (Fang and Yuste, 2017; M. Wenzel et al., 2019), the reliabitiy/flexibility of ensemble groups across time (Hamm et al., 2020), the spatial distribution of ensembles (Ikegaya et al., 2004), or the functional overlap of ensemble groupings (Cai et al., 2016; Miller et al., 2014). These measures could be compared between two timepoints, regions, or animals, or they could be analysed in relation to different stimulation or behavioral conditions, for instance; an ultimate goal is to arrive at a reliable estimate for each experiment which can be replicated in subsequent work. Figure 1D–F describes a pipeline wherein the ensemble activity can be compared between two cases (using the same or different neurons; in the same or different mice). First, some similarity metric among population activity states is arrived at (Figure 1D), and frame-frame similarity matrix is computed (Figure 1E). From here, similarities can be examined as they are distributed aross frame-frame pairs (without clustering and/or identification of recurrent ensemble activation states) to identify differences in the distributions of frame-frame similarities (Figure 1F, left; testable e.g. with Komolgorov-Smirnov statistics). A Gaussian distribution suggests that no clearly repeating ensemble activation exist, where a positively skewed distribution would be expected if distinct activity states were recurrent across the imaging paradigm. After identifying the stable “activity states” or ensemble activations, the similarity values (e.g. from Figure 1E) can be averaged among frames within and between “clusters” (Figure 1F, right) and then between conditions (e.g. with a mixed measures ANOVA).

For understanding how neuronal populations function as ensembles, both cell-cell and frame-frame co-activity distributions provide meaningful starting points, and it is encouraged that analyses employ both to fully understand ensemble dynamics. Important effects could be identified with both: for instance, in layer 2/3 cortical circuits of Setd1a$^{+/-}$ mice (a mouse strain modeling the human genotype with a high risk for schizophrenia), the slope of the spatial distribution of cell-cell similarities is shallower, while frame-frame similarities index a less distinct set of repeating population-wide activation patterns (Figure 1 G) (Hamm et al., 2020). These findings together were complementary (e.g. activation patterns did not have a spatial component), revealing circuit pathology more comprehsively. Although not typically anticipated, it is possible that different clustering or factor analyses could yield different estimates of effect significance, or even different effects entirely, in the same dataset. For instance, k-means clustering may identify clusters more conservatively than watershed segmentation, leading to a condition difference in ensemble similarity with one technique and ensemble number with the other. Interpreting such discrepancies is ultimately challenging. First, comparing the algorithms carefully by comparing the face validity of the clustering solutions, systematically varying algorithmic parameters, assessing solution stability across partitioned datasets, and even using a “clamp” approach wherein the analysis has a predictable solution (e.g. ensembles activated by different visual orientations)

in order to evaluate the most stable algorithm for the current dataset may be all constitute valid steps. Ultimately, it may be worthwhile to report both results if systematic comparison is inconclusive.

An alternative route to quantifying ensemble activity is graph theoretical network analyses of topology (Bullmore and Sporns, 2009). Here, researchers can analyse a cell-cell connectivity matrix to estimate of “clustering” and “modularity”, which provide quantitative estimates of the degree to which a measured functional network exhibits reliable, distinct ensembles as well as other meaningful metrics of network structure and topology, including “assortativity” (e.g. the degree to which highly connected neurons are connected/disconnected with each other). These measures carry the advantage of being simply derived and highly standardized in the literature. While functional magnetic resonance imaging (fMRI) studies of macro-scale functional networks have widely adopted these approaches (He et al., 2012; Sporns and Betzel, 2016; van den Heuvel et al., 2010; Zhu et al., 2016), they are less common in focal optical imaging of microcircuits (Betzel et al., 2019; Malmersjö et al., 2013), perhaps because incomplete measurement of the connected network may unevenly and unpredictably bias the results. Such a technique does not involve the automatic or manual identification or delineation of discrete individual ensembles or repeating activation patterns, and in some sense could be considered a less biased and/or complimentary to the analyses above. As with other methods, the quantification method for activity and co-activity will significantly influence such metrics (see above). Further, decisions as to what is considered a “connection” between cells (or “nodes” in the network) are critical for analysing the network; ideally, clustering, modularity, and other metrics should be calculated at many co-activity cutoffs, and curves should be compared between conditions or mice (e.g. see (Zhu et al., 2016)).

Although intrinsic or spontaneous ensemble activity can and has been meaningfully studied during “rest” paradigms, including a sensory stimulus, recording a motor output, or requiring a cognitive task adds important context for understanding ensemble activity in living, intact brain systems. While a comprehensive discussion of such paradigms is outside the scope of this paper, an important point to consider is that such experimental conditions (e.g. different visual stimuli (Hamm et al., 2017), successful vs. failed tasks (Carrillo-Reid et al., 2019), unstructured motor output (Musall et al., 2019)) provide a type of “clamp” for understanding how ensemble activity (and related metrics, e.g. state-reliability, number of distinct states, or spatial correlations) is stable or is meaningfully changed by conditions or mental status (Figure 1 G,H). Decoding behavior or stimulus presentation from neural population data is also a useful tool for determining the degree to which the group-level dynamics play emergent computational roles beyond that of individual neurons (Morcos and Harvey, 2016). One possible technique to quantify the stability of ensemble encoding of sensory stimuli employs the use of support vector machine algorithms (SVM) to compute multineuronal decoders on half of stimulation trials, and then testing the performance of decoder models on the remaining half, comparing this performance between conditions (Agetsuma et al., 2017; Hamm et al., 2020).

It is important to keep in mind: differences between recordings, conditions, or neuronal populations in lower-order measures, such as average neuronal activity rates (e.g. average

Δf/f) or global average co-activity (for graph-theory metrics, “average degree” or degree distribution), should be noted in any report of “ensemble-level” differences. If a truly emergent ensemble-effect is present, it should come in the absence or show some independence of these lower level effects. This isn’t to say that, e.g., a shift in ensemble reliability or overlap resulting from a general increase in neuronal activity is not important to report, but reporting both levels is key to understanding the nature of the effects. On the other hand, if, for instance, differences between conditions are only seen consistently at the ensemble level, this is evidence for a primacy of this systems-biological scale for the phenomena under investigation.

Finally, as with estimating co-activity, it is highly useful to come to some estimate of baseline or “random” levels of ensemble activity, perhaps through reshuffling or bootstrapping (e.g. Figure 1F–H). Statistically identified “differences” between two conditions are suspect and difficult to interpret when neither of the conditions substantially deviate from ensemble measures that could be expected from randomly generated co-activity matrices.

Conclusion.

Here we have provided a roadmap of considerations and a set of example pipelines for the processing of optical imaging datasets for the quantification and identification of neuronal ensembles. Ensembles, as functionally defined, are locally co-active neuronal groups which may represent a critical level of function for cortical circuits (Cossart et al., 2003; Hopfield, 1982), as well as a critical point of disease-pathway convergence for understanding the core pathophysiology of neuropsychiatric or neurological disease (Fang and Yuste, 2017; Hamm et al., 2020, 2017; Liang et al., 2018; M. Wenzel et al., 2019; Wenzel et al., 2017). Currently, algorithms and analysis pipelines for quantifying ensemble activity are numerous. Cataloguing such approaches is beyond the scope of this paper, and may exceed the scope of most journal articles. Here we provide a practical background and a list of important considerations for a researcher new or intermediate to the field of optical imaging of neural populations (summarized in table 2). As methods for rapid single-cell manipulation of experiment-defined ensemble patterns advance and become more widely available (Mardinly et al., 2018; Yang and Yuste, 2018), the ability to directly test the coactivity structures identified with the analyses described herein will serve as a valuable validation tool.

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Bibliography.

Abdelfattah AS, Kawashima T, Singh A, Novak O, Liu H, Shuai Y, Huang YC, Campagnola L, Seeman SC, Yu J, Zheng J, Grimm JB, Patel R, Friedrich J, Mensh BD, Paninski L, Macklin JJ, Murphy GJ, Podgorski K, Lin BJ, Chen TW, Turner GC, Liu Z, Koyama M, Svoboda K, Ahrens MB, Lavis LD, Schreiter ER, 2019. Bright and photostable chemigenetic indicators for extended in vivo voltage imaging. Science 10.1126/science.aav6416

Abeles M, 1991. Corticonics, Corticonics 10.1017/cbo9780511574566

Agetsuma M, Hamm JP, Tao K, Fujisawa S, Yuste R, 2017. Parvalbumin-Positive Interneurons Regulate Neuronal Ensembles in Visual Cortex. Cereb. Cortex 6, 1–15. 10.1093/cercor/bhx169

Alrabea A, Senthilkumar AV, Al-Shalabi H, Bader A, 2013. Enhancing K-Means Algorithm with Initial Cluster Centers Derived from Data Partitioning along the Data Axis with PCA. J. Adv. Comput. Networks 137–142. 10.7763/jacn.2013.v1.28

Andermann ML, Gilfoy NB, Goldey GJ, Sachdev RNS, Wölfel M, McCormick DA, Reid RC, Levene MJ, 2013. Chronic Cellular Imaging of Entire Cortical Columns in Awake Mice Using Microprisms. Neuron 10.1016/j.neuron.2013.07.052

Aronov D, Nevers R, Tank DW, 2017. Mapping of a non-spatial dimension by the hippocampalentorhinal circuit. Nature 10.1038/nature21692

Baird-Daniel E, Daniel AGS, Wenzel M, Li D, Liou J-Y, Laffont P, Zhao M, Yuste R, Ma H, Schwartz TH, 2017. Glial Calcium Waves are Triggered by Seizure Activity and Not Essential for Initiating Ictal Onset or Neurovascular Coupling. Cereb. Cortex 27. 10.1093/cercor/bhx072

Bando Y, Grimm C, Cornejo VH, Yuste R, 2019a. Genetic voltage indicators. BMC Biol 10.1186/s12915-019-0682-0

Bando Y, Sakamoto M, Kim S, Ayzenshtat I, Yuste R, 2019b. Comparative Evaluation of Genetically Encoded Voltage Indicators. Cell Rep 10.1016/j.celrep.2018.12.088

Berens P, Freeman J, Deneux T, Chenkov N, McColgan T, Speiser A, Macke JH, Turaga SC, Mineault P, Rupprecht P, Gerhard S, Friedrich RW, Friedrich J, Paninski L, Pachitariu M, Harris KD, Bolte B, Machado TA, Ringach D, Stone J, Rogerson LE, Sofroniew NJ, Reimer J, Froudarakis E, Euler T, Román Rosón M, Theis L, Tolias AS, Bethge M, 2018. Community-based benchmarking improves spike rate inference from two-photon calcium imaging data. PLoS Comput. Biol 14. 10.1371/journal.pcbi.1006157

Berger H, 1929. Über das Elektroenkephalogramm des Menschen. Arch. Psychiatr. Nervenkr 87, 527–570.

Betzel RF, Wood KC, Angeloni C, Geffen MN, Bassett DS, 2019. Stability of spontaneous, correlated activity in mouse auditory cortex. PLoS Comput. Biol 15. 10.1371/journal.pcbi.1007360

Braitenberg V, 1978. Cell Assemblies in the Cerebral Cortex 10.1007/978-3-642-93083-6_9

Broussard GJ, Liang Y, Fridman M, Unger EK, Meng G, Xiao X, Ji N, Petreanu L, Tian L, 2018. In vivo measurement of afferent activity with axon-specific calcium imaging. Nat. Neurosci 10.1038/s41593-018-0211-4

Bullmore E, Sporns O, 2009. Complex brain networks: graph theoretical analysis of structural and functional systems. Nat. Rev. Neurosci 10, 186–98. 10.1038/nrn2575 [PubMed: 19190637]

Buzsaki G, 2009. Rhythms of the Brain Oxford University Press, New York.

Buzsáki G, 2010. Neural Syntax: Cell Assemblies, Synapsembles, and Readers. Neuron 68, 362–385. 10.1016/j.neuron.2010.09.023 [PubMed: 21040841]

Buzsáki G, Draguhn A, 2004. Neuronal olscillations in cortical networks. Science 10.1126/science.1099745

Cai DJ, Aharoni D, Shuman T, Shobe J, Biane J, Song W, Wei B, Veshkini M, La-Vu M, Lou J, Flores SE, Kim I, Sano Y, Zhou M, Baumgaertel K, Lavi A, Kamata M, Tuszynski M, Mayford M, Golshani P, Silva AJ, 2016. A shared neural ensemble links distinct contextual memories encoded close in time. Nature 534, 115–118. 10.1038/nature17955 [PubMed: 27251287]

Cammarota M, Losi G, Chiavegato A, Zonta M, Carmignoto G, 2013. Fast spiking interneuron control of seizure propagation in a cortical slice model of focal epilepsy. J Physiol 591, 807–822. 10.1113/jphysiol.2012.238154 [PubMed: 23207591]

Cardin JA, Carlén M, Meletis K, Knoblich U, Zhang F, Deisseroth K, Tsai L-H, Moore CI, 2009. Driving fast-spiking cells induces gamma rhythm and controls sensory responses. Nature 459, 663–7. 10.1038/nature08002 [PubMed: 19396156]

Carillo-Reid L, Yuste R, Carillo-Reid L, Yuste R, 2020. What Is a Neuronal Ensemble?, in: Oxford Research Encyclopedia of Neuroscience Oxford University Press. 10.1093/acrefore/9780190264086.013.298

Carrillo-Reid L, Han S, Yang W, Akrouh A, Yuste R, 2019. Controlling Visually Guided Behavior by Holographic Recalling of Cortical Ensembles. Cell 178, 447–457.e5. 10.1016/j.cell.2019.05.045 [PubMed: 31257030]

Carrillo-Reid Luis, Kang J-E, Hamm JP, Jackson J, Yuste R, Miller J-EK, Hamm JP, Jackson J, Yuste R, 2015a. Endogenous sequential cortical activity evoked by visual stimuli. J. Neurosci 35, 8813–28. 10.1523/JNEUROSCI.5214-14.2015 [PubMed: 26063915]

Carrillo-Reid L, Miller J. -e. K., Hamm JP, Jackson J, Yuste R, 2015. Endogenous Sequential Cortical Activity Evoked by Visual Stimuli. J. Neurosci 35, 8813–8828. 10.1523/JNEUROSCI.5214-14.2015 [PubMed: 26063915]

Carrillo-Reid L, Yang W, Bando Y, Peterka DS, Yuste R, 2016. Imprinting Cortical Ensembles. Science 353, 691–694. 10.1126/science.aaf7560 [PubMed: 27516599]

Carrillo-Reid L, Yang W, Kang Miller J, Peterka DS, Yuste R, 2017. Imaging and Optically Manipulating Neuronal Ensembles. Annu. Rev. Biophys 46, 271–293. 10.1146/annurev-biophys-070816-033647 [PubMed: 28301770]

Carrillo-Reid L, Yuste R, 2020. Playing the piano with the cortex: role of neuronal ensembles and pattern completion in perception and behavior. Curr. Opin. Neurobiol 10.1016/j.conb.2020.03.014

Chen TW, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, Schreiter ER, Kerr RA, Orger MB, Jayaraman V, Looger LL, Svoboda K, Kim DS, 2013. Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499, 295–300. 10.1038/nature12354 [PubMed: 23868258]

Cheng A, Gonçalves JT, Golshani P, Arisaka K, Portera-Cailliau C, 2011. Simultaneous two-photon calcium imaging at different depths with spatiotemporal multiplexing. Nat. Methods 10.1038/nmeth.1552

Chklovskii DB, Schikorski T, Stevens CF, 2002. Wiring optimization in cortical circuits. Neuron 10.1016/S0896-6273(02)00679-7

Cossart R, Aronov D, Yuste R, 2003. Attractor dynamics of network UP states in the neocortex. Nature 423, 283–8. 10.1038/nature01614 [PubMed: 12748641]

Dana H, Chen TW, Hu A, Shields BC, Guo C, Looger LL, Kim DS, Svoboda K, 2014. Thy1-GCaMP6 transgenic mice for neuronal population imaging in vivo. PLoS One 9, e108697. 10.1371/journal.pone.0108697 [PubMed: 25250714]

Dana H, Novak O, Guardado-Montesino M, Fransen JW, Hu A, Borghuis BG, Guo C, Kim DS, Svoboda K, 2018. Thy1 transgenic mice expressing the red fluorescent calcium indicator jRGECO1a for neuronal population imaging in vivo. PLoS One 10.1371/journal.pone.0205444

Dana H, Sun Y, Mohar B, Hulse BK, Kerlin AM, Hasseman JP, Tsegaye G, Tsang A, Wong A, Patel R, Macklin JJ, Chen Y, Konnerth A, Jayaraman V, Looger LL, Schreiter ER, Svoboda K, Kim DS, 2019. High-performance calcium sensors for imaging activity in neuronal populations and microcompartments. Nat. Methods 10.1038/s41592-019-0435-6

Deisseroth K, 2011. Optogenetics. Nat. Methods 8, 26–29. 10.1038/nmeth.f.324 [PubMed: 21191368]

Denk W, Strickler JH, Webb WW, 1990. Two-photon laser scanning fluorescence microscopy. Science (80−.) 248, 73–76. 10.1126/science.2321027

Diba K, Amarasingham A, Mizuseki K, Buzsáki G, 2014. Millisecond timescale synchrony among hippocampal neurons. J. Neurosci 10.1523/JNEUROSCI.1091-14.2014

Dubbs A, Guevara J, Yuste R, 2016. moco: Fast Motion Correction for Calcium Imaging. Front. Neuroinform 10, 6. 10.3389/fninf.2016.00006 [PubMed: 26909035]

Duemani Reddy G, Kelleher K, Fink R, Saggau P, 2008. Three-dimensional random access multiphoton microscopy for functional imaging of neuronal activity. Nat. Neurosci 11, 713–720. 10.1038/nn.2116 [PubMed: 18432198]

Engel A, Konig P, Kreiter A, Singer W, 1991. Interhemispheric synchronization of oscillatory neuronal responses in cat visual cortex. Science 252, 1177–1179. 10.1126/science.252.5009.1177 [PubMed: 2031188]

Fang WQ, Yuste R, 2017. Overproduction of Neurons Is Correlated with Enhanced Cortical Ensembles and Increased Perceptual Discrimination. Cell Rep 10.1016/j.celrep.2017.09.040

Fino E, Yuste R, 2011. Dense inhibitory connectivity in neocortex. Neuron 69, 1188–203. 10.1016/j.neuron.2011.02.025 [PubMed: 21435562]

Flusberg BA, Nimmerjahn A, Cocker ED, Mukamel EA, Barretto RPJ, Ko TH, Burns LD, Jung JC, Schnitzer MJ, 2008. High-speed, miniaturized fluorescence microscopy in freely moving mice. Nat. Methods 10.1038/nmeth.1256

Frey BJ, Dueck D, 2007. Clustering by passing messages between data points. Science 315, 972–976. 10.1126/science.1136800 [PubMed: 17218491]

Friedrich J, Zhou P, Paninski L, 2017. Fast online deconvolution of calcium imaging data. PLoS Comput. Biol 13. 10.1371/journal.pcbi.1005423

Ghosh KK, Burns LD, Cocker ED, Nimmerjahn A, Ziv Y, El Gamal A, Schnitzer MJ, 2011. Miniaturized integration of a fluorescence microscope. Nat. Methods 10.1038/nmeth.1694

Glickfeld LL, Andermann ML, Bonin V, Reid RC, 2013. Cortico-cortical projections in mouse visual cortex are functionally target specific. Nat. Neurosci 16, 219–226. 10.1038/nn.3300 [PubMed: 23292681]

Hamm JP, Peterka DS, Gogos JA, Yuste R, 2017. Altered Cortical Ensembles in Mouse Models of Schizophrenia. Neuron 94, 153–167.e8. 10.1016/j.neuron.2017.03.019 [PubMed: 28384469]

Hamm JP, Shymkiv Y, Han S, Yang W, Yuste R, 2018. Cortical subnetworks encode context of a visual stimulus. bioRxiv 452219. 10.1101/452219

Hamm JP, Shymkiv Y, Mukai J, Gogos JA, Yuste R, 2020. Aberrant Cortical Ensembles and Schizophrenia-like Sensory Phenotypes in Setd1a+/− Mice. Biol. Psychiatry 88, 215–233. 10.1016/j.biopsych.2020.01.004 [PubMed: 32143831]

Hamm JP, Yuste R, 2016. Somatostatin Interneurons Control a Key Component of Mismatch Negativity in Mouse Visual Cortex. Cell Rep 16, 597–604. 10.1016/j.celrep.2016.06.037 [PubMed: 27396334]

Han S, Yang W, Yuste R, 2019. Two-Color Volumetric Imaging of Neuronal Activity of Cortical Columns. Cell Rep 27, 2229–2240.e4. 10.1016/j.celrep.2019.04.075 [PubMed: 31091458]

Harris KD, Csicsvari J, Hirase H, Dragoi G, Buzsáki G, 2003. Organization of cell assemblies in the hippocampus. Nature 10.1038/nature01834

He H, Sui J, Yu Q, Turner JA, Ho BC, Sponheim SR, Manoach DS, Clark VP, Calhoun VD, 2012. Altered small-world brain networks in schizophrenia patients during working memory performance. PLoS One 10.1371/journal.pone.0038195

He M, Huang ZJ, 2018. Genetic approaches to access cell types in mammalian nervous systems. Curr. Opin. Neurobiol 50, 109–118. 10.1016/j.conb.2018.02.003 [PubMed: 29471215]

Hebb DO, 1949. The Organization of Behavior, The Organization of Behavior 10.2307/1418888

Helmchen F, Fee MS, Tank DW, Denk W, 2001. A miniature head-mounted two-photon microscope: High-resolution brain imaging in freely moving animals. Neuron 10.1016/S0896-6273(01)00421-4

Hess GP, Lewis RW, Chen Y, 2014. Caged neurotransmitters and other caged compounds: Design and application. Cold Spring Harb. Protoc 10.1101/pdb.top084152

Holtmaat A, Bonhoeffer T, Chow DK, Chuckowree J, De Paola V, Hofer SB, Hübener M, Keck T, Knott G, Lee WCA, Mostany R, Mrsic-Flogel TD, Nedivi E, Portera-Cailliau C, Svoboda K, Trachtenberg JT, Wilbrecht L, 2009. Long-term, high-resolution imaging in the mouse neocortex through a chronic cranial window. Nat. Protoc 10.1038/nprot.2009.89

Hopfield JJ, 1982. Neural networks and physical systems with emergent collective computational abilities. Proc. Natl. Acad. Sci 79, 2554–2558. 10.1073/pnas.79.8.2554 [PubMed: 6953413]

Hu S, Ciliberti D, Grosmark AD, Michon F, Ji D, Penagos H, Buzsáki G, Wilson MA, Kloosterman F, Chen Z, 2018. Real-Time Readout of Large-Scale Unsorted Neural Ensemble Place Codes. Cell Rep 25, 2635–2642.e5. 10.1016/j.celrep.2018.11.033 [PubMed: 30517852]

Ikegaya Y, Aaron G, Cossart R, Aronov D, Lampl I, Ferster D, Yuste R, 2004. Synfire chains and cortical songs: temporal modules of cortical activity. Science 304, 559–64. 10.1126/science.1093173 [PubMed: 15105494]

Jáidar O, Carrillo-Reid L, Hernández A, Drucker-Colín R, Bargas J, Hernández-Cruz A, 2010. Dynamics of the Parkinsonian striatal microcircuit: Entrainment into a dominant network state. J. Neurosci 10.1523/JNEUROSCI.1380-10.2010

Jayant K, Wenzel M, Bando Y, Hamm JP, Mandriota N, Rabinowitz JH, Plante IJ-L, Owen JS, Sahin O, Shepard KL, Yuste R, 2019. Flexible Nanopipettes for Minimally Invasive Intracellular Electrophysiology In Vivo. Cell Rep 26. 10.1016/j.celrep.2018.12.019

Josselyn SA, Tonegawa S, 2020. Memory engrams: Recalling the past and imagining the future. Science 10.1126/science.aaw4325

Jun JJ, Steinmetz NA, Siegle JH, Denman DJ, Bauza M, Barbarits B, Lee AK, Anastassiou CA, Andrei A, Aydin Ç, Barbic M, Blanche TJ, Bonin V, Couto J, Dutta B, Gratiy SL, Gutnisky DA, Häusser M, Karsh B, Ledochowitsch P, Lopez CM, Mitelut C, Musa S, Okun M, Pachitariu M, Putzeys J, Rich PD, Rossant C, Sun WL, Svoboda K, Carandini M, Harris KD, Koch C, O'Keefe J, Harris TD, 2017. Fully integrated silicon probes for high-density recording of neural activity. Nature 551, 232–236. 10.1038/nature24636 [PubMed: 29120427]

Kampa BM, Roth MM, Göbel W, Helmchen F, 2011. Representation of visual scenes by local neuronal populations in layer 2/3 of mouse visual cortex. Front. Neural Circuits 10.3389/fncir.2011.00018

Kannan M, Vasan G, Huang C, Haziza S, Li JZ, Inan H, Schnitzer MJ, Pieribone VA, 2018. Fast, in vivo voltage imaging using a red fluorescent indicator. Nat. Methods 10.1038/s41592-018-0188-7

Karnani MMM, Jackson J, Ayzenshtat I, Tucciarone J, Manoocheri K, Snider WGG, Yuste R, 2016. Cooperative Subnetworks of Molecularly Similar Interneurons in Mouse Neocortex. Neuron 10.1016/j.neuron.2016.02.037

Khodagholy D, Gelinas JN, Thesen T, Doyle W, Devinsky O, Malliaras GG, Buzsáki G, 2015. NeuroGrid: Recording action potentials from the surface of the brain. Nat. Neurosci 18, 310–315. 10.1038/nn.3905 [PubMed: 25531570]

Knöpfel T, Song C, 2019. Optical voltage imaging in neurons: moving from technology development to practical tool. Nat. Rev. Neurosci 10.1038/s41583-019-0231-4

Koch C, Rapp M, Segev I, 1996. A brief history of time (constants). Cereb. Cortex 10.1093/cercor/6.2.93

Kuchibhotla KV, Wegmann S, Kopeikina KJ, Hawkes J, Rudinskiy N, Andermann ML, Spires-Jones TL, Bacskai BJ, Hyman BT, 2014. Neurofibrillary tangle-bearing neurons are functionally integrated in cortical circuits in vivo. Proc. Natl. Acad. Sci. U. S. A 10.1073/pnas.1318807111

Laughlin SB, Sejnowski TJ, 2003. Communication in neuronal networks. Science (80−.) 10.1126/science.1089662

Lee J, Park C, Dyckman K. a, Lazar N. a, Austin BP, Li Q, McDowell JE, 2012. Practice-related changes in neural activation patterns investigated via wavelet-based clustering analysis. Hum. Brain Mapp 000. 10.1002/hbm.22066

Liang B, Zhang L, Barbera G, Fang W, Zhang J, Chen X, Chen R, Li Y, Lin D-T, 2018. Distinct and Dynamic ON and OFF Neural Ensembles in the Prefrontal Cortex Code Social Exploration. Neuron 10.1016/J.NEURON.2018.08.043

Lillis KP, Wang Z, Mail M, Zhao GQ, Berdichevsky Y, Bacskai B, Staley KJ, 2015. Evolution of Network Synchronization during Early Epileptogenesis Parallels Synaptic Circuit Alterations. J Neurosci 35, 9920–9934. 10.1523/JNEUROSCI.4007-14.2015 [PubMed: 26156993]

Liou J-Y, Ma H, Wenzel M, Zhao M, Baird-Daniel E, Smith EH, Daniel A, Emerson R, Yuste R, Schwartz TH, Schevon CA, 2018. Role of inhibitory control in modulating focal seizure spread. Brain 141. 10.1093/brain/awy116

Little WA, 1974. The existence of persistent states in the brain. Math. Biosci 19, 101–120. 10.1016/0025-5564(74)90031-5

Lorente de No R, 1938. Analysis of the activity of the chains of internuncial neurons. J. Neurophysiol 207–244. 10.1152/jn.1938.1.3.207

Lu R, Liang Y, Meng G, Zhou P, Svoboda K, Paninski L, Ji N, 2020. Rapid mesoscale volumetric imaging of neural activity with synaptic resolution. Nat. Methods 10.1038/s41592-020-0760-9

Luczak A, Barthó P, Harris KD, 2009. Spontaneous events outline the realm of possible sensory responses in neocortical populations. Neuron 62, 413–25. 10.1016/j.neuron.2009.03.014 [PubMed: 19447096]

Malmersjö S, Rebellato P, Smedler E, Uhlén P, 2013. Small-world networks of spontaneous ca2+ activity. Commun. Integr. Biol 6. 10.4161/cib.24788

Mao BQ, Hamzei-Sichani F, Aronov D, Froemke RC, Yuste R, 2001. Dynamics of spontaneous activity in neocortical slices. Neuron 32, 883–898. 10.1016/S0896-6273(01)00518-9 [PubMed: 11738033]

Mardinly AR, Oldenburg IA, Pégard NC, Sridharan S, Lyall EH, Chesnov K, Brohawn SG, Waller L, Adesnik H, 2018. Precise multimodal optical control of neural ensemble activity. Nat. Neurosci 21, 881–893. 10.1038/s41593-018-0139-8 [PubMed: 29713079]

Marinković P, Blumenstock S, Goltstein PM, Korzhova V, Peters F, Knebl A, Herms J, 2019. In vivo imaging reveals reduced activity of neuronal circuits in a mouse tauopathy model. Brain 10.1093/brain/awz035

Menendez de la Prida L, Trevelyan AJ, 2011. Cellular mechanisms of high frequency oscillations in epilepsy: On the diverse sources of pathological activities. Epilepsy Res 10.1016/j.eplepsyres.2011.02.009

Meng G, Liang Y, Sarsfield S, Jiang WC, Lu R, Dudman JT, Aponte Y, Ji N, 2019. High-throughput synapse-resolving two-photon fluorescence microendoscopy for deep-brain volumetric imaging in vivo. Elife 10.7554/eLife.40805

Merricks EM, Smith EH, McKhann GM, Goodman RR, Bateman LM, Emerson RG, Schevon CA, Trevelyan AJ, 2015. Single unit action potentials in humans and the effect of seizure activity. Brain 10.1093/brain/awv208

Miller J. -e. K., Ayzenshtat I, Carrillo-Reid L, Yuste R, 2014. Visual stimuli recruit intrinsically generated cortical ensembles. Proc. Natl. Acad. Sci 111, E4053–E4061. 10.1073/pnas.1406077111 [PubMed: 25201983]

Mollinedo-Gajate I, Song C, Knöpfel T, 2019. Genetically encoded fluorescent calcium and voltage indicators, in: Handbook of Experimental Pharmacology 10.1007/164_2019_299

Morcos AS, Harvey CD, 2016. History-dependent variability in population dynamics during evidence accumulation in cortex. Nat. Neurosci 19, 1672–1681. 10.1038/nn.4403 [PubMed: 27694990]

Muldoon SF, Villette V, Tressard T, Malvache A, Reichinnek S, Bartolomei F, Cossart R, 2015. GABAergic inhibition shapes interictal dynamics in awake epileptic mice. Brain 10.1093/brain/awv227

Musall S, Kaufman MT, Juavinett AL, Gluf S, Churchland AK, 2019. Single-trial neural dynamics are dominated by richly varied movements. Nat. Neurosci 22, 1677–1686. 10.1038/s41593-019-0502-4 [PubMed: 31551604]

Nguyen QT, Callamaras N, Hsieh C, Parker I, 2001. Construction of a two-photon microscope for video-rate Ca2+ imaging. Cell Calcium 30, 383–393. 10.1054/ceca.2001.0246 [PubMed: 11728133]

Niediek J, Boström J, Elger CE, Mormann F, 2016. Reliable analysis of single-unit recordings from the human brain under noisy conditions: Tracking neurons over hours. PLoS One 10.1371/journal.pone.0166598

Nikolenko V, Watson BO, Araya R, Woodruff A, Peterka DS, Yuste R, 2008. SLM microscopy: Scanless two-photon imaging and photostimulation with spatial light modulators. Front. Neural Circuits 10.3389/neuro.04.005.2008

Ozden I, Lee HM, Sullivan MR, Wang SSH, 2008. Identification and clustering of event patterns from in vivo multiphoton optical recordings of neuronal ensembles. J. Neurophysiol 100, 495–503. 10.1152/jn.01310.2007 [PubMed: 18497355]

Palm G, 1981. Towards a theory of cell assemblies. Biol. Cybern 39, 181–194. 10.1007/BF00342771 [PubMed: 7248336]

Palm G, Knoblauch A, Hauser F, Schüz A, 2014. Cell assemblies in the cerebral cortex. Biol. Cybern 108, 559–572. 10.1007/s00422-014-0596-4 [PubMed: 24692024]

Paninski L, Cunningham JP, 2018. Neural data science: accelerating the experiment-analysis-theory cycle in large-scale neuroscience. Curr. Opin. Neurobiol 50, 232–241. 10.1016/j.conb.2018.04.007 [PubMed: 29738986]

Pedreira C, Martinez J, Ison MJ, Quian Quiroga R, 2012. How many neurons can we see with current spike sorting algorithms? J. Neurosci. Methods 10.1016/j.jneumeth.2012.07.010

Pnevmatikakis EA, Giovannucci A, 2017. NoRMCorre: An online algorithm for piecewise rigid motion correction of calcium imaging data. J. Neurosci. Methods 10.1016/j.jneumeth.2017.07.031

Pnevmatikakis EA, Soudry D, Gao Y, Machado TA, Merel J, Pfau D, Reardon T, Mu Y, Lacefield C, Yang W, Ahrens M, Bruno R, Jessell TM, Peterka DS, Yuste R, Paninski L, 2016. Simultaneous

Denoising, Deconvolution, and Demixing of Calcium Imaging Data. Neuron 89, 285–299. 10.1016/j.neuron.2015.11.037 [PubMed: 26774160]

Pouget A, Dayan P, Zemel R, 2000. Information processing with population codes. Nat. Rev. Neurosci 10.1038/35039062

Prut Y, Vaadia E, Bergman H, Haalman I, Slovin H, Abeles M, 1998. Spatiotemporal structure of cortical activity: Properties and behavioral relevance. J. Neurophysiol 79, 2857–2874. 10.1152/jn.1998.79.6.2857 [PubMed: 9636092]

Reznichenko L, Cheng Q, Nizar K, Gratiy SL, Saisan PA, Rockenstein EM, González T, Patrick C, Spencer B, Desplats P, Dale AM, Devor A, Masliah E, 2012. In vivo alterations in Calcium buffering capacity in transgenic mouse model of synucleinopathy. J. Neurosci 10.1523/JNEUROSCI.1270-12.2012

Scannell JW, Blakemore C, Young MP, 1995. Analysis of connectivity in the cat cerebral cortex. J. Neurosci 10.1523/jneurosci.15-02-01463.1995

Schüz A, Chaimow D, Liewald D, Dortenman M, 2006. Quantitative aspects of corticocortical connections: A tracer study in the mouse. Cereb. Cortex 10.1093/cercor/bhj085

Sessolo M, Marcon I, Bovetti S, Losi G, Cammarota M, Ratto GM, Fellin T, Carmignoto G, 2015. Parvalbumin-Positive Inhibitory Interneurons Oppose Propagation But Favor Generation of Focal Epileptiform Activity. J Neurosci 35, 9544–9557. 10.1523/JNEUROSCI.5117-14.2015 [PubMed: 26134638]

Sharma G, Annetta N, Friedenberg D, Blanco T, Vasconcelos D, Shaikhouni A, Rezai AR, Bouton C, 2015. Time Stability and Coherence Analysis of Multiunit, Single-Unit and Local Field Potential Neuronal Signals in Chronically Implanted Brain Electrodes. Bioelectron. Med 10.15424/bioelectronmed.2015.00010

Shemesh O, Linghu C, Piatkevich K, Goodwin D, Gritton H, Romano M, Siciliano C, Gao R, Yu C-C, Tseng H-A, Bensussen S, Narayan S, Yang C-T, Freifeld L, Gupta I, Noamany H, Pak N, Yoon Y-G, Ullmann J, Guner-Ataman B, Sheinkopf Z, Park WM, Asano S, Keating A, Trimmer J, Reimer J, Tolias A, Tye K, Han X, Ahrens M, Boyden E, 2020. Precision calcium imaging of dense neural populations via a cell body-targeted calcium indicator. Neuron 10.1101/773069

Silva AJ, 2017. Miniaturized two-photon microscope: Seeing clearer and deeper into the brain. Light Sci. Appl 10.1038/lsa.2017.104

Siniscalchi MJ, Wang H, Kwan AC, 2019. Enhanced Population Coding for Rewarded Choices in the Medial Frontal Cortex of the Mouse. Cereb. Cortex 29, 4090–4106. [PubMed: 30615132]

Skocek O, Nöbauer T, Weilguny L, Martínez Traub F, Xia CN, Molodtsov MI, Grama A, Yamagata M, Aharoni D, Cox DD, Golshani P, Vaziri A, 2018. High-speed volumetric imaging of neuronal activity in freely moving rodents. Nat. Methods 15, 429–432. 10.1038/s41592-018-0008-0 [PubMed: 29736000]

Sofroniew NJ, Flickinger D, King J, Svoboda K, 2016. A large field of view two-photon mesoscope with subcellular resolution for in vivo imaging. Elife 5. 10.7554/eLife.14472

Sporns O, Betzel RF, 2016. Modular brain networks. Annu. Rev. Psychol 10.1146/annurev-psych-122414-033634

Thévenaz P, Ruttimann UE, Unser M, 1998. A pyramid approach to subpixel registration based on intensity. IEEE Trans. Image Process 7, 27–41. 10.1109/83.650848 [PubMed: 18267377]

Trevelyan AJ, Sussillo D, Watson BO, Yuste R, 2006. Modular Propagation of Epileptiform Activity: Evidence for an Inhibitory Veto in Neocortex, Journal of Neuroscience

Turcotte R, Liang Y, Tanimoto M, Zhang Q, Li Z, Koyama M, Betzig E, Ji N, 2019. Dynamic super-resolution structured illumination imaging in the living brain. Proc. Natl. Acad. Sci. U. S. A 10.1073/pnas.1819965116

van den Heuvel MP, Mandl RCW, Stam CJ, Kahn RS, Hulshoff Pol HE, 2010. Aberrant frontal and temporal complex network structure in schizophrenia: a graph theoretical analysis. J. Neurosci 30, 15915–26. 10.1523/JNEUROSCI.2874-10.2010 [PubMed: 21106830]

van der Maaten L, Hinton G, 2008. Visualizing high-dimensional data using t-sne. J. Mach. Learn. Res 9, 2579–2605.

Wenzel, Hamm JP, Peterka DS, Yuste R, 2019a. Acute focal seizures start as local synchronizations of neuronal ensembles. J. Neurosci 3118–3176. 10.1523/JNEUROSCI.3176-18.2019

Wenzel, Leunig A, Han S, Peterka DS, Yuste R, 2019b. Prolonged anesthesia alters brain synaptic architecture. biorxiv 10.1101/862334

Wenzel M, Hamm JP, Peterka DS, Yuste R, 2017. Reliable and Elastic Propagation of Cortical Seizures In Vivo. Cell Rep 19. 10.1016/j.celrep.2017.05.090

Wenzel M, Han S, Smith EH, Hoel E, Greger B, House PA, Yuste R, 2019. Reduced Repertoire of Cortical Microstates and Neuronal Ensembles in Medically Induced Loss of Consciousness. Cell Syst 8. 10.1016/j.cels.2019.03.007

Xin Y, Zhong L, Zhang Y, Zhou T, Pan J, Xu N. long, 2019. Sensory-to-Category Transformation via Dynamic Reorganization of Ensemble Structures in Mouse Auditory Cortex. Neuron 103, 909–921.e6. 10.1016/j.neuron.2019.06.004 [PubMed: 31296412]

Xu Y, Zou P, Cohen AE, 2017. Voltage imaging with genetically encoded indicators. Curr. Opin. Chem. Biol 10.1016/j.cbpa.2017.04.005

Yang G, Pan F, Gan WB, 2009. Stably maintained dendritic spines are associated with lifelong memories. Nature 462, 920–924. 10.1038/nature08577 [PubMed: 19946265]

Yang W, Carrillo-Reid L, Bando Y, Peterka DS, Yuste R, 2018. Simultaneous two-photon imaging and two-photon optogenetics of cortical circuits in three dimensions. Elife 10.7554/eLife.32671

Yang W, Miller J. eun K., Carrillo-Reid L, Pnevmatikakis E, Paninski L, Yuste R, Peterka DS, 2016. Simultaneous Multi-plane Imaging of Neural Circuits. Neuron 10.1016/j.neuron.2015.12.012

Yang W, Yuste R, 2018. Holographic imaging and photostimulation of neural activity. Curr. Opin. Neurobiol 50, 211–221. 10.1016/j.conb.2018.03.006 [PubMed: 29660600]

Yang W, Yuste R, 2017. In vivo imaging of neural activity. Nat. Methods 14, 349–359. 10.1038/nmeth.4230 [PubMed: 28362436]

Yuste R, Denk W, 1995. Dendritic spines as basic functional units of neuronal integration. Nature 375, 682–684. 10.1038/375682a0 [PubMed: 7791901]

Zaremba JD, Diamantopoulou A, Danielson NB, Grosmark AD, Kaifosh PW, Bowler JC, Liao Z, Sparks FT, Gogos JA, Losonczy A, 2017. Impaired hippocampal place cell dynamics in a mouse model of the 22q11.2 deletion. Nat. Neurosci 20, 1612–1623. 10.1038/nn.4634 [PubMed: 28869582]

Zhu J, Zhuo C, Liu F, Qin W, Xu L, Yu C, 2016. Distinct disruptions of resting-state functional brain networks in familial and sporadic schizophrenia. Sci. Rep 6, 23577. 10.1038/srep23577 [PubMed: 27032817]

Zong W, Wu R, Li M, Hu Y, Li Y, Li J, Rong H, Wu H, Xu Y, Lu Y, Jia H, Fan M, Zhou Z, Zhang Y, Wang A, Chen L, Cheng H, 2017. Fast high-resolution miniature two-photon microscopy for brain imaging in freely behaving mice. Nat. Methods 10.1038/nmeth.4305

Highlights

- Cellular scale imaging represents an ideal tool to study neuronal ensembles
- Methods of ensemble analysis in imaging studies are highly varied across the field
- Comprehensive overview of ensemble identification and quantification
- Pipeline of considerations for ensemble identification and quantification

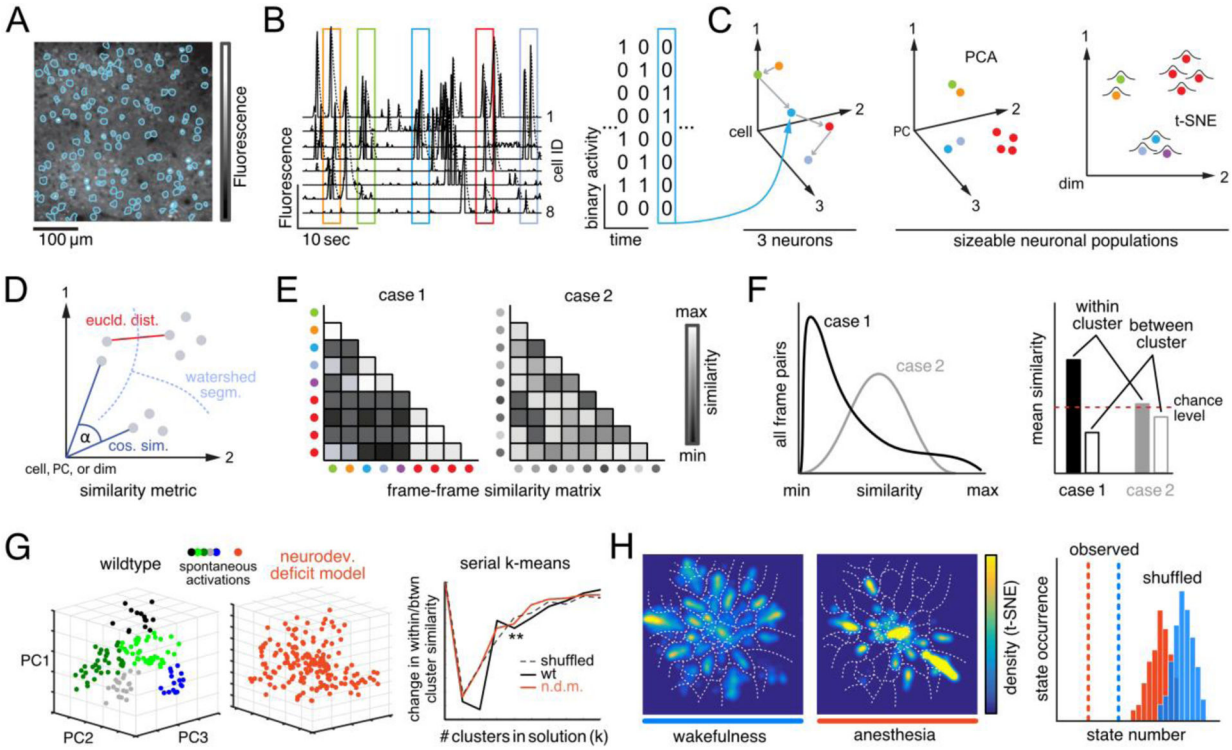


Figure 1. Example pipeline for the analysis of functional neuronal ensembles

A) Regions of interest (e.g. cell bodies) identified in optical imaging datasets are **B)** scored for activity based on fluorescence changes. Timepoints (imaging frames) of continuous neuronal Ca^{2+} transients (left) meeting some threshold of population-wide activity are isolated and binarized (right) or normalized across cells or time. **C)** These "ensemble activations" (e.g. high activity frames) can be analysed as a functional trajectory or set of activations in cell-space (left), or reduced to a lower dimensional space of linear combinations of cells with principal components analysis (PCA; middle) or of non-linear combinations of cells with t-distributed stochastic neighbor embedding (t-SNE) space optimized for visualizing clusters (or recurring) of ensemble activations (right). **D)** From here, similarities between ensemble activations can be assessed with multiple distance metrics such as cosine similarity, Euclidean distance or watershed segmentation. **E)** These metrics are compared here in two different cases (1 and 2). Similarity metrics are calculated for all pairs of activations (e.g. frames). **F)** Left: Histograms of all ensemble-activation pairs (left). As opposed to case 2, a non-gaussian distribution in case 1 is found, suggestive of subsets of highly similar and highly dissimilar ensemble activations. (right) After utilizing k-means or watershed segmentation to group observed ensemble activations into "clusters", similarity values between ensemble activations within the same vs different clusters are averaged to arrive at one value for each imaging dataset or mouse. Case 1 shows a significant within vs between cluster similarity difference, while case 2 shows no significant diffference in this comparison. Also, within cluster similarity for case 2 is not greater than the average value of similarities from an activity shuffled surrogate (dotted line; e.g. shuffling data at step B (right), suggesting that the manipulation carried out for case 2 significantly disrupted ensemble distinctness. **G)** In an example from a dataset comparing

spontaneous (resting) activity between a wildtype mouse ('wt', left) and a genetic model of neurodevelopmental deficits ('n.d.m.', middle, e.g. 22q11.2 microdeletion), population activations (dots in left / middle) are reduced to a low-dimensional space with PCA and then (right) k-means clustering analysis is carried out with multiple values of 'k' (e.g. clusters) for each condition. The point of the last significant decrease in within-cluster distances (relative to a temporally shuffled dataset, dotted line) is suggestive of the inherent number of activation "states" in the dataset. The relative disorganization of population states is quantifiable at this step in the 22q11.2 model. **H)** In another example experiment, discriminable activity patterns of coactive ensembles are compared between wakefulness, and anesthesia. Left: After dimensionality reduction of the continuous Ca^{2+} signals of all identified neurons using t-stochastic neighbor embedding (t-SNE, 1000 repetitions) across a range of perplexity values, and initial components, watershed segmentation (dotted line) is carried out on the 2-dimensional t-SNE density plot using a range of granularity values to optimally discern regions of similar activity ("microstates"). Right: for each condition, the observed number of microstates is compared against a shuffled surrogate distribution to test non-randomness of the observed microstates (here, within-frame shuffling disrupts recurrent ensemble co-activity patterns in the observed data resulting in a significantly higher number of discriminable microstates in the shuffled data). From here, quantitative ensemble analysis can be carried out. T-SNE density spaces plotted here originate from calcium imaging data that was used in Wenzel et al., 2019a, but not displayed, or analyzed, in its continuous format. Watershed segmentation/dotted lines shown here represent a purely schematic depiction (left), as are observed vs. shuffled data (right).

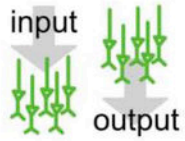
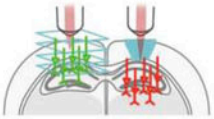
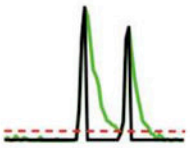
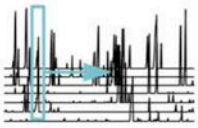
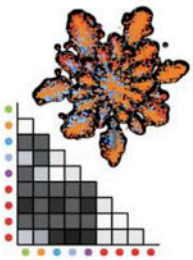
Table 1:

Direct comparison of cellular scale optical imaging and electrophysiology regarding key parameters for a planned experiment.

	Cellular scale optical Imaging	Cellular scale electrophysiology
Temporal resolution	⊖ Current Ca^{2+} sensors can indirectly detect even single APs, yet their rise time kinetics range in the dozens of ms, their decay time in the range of hundreds of ms, at best. Yet, this lack of temporal resolution as compared to real world neuronal dynamics may be overcome soon due to improved fluorescent voltage indicators, although their signal to noise ratio is still poor, especially in *in vivo* experiments.	✚ The only restriction to the temporal resolution of recorded electrical signals is the rate of digitization. Thus, neuronal firing can be captured at real world temporal resolution.
Spatial resolution	✚ Intracellular signals can be imaged at synaptic resolution.	⊖ Cellular resolution can only be indirectly achieved through algorithmic sorting of extracellularly recorded action potentials. Fidelity of unit assignment is inversely correlated to distance of the neuronal unit to the recording electrode.
Tissue depth of recording	⊖ Scattering brain tissue limits fluorescent imaging depth. However, fiber-endoscopic, or 3-photon, or prism-assisted imaging partially alleviates this problem (with some additional complications).	✚ There is practically no depth limit to electrical neural recordings using linear shank electrode.
Sampling density	✚ Imaging is possible at near real-world density	⊖ Recording density is constantly improved by ever denser electrode arrays, but is typically worse than in optical imaging.
Scalability of sampled area	✚ Sampled area is restricted mainly by microscopy limitations that are improving rapidly. Theoretically, large portions of a rodent brain can be imaged at cellular resolution without increase of sampling invasiveness.	⊖ Usually, cellular scale multielectrode arrays are planar, or linear. There is a limit as to how many linear probes can be inserted into a brain. Planar electrode arrays are typically limited towards more superficial brain structures.
Neural subtype specificity	✚ Theoretically, all neuronal and non-neuronal cell types can be imaged, limited only by the availability of molecularly defined fluorescent labeling.	⊖ Limited usually to regular spiking versus fast spiking neurons. Opto-tagging enables some neuron-type specificity but is low throughput and resource intensive. Non-neuronal cell types cannot be monitored.
Invasiveness of recording method	✚ Increasing the area of sampled tissue does not necessarily increase invasiveness of recording.	⊖ Increasing the area of sampled tissue typically goes along with an increase of invasiveness of recording.
Duration of recording	⊖ Continuous recording duration is usually limited to several hours, due to photobleaching, and tissue warming.	✚ Electrical signals can be continually recorded for months, limited only by reactive glial scarring around the electrodes that acts like an insulator.
Stability of recording	✚ Even though neurons can only be imaged for several hours per imaging session, they can faithfully be monitored for months across repeated imaging sessions.	⊖ Although possible, slight electrode movements severely complicate monitoring an algorithmically identified single unit across hours, sessions, or days.
Cost of recording method	= Cellular scale population imaging technology is becoming increasingly affordable. Basic cellular resolution recordings can be achieved relatively easily.	= Classic cellular scale recordings can be achieved at relatively low cost, but increasing recording density to Ca^{2+} imaging levels comes with increased cost.
Combination with other prevalent methods	= Combination with electrophysiology is unproblematic. Combination with other optical tools such as optogenetics requires careful consideration of excitation/emission spectra.	= Combination with optical techniques is generally unproblematic. However, ensure proper shielding of electrodes to minimize photoelectric effects.

Table 2:

Pipeline of considerations for identification and quantification of neuronal ensembles in optical imaging experiments

	**I** Experimental considerations	**General:** e.g. input- vs. output- vs. condition-centric, **Time:** temporal window / synchrony, activity timing, **Space:** overlap of ensembles, short- vs. long-range connectivity, microscale vs. mesoscale vs. macroscale imaging
	**II** Imaging	Choice of indicator, fluophore, indicator expression (e.g. Ca^{2+} vs. voltage imaging, GFP vs. RFP, dye vs. AAV-mediated vs. transgenic expression, SNR), acute vs. chronic imaging, single- vs. multi-plane, single- vs. multicolor, 1- vs. 2-photon, anesthetized or awake
	**III** Scoring activity	Motion correction, ROI identification, noise reduction, signal deconvolution, inferred vs. non-inferred transients, f vs. Δf/f vs. Δf/std, normalization (e.g. max.), amplitude thresholding, indicator decay removal, continuous vs. spike inference
	**IV** Scoring co-activity	Choice of temporal window (e.g. membrane time constants), dependence on recording modality (e.g. Ca^{2+} vs. voltage imaging), exclusion of neuronal inactivity / thresholding of per-frame-activity, normalization/weighting of neuronal activity (e.g. accounting for highly active neurons).
	**V** Analysis of ensembles	**Identification:** cell-cell or frame-frame, dimensionality reduction (e.g. PCA, t-SNE), unsupervised vs. supervised similarity metric (e.g. cosine, Euclidian distance), ensemble/state identification (k-means, watershed segmentation, affinity propagation). Spontaneous vs stimulus/behavioral clamp; SVM, multiregression. **Quantification:** Contextual ensemble activation, ensemble formation / size / stability / overlap, physiological vs. pathology-related ensemble dynamics, functional connectivity (e.g. graph theory). Comparison observed data vs. shuffled null distributions, comparison within-cluster vs. between-cluster distance