

Quantifying the effect of staining methods on extracted neuron morphology

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Abstract

The process through which neurons are probed and labeled for imaging is a key methodological choice in quantifying neuron morphology. However, little is known about how this choice may create biases in morphometrics. Here, we compare a large number of morphological features amongst samples collected from the same species, brain region, and cell-type. This allows us to identify differences in morphologies that can only be attributed to differences in experimental methods. In particular, we select four popular staining methods and compare each pair of them across different regions of the adult mouse brain. We find that all methods disagree in some respects when we look at the morphometry of the neurons. We propose that one explanation for this difference is the bias toward some cell types of neurons when one particular staining method is being used. Our result indicates the importance of the choice of staining methods for studying neuronal morphology, and suggests that more experimental detail is needed to allow analysis across large datasets of neuron morphology.

Keywords: Morphology; Staining methods; Cell Classification

Introduction

Imaging methods can reveal a lot about the characteristics of neuron morphology. There are numerous techniques through which a neuron may be imaged and studied. Functionally, fluorescent dyes may be used to interrogate calcium activity, a proxy for the voltage activity of a neuron. While new techniques such as expansion microscopy allow for detailed pictures of a cell's internal makeup and structure (Chen, Tillberg, & Boyden, 2015). Techniques used to identify a neuron's morphology generally rely on stains or dyes to target and render visible a neuron's structure. These imaging techniques provide massive amounts of data that promise to give insight into the complicated structure of neuron morphology.

Staining methods remain one of the key imaging techniques. The oldest method, Golgi staining, is a silver staining technique that randomly labels neurons in their entirety

(Koyama, 2013). Today, Golgi staining can be divided into three methods: Rapid Golgi, Golgi-kopsch and Golgi-Cox (Koyama, 2013). Golgi staining must be performed after the sample has been fixed, meaning that in vivo or in vitro recordings cannot be connected to histology. The stain is then imaged through light microscopy. Alternatively, immunostaining allows neurons to be labeled in vivo or in slice samples, meaning that electrophysiology can be related to morphology. Immunostaining uses antibodies to target neuronal cell-type markers, which can be labeled with fluorescent or chromogenic tags for imaging. One popular class relies on biotin variants, such as biocytin or neurobiotin, being conjugated to an antibody. A complex of biocytin and its binding partner, avidin, are tagged with a fluorescent or colored label that can then be imaged. The avidin-biotin complex is beneficial for imaging, and may be imaged through either light, fluorescent, or EM microscopy depending on the label. Finally, fluorescent proteins such as GFP can be introduced transgenically to be expressed in neurons and then imaged through fluorescent microscopy. (Marshall, Molloy, Moss, Howe, & Hughes, 1995) The above methods are all used today to characterize morphology, depending on the needs of the researcher.

Although large databases of neuron morphologies exist and provide a useful resource for studying morphology, little is known about the effect of the experimental process on resulting neuron morphology. Previous studies have shown differences due to methodology over labs within a same class-type neuron. For example (Scorcioni, Lazarewicz, & Ascoli, 2004) studied CA1 and CA3 neurons extracted by different labs, and demonstrated that many morphometric features show artifacts as a result of inter-lab variation and experimental methodology. These artifacts also obstruct accurate classification of neuronal cell types (Vasques, Vanel, Villette, & Cif, 2016). Hence we need to understand the source of this variability to better utilize these databases for studying morphology.

Here we focus on variation in neuron morphology related to the staining, or the fluorescent labeling method used, though we will refer to both of these as staining method. We analyze mouse data that has been uploaded by various labs to the public morphology repository neuromorpho.org (Ascoli, 2006) and group them based on the biological attributes and the

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staining methods. By matching on biological attributes and computing the morphometry of each group we identify the variation that can be explained by different staining methods.

Methods

Data Acquisition

To compare neurons from different staining methods, we used data from neuromorpho.org (v7.3), a publicly available database of morphology. We first searched for neurons from adult mouse that represent the control group of the source literature. Then, we separated neurons based on their biological attribute – the primary and secondary cell class (e.g. pyramidal and interneuron) and primary, secondary and tertiary brain regions (e.g. neocortex, frontal, layer 3). We only used neurons with complete dendritic trees, and extracted their dendrites. These criteria specify around 10,000 neurons (out of the 80,000 neurons comprising the entire neuromorpho database). Next we compared neurons that have been extracted with four different staining methods: Golgi staining, Golgi-Cox staining, GFP and biocytin immunostaining. At the end of this step we found eight brain regions that have at least two of these staining methods (Figure 1). The number of neurons in these groups range between 80 to 120. We use the naming convention for each brain region of the Allen Institute mouse brain atlas (Figure 1a). Once these groups were identified the original research papers were double checked for accuracy and relevance. The ultimate dataset comprises neurons collected from 31 separate research articles (Table 1), the earliest of which is from 2006 (Figure 1b). These groups are used to quantify the effect of the staining methods.

Morphological features

To compare neuron morphology we need to quantify them. The morphology of a neuron is modeled by a geometric graph, where the nodes and edges represent the point on the morphology and links between the points, respectively (Stockley, Cole, Brown, & Wheal, 1993). We used a set of features that are previously described as L-measure (Scorcioni, Polavaram, & Ascoli, 2008). In L-measure every feature is summarized to one scalar value. Using these feature set helps to compare morphologies.

Statistical testing

We sought to test for an effect of staining method on morphology above effects that are explained through biological attributes. The morphological features are generally continuous valued, while the biological and non-biological features are generally categorical. Further, the morphological features generally follow a non-Gaussian distribution (data not shown). This requires using non-parametric tests. We use the Wilcoxon rank sum test. That is, for biological attribute $b \in \mathcal{B}$, we test:

$$H_0(B = b) : \mu_{1/2}(M|B = b, S = s_1) = \mu_{1/2}(M|B = b, S = s_0), \quad (1)$$

for all $b \in \mathcal{B}$, where $\mu_{1/2}$ represents the median, S the staining method, and M the morphological feature. The hypothesis that no overall effect exists for a given morphological feature is

$$H_0 : \bigcap_{i=1}^{|\mathcal{B}|} H_0(B = b_i),$$

for the N levels in B . To correct for multiple testing we use the Bonferroni correction.

Average effects

The differences in morphology between staining methods can also be quantified over brain regions by considering the difference in means:

$$\beta_j = \mathbb{E}(\mathbb{E}(M_j|B, S = S_1) - \mathbb{E}(M_j|B, S = S_0)), \quad (2)$$

which summarizes the average difference in morphological feature M_j . This corresponds to the average treatment effect in the causal inference literature (Pearl, 2009), although we make no strong claims about causality here. A null distribution for each β_j is generated by repeated permutation of staining label, allowing us to determine significance levels.

Results

To make a database for comparing staining methods, we selected four popular staining methods and identified brain regions in adult mice where data existed from which we could make at least one comparison. We found eight distinct regions that cover data from more than 30 recent papers. In Figure 1 the areas of the brain studied are shown. The source papers are listed in the Table 1. We then plot a few samples of each group (Figure 2). Having this table lets us to find the comparison groups between two staining methods.

To compare two staining methods for one particular morphological feature, we compare the distribution of the feature for neurons in each staining method. As an example we compare morphologies that have been extracted by Golgi and Golgi-Cox methods. Figure 3 (top) shows that, except for one area (CA1), all the features have a significantly different distribution. Moreover, we show the quantitative difference by computing the absolute difference in the the mean of each feature averaged over brain regions, we observed that, except for two features, all of them are significantly different. Using this approach we can test whether a morphological feature is significantly different for two methods.

For each pair of staining method, we then asked whether they have generated statistically similar neurons. To test this hypothesis, we used the significant level for each feature and counted the number of features that are (highly) significant for all the regions tested and features recorded (Table 2). For all the comparison pairs there are feature that are highly different. Interestingly, we observed that among these four staining methods, Golgi-Cox has highest percentage of different features.

Discussion

Here we focused on the effect of the staining methods and showed a significant difference between neurons that were extracted from the same biological attribute but by two different staining methods. Although this analysis was performed for the staining method, a similar approach could be taken to study the effect of other non-biological attributes such as reconstruction software or objective type on the final morphology. Understanding the source of these artifact helps us to generate databases that reflect an accurate picture of the variation of neurons in the brain.

While our analysis highlights differences amongst ostensibly equivalent brain regions and cell types, there are a number of ways of accounting for the differences in morphology we observe. First, there may exist procedural differences between labs, coming from preferences for particular sub-regions or cell types or other preparation details not reported. Second, there may exist differences caused by other methodological details that happen to be correlated with the staining method, not because the method goes in hand with the staining method, but just by chance in the data we analyzed. For example if the objective type used in the microscopy correlates with different staining methods then this is a potential confound. Finally, differences may be due to the focus of our study: there may exist differences owing to artifacts inherent to the different staining methods. By performing the same comparison over lab groups and brain regions, we mitigate these confounding effects to some extent, and thus better measure differences particular to staining method. But these other explanations can not be ruled out entirely without more controlled comparisons. This is challenging, even with a large database such as neuromorpho.org. Specific experiments would have to be performed to settle these questions.

Why might the staining method affect morphology? There are a few possible explanations: 1) the staining methods represent different physical and biochemical reactions that may interact differently with different neuronal elements; 2) different methods rely on different types of microscopy that also may highlight different morphological features; and 3) researchers may have a preference for the cleanest examples of neuron morphology which may be different for different methods.

The features set used here is often used for the basis of cell classification (Vasques et al., 2016). In this regard, our results suggest the need to standardize and carefully characterize these artifacts. Alternatively, although some features are changed by different staining method, there are some less affected by them (Figure 3). One possibility is to then use these features, that vary most by cell type and least by staining method, as the basis of classification.

Fully characterizing neuronal morphology and its relation to function relies on the generation and analysis of vast amounts of data. Across neuroscience, many-institution collaborative efforts to understand neuroscience questions are now common (e.g. (International Brain Laboratory. Electronic ad-

dress: churchland@cshl.edu & International Brain Laboratory, 2017)). Amongst the wealth of datasets available, the need for understanding variability due to the data generation process is important for drawing inferences and analyzing data across disparate sources. It is important to understand this variability when studying neuronal morphology.

Brain-Region	Golgi	Golgi-Cox	GFP	Biocytin
CA1	[1,2]	[3,4]	[5]	[6,7,8,9]
VIS2/3		[10]	[11, 12, 13]	
DG-sg	[14,15]	[16]		[17,18,19]
SS2/3	[20]	[21]		
mPFC2/3	[22]	[23,24]		
MOBgr		[25]	[26,27]	
FC3	[28]	[29]		[30]
MSNs	[31]	[31]		[31]

Table 1: Sources of morphological data submitted to neuro-morpho.org and used in analysis, by brain region and staining method. Search criteria to identify these papers provided in Methods. 1. (Nobili et al., 2017), 2.(Wang et al., 2016), 3. (Morelli et al., 2014), 4. (F. H. Lee et al., 2011), 5. (Sauer et al., 2015) 6. (Zhang et al., 2016), 7. (S.-H. Lee et al., 2017), 8. (Tyan et al., 2014), 9. (Basu et al., 2013), 10. (Vannini et al., 2016), 11. (DSouza et al., 2016), 12. (Jiang et al., 2015), 13. (Longordo et al., 2013),14. (Yanpallewar et al., 2012),15.(Qin et al., 2014), 16. (Carim-Todd et al., 2009), 17.(Chancey et al., 2013), 18.(Gonaves et al., 2016), 19. (Walter et al., 2007) 20. ,(Alpr et al., 2006), 21. (Smit-Rigter et al., 2012),22. (Juan et al., 2014),23. (Nashed et al., 2015), 24.(Errico et al., 2014), 25. (McDole et al., 2015), 26. (Breton-Provencher et al., 2016), 27. (Belnoue et al., 2016), 28. (Karlsson et al., 2016), 29. (F. H. Lee et al., 2011),30. (Rocher et al., 2010), 31. (Cazorla et al., 2012).

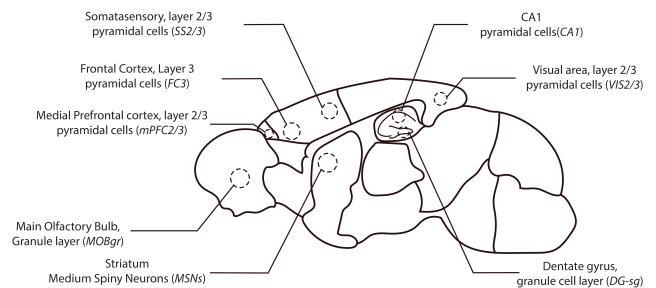


Figure 1: Brain Regions for Comparing Staining Methods. Sagittal view of the mouse adult brain is shown. Existing brain regions for comparison are identified by the mouse brain atlas (Allen institute)

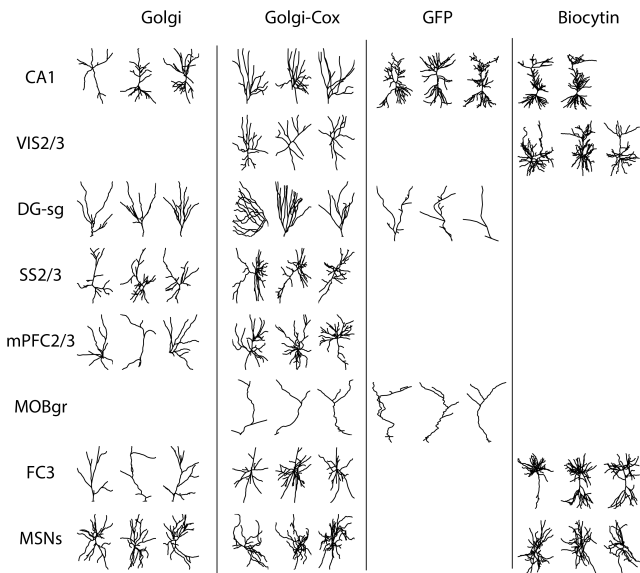


Figure 2: Example neurons across different brain regions and staining methods. If there exists morphological, three sample neurons are plotted.

Staining Method 1	Staining Method 2	% significant	highly significant
Golgi	Golgi-Cox	62	56
Golgi	GFP	15	14
Golgi	Biocytin	19	16
Golgi-Cox	GFP	34	28
Golgi-Cox	Biocytin	33	23
GFP	Biocytin	13	3

Table 2: For the staining method 1 vs. 2, percentage of features that are significantly ($p < .05$) or highly significantly ($p < .001$) amongst all the feature set and brain regions is shown on the third and fourth columns, respectively.

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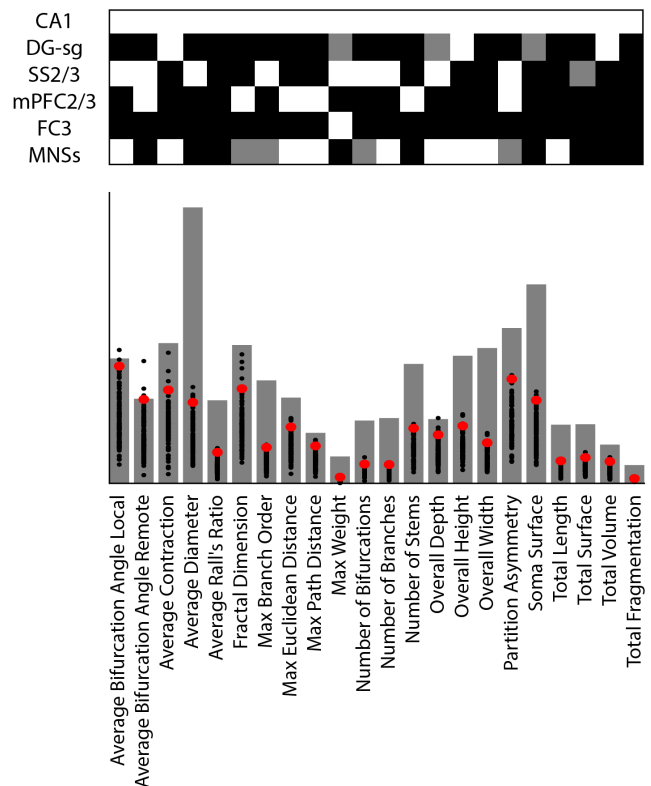


Figure 3: Comparing morphological features quantified by Golgi and Golgi-Cox staining methods. a) Significance level (white = not significant; gray = significant, $0.001 < p \leq 0.05$; black = highly significant, $p < 0.001$) shown by feature and brain region. Wilcoxon rank sum test, Bonferroni corrected for hypotheses over all brain regions and features. b) Average absolute difference in morphological features over brain regions (gray bar), compared with a null-distribution generated through permutation (samples = black dots, red dot = 95th percentile). Features are normalized by their standard deviation.

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