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Semi-automated quantification and neuroanatomical mapping of heterogeneous cell populations

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ABSTRACT

Background: Our group studies the interactions between cells of the brain and the neurotropic parasite *Toxoplasma gondii*. Using an in vivo system that allows us to permanently mark and identify brain cells injected with *Toxoplasma* protein, we have identified that *Toxoplasma*-injected neurons (TINs) are heterogeneously distributed throughout the brain. Unfortunately, standard methods to quantify and map heterogeneous cell populations onto a reference brain atlas are time consuming and prone to user bias.

New method: We developed a novel MATLAB-based semi-automated quantification and mapping program to allow the rapid and consistent mapping of heterogeneously distributed cells on to the Allen Institute Mouse Brain Atlas. The system uses two-threshold background subtraction to identify and quantify cells of interest.

Results: We demonstrate that we reliably quantify and neuroanatomically localize TINs with low intra- or inter-observer variability. In a follow up experiment, we show that specific regions of the mouse brain are enriched with TINs.

Comparison with existing methods: The procedure we use takes advantage of simple immunohistochemistry labeling techniques, use of a standard microscope with a motorized stage, and low cost computing that can be readily obtained at a research institute. To our knowledge there is no other program that uses such readily available techniques and equipment for mapping heterogeneous populations of cells across the whole mouse brain.

Conclusion: The quantification method described here allows reliable visualization, quantification, and mapping of heterogeneous cell populations in immunolabeled sections across whole mouse brains.

1. Introduction

Given the intimate link between neuroanatomic location and function, there has always been an interest in mapping and quantifying cells and disease processes throughout the brain. Until recently, processing these large sets of data has been time consuming and labor intensive as it required manual counting and neuroanatomic localization. To address this issue, various quantification methods have been developed. Stereological methods quantify cells in a precise stereotyped manner (Sterio, 1984; Howard and Reed, 1998; Gundersen, 1986; West et al., 1991 Hedreen, 1998). These techniques were developed to count cells in relatively small sample regions of pre-identified neuroanatomic locations and in regions with uniform cell densities (Schmitz and Hof, 2005). However, in certain scenarios, like infectious models of disease, the cells of interest can be heterogeneously distributed throughout the brain and idiosyncratically vary between mice, thus making proper stereology quantification difficult to do. To address this gap, there has been growing focus on imaging of whole brains or thick serial sections using a variety of imaging techniques such as confocal, 2-photon, or light-sheet microscopy, and micro-optical sectioning tomography. These imaging techniques have been used in combination with complex algorithms and machine learning techniques to localize and quantify

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cells (Peng et al., 2017;Inglis et al., 2008). The equipment used and methods of quantification are not readily available at many universities. Here, we describe a semi-automated method that can be utilized by most researchers because it relies on standard immunohistochemistry and basic light microscopy coupled to a MATLAB (Mathworks, Inc., Natick, MA)-based program to count and map cells of interest onto the Allen Institute Mouse Brain Atlas (http://www.brain-map.org/).

We developed this methodology to define neurons and CNS regions that are targeted by the protozoan parasite, *Toxoplasma gondii*. *T. gondii* is an intracellular parasite that naturally infects a wide range of warm blooded hosts, including humans and rodents (Dubey et al., 1998; Dubey, 2008). In most hosts, *T. gondii* is able to establish a life-long infection in specific tissues. In humans and rodents, the central nervous system is the major organ for *T. gondii* persistence (Dubey et al., 1998; Remington and Cavanaugh, 1965). This tropism for and persistence in the CNS underlies the devastating symptomatic disease *T. gondii* causes in those with under-developed immune responses (e.g. fetus, AIDS patients) (Luft and Remington, 1992; McLeod et al., 2009). The persistent form of the parasite, the bradyzoite, is a slow-growing form that establishes intracellular cysts, primarily in neurons (Ferguson and Hutchinson, 1987; Melzer et al., 2010; Cabral et al., 2016). Thus, understanding the *T. gondii*-neuron interaction is essential to understanding symptomatic toxoplasmosis.

Until recently, the only way to identify infected brain regions was by cyst location. Several studies found that encystment is highest in the neocortex, thalamus, and striatum, along with other forebrain structures (Berenreiterová et al., 2011; Evans et al., 2014). However, cyst location does not necessarily give an accurate representation of which neurons are infected, as cysts can be located in distal processes > 100 microns from the neuron soma (Cabral et al., 2016). The recent development of a system in which permanent host cell GFP-expression is triggered by injection of a parasite protein significantly changed this landscape (Koshy et al., 2012; Cabral et al., 2016). First, GFP expression enables visualization of the whole neuron (both cyst location and soma) (Koshy and Cabral, 2014). Second, as this system is dependent upon injection of parasite protein, not active infection, it revealed that *T. gondii* injects its effector proteins— parasite proteins that hijack host cell processes and signals— into far more host cells than it productively infects (Koshy et al., 2012). This finding is particularly pronounced in the CNS, where *T. gondii*-injected neurons (TINs) out-number cysts up to 50-fold (Koshy et al., 2012). Thus, by mapping TINs, we can determine if cysts are commonly found in specific brain regions because these regions are particularly susceptible to *T. gondii* infection or if neurons display regional differences in the capability to clear intracellular parasites, as has been suggested for West Nile Virus (Cho et al., 2013). To test this hypothesis, we need to reliably quantify and neuroanatomically localize TINs throughout the entire brain.

Unfortunately, TIN location in the brain widely varies by mouse and TINs are non-uniformly dispersed throughout the brain. Manual counting of these large populations of TINs would be time-consuming and inefficient. Thus, the lack of large-scale, efficient methods to quantify and localize idiosyncratic cell distributions throughout a whole mouse brain section was an impediment to moving this work forward. To overcome this barrier, we developed a semi-automated, MATLAB-based method that allows rapid quantification and neuroanatomical localization across whole-brain histological images. While the program has been developed for identifying TINs, it can be applied to count and localize a wide variety of cells or processes within histological images of sagittal mouse brain sections.

2. Methods

2.1. Animal and parasite model

The animal model used for these experiments is B6.Cg-Gt(ROSA) 26Sort$^{m6(CAG-ZsGreen1)Hze}$/J on a C57BL/6J background (Madisen et al., 2010). The cells of these Cre reporter mice only express a green fluorescent protein (ZsGreen) after Cre-recombinase mediated genetic recombination. The *T.gondii* parasite strain used for this study was engineered to inject Cre into host cells concomitantly with parasite effector proteins (parasite proteins used to manipulate host cells) (Koshy et al., 2010; Koshy et al., 2012).

Parasites were maintained via serial passage through human foreskin fibroblasts using DMEM supplemented with 2 mM glutagro, 10% fetal bovine serum, and 100 I.U./ml penicillin/ 100 µg/ml streptomycin (Cabral et al., 2016). Mice were infected at 2–3 months of age via intraperitoneal (IP) injection with freshly syringe-released parasites. Mice were inoculated with 10,000 parasites per 200 µl volume in USP grade PBS. At 3 weeks post infection, animals were sedated with a ketamine/xylazine cocktail, intracardially perfused with saline followed by 4% paraformaldehyde, after which brains were harvested.

2.2. Immunohistochemistry

Left and right brain hemispheres were isolated and 40 µm-thick sagittal sections were generated using a freezing sliding microtome (Microm HM 430). Sections were sampled every 200 microns to obtain a set of 20 sections, that would increase the likelihood of matching sections to the Allen Brain Mouse Atlas (ABA). Sections were premounted on slides before immunohistochemical labeling (Fig. 1).

To insure adhesion of tissue onto slides, tissue was allowed to air-dry onto slides overnight followed by dehydrated using increasing then decreasing concentrations of 50%, 75%, 95%, and 100% ethanol. Slides were washed with TBS, peroxidases inactivated (3%H_2O_2/10% methanol), permeabilized (0.6% Triton X-100), blocked (1.5% BSA and 1.5% goat serum), and incubated with Rabbit anti-ZsGreen (Clontech, Cat. No. 632474, 1:10,000) for 15–18 hrs. Next, slides were incubated in Goat anti-rabbit polyclonal biotinylated conjugated antibody (Vector Labs, Cat. No. BA-1000, 1:500) for 2 h, incubated with avidin-biotin complex kit (ThermoFischer Scientific, Cat. No. 32,020) for 2 h and visualized with a 3,3′-diaminobenzidine kit (Vectastain, Vector Labs Cat. No. SK-4100). Sections were then counterstained with cresyl violet for Nissl labeling (Dorph Petersen et al., 2001). Although the Nissl counterstain is not part of the colorimetric thresholds used in cell detection, it increased the contrast between TINs and surrounding tissue, which improved the consistency of automated cell detection. After processing tissue sections were estimated to shrink by over 50%.

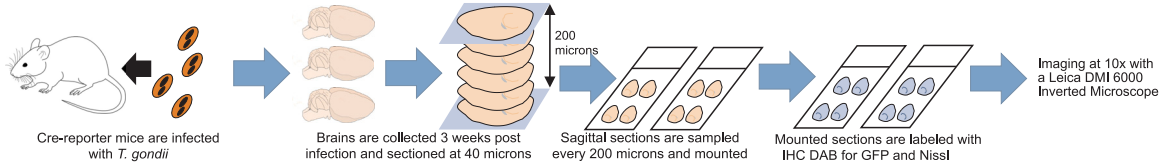

Fig. 1. Tissue processing workflow and mapping of sections onto Allen atlas reference.
Three weeks after intraperitoneal infection with parasites, brains are harvested, sectioned, and then sampled every 200 µm. Slides are premounted onto charged slides, immunloabled for a green fluorescent protein (ZsGreen), counterstained with Cresyl violet and imaged.