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***Toxoplasma gondii* tissue cyst purification using Percoll gradients**

Elizabeth A. Watts[^], Animesh Dhara, and Anthony P. Sinai

Department of Microbiology Immunology and Molecular Genetics, University of Kentucky College of Medicine, Lexington, Kentucky

Abstract

The protozoan parasite *Toxoplasma gondii* is capable of infecting all warm blooded animals and humans. Infectious, transmissible forms of the parasite include oocysts produced by the sexual cycle within the definitive feline host and tissue cysts that form *Toxoplasma* in the CNS and muscle during the asexual cycle within all chronically infected warm-blooded hosts. These tissue cysts are populated with slow growing bradyzoites which have been until recently thought to be dormant entities in the context of immune sufficiency. Reactivation to active growth during immune suppression is of critical clinical importance. Yet we know little about tissue cysts or the bradyzoites they house as the diversity of tissue cysts cannot be replicated in cell culture systems. Our optimization of tissue cyst purification from the brains of infected mice using Percoll gradients provides an efficient means to recover *in vivo* derived tissue cysts that can be applied to imaging, cell-biologic, biochemical, transcriptomic and proteomic analyses.

Keywords

Toxoplasma gondii; tissue cyst; bradyzoite; Percoll

Introduction

Despite their critical role in the pathogenesis of *Toxoplasma* infections in the context of immune suppression, chronic toxoplasmosis remains grossly understudied (reviewed in (Sinai et al., 2016)). This phase, mediated by bradyzoites within tissue cysts presents significant challenges, not the least of which is the fact that true tissue cysts have to be derived from *in vivo* sources. In the murine model, tissue cysts are typically obtained from the brains of chronically infected animals as the brain is a primary site for cyst formation (Ferguson, Graham, & Hutchison, 1991; Ferguson, Huskinson-Mark, Araujo, & Remington, 1994) and can be isolated and recovered relatively easily (Cornelissen, Overdulse, & Hoenderboom, 1981). In addition, processing of infected brains is aided by the relative ease of homogenization that allows for the selective disruption of host tissue while keeping the tissue cysts intact (Cornelissen et al., 1981). The yield of tissue cysts from infected animals is affected greatly by both the strain of parasite used and genetic background of the host

Tel: 859-323-6680; Fax: 859-257-8994 sinai@uky.edu.

[^]Current Address: Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia.

(reviewed in (Sinai et al., 2016)). The combination of the prototypical Type II reference strain ME49 (available from the AIDS Resource Center) and CBA/J mice (Jackson Laboratories) appear to be optimal for cyst yields. It should be noted however that yields can be highly variable despite using an established parasite strain and an in-bred host. This is what we found in the compiled data from 99 independent tissue cyst purifications over a 2 year period where yields per mouse covered the range from around 500 to over 15,000 cysts per brain (Watts et al., 2015). The average yield tended to be around 3000 cysts per brain (Watts et al., 2015). Such numbers allow for detailed imaging studies, biochemical and molecular biological analyses.

The chronic phase in the mouse infection model is established roughly 3 weeks post infection where stable tissue cysts can be reproducibly recovered (see (Watts et al., 2015)). While cysts can be recovered between 2 and 3 weeks post infection they tend to have extremely thin cyst walls and are extremely fragile with low yields suggesting the loss of integrity during the purification (Watts et al., 2015). The yields of tissue cysts between weeks 3 and 8 post infection are fairly uniform as is their size (diameter) distribution (Watts et al., 2015). What does appear to change however, are their physical characteristics in Percoll gradients indicating a change in their density and by extension their composition (Watts et al., 2015). Indeed, the relationship between tissue cyst size and occupancy, the packing density was found to be variable and likely accounts for the differences in their hydrodynamic densities (Watts et al., 2015). Thus, in addition to providing a means to purify tissue cysts from bulk brain material, the density in Percoll gradients can be used analytically to establish evidence for structural changes in the physical properties of tissue cysts useful for instance in the analysis of mutant phenotypes or the assessment of the effects of drug treatment. For this reason, reproducibility in the establishment and running of Percoll gradients is essential, as is the periodic calibration of gradients using density calibration beads as discussed below.

CAUTION: <*Toxoplasma gondii*> is a Biosafety Level 2 (BSL2) pathogen. Follow appropriate biosafety guidelines and regulations for the use and handling of pathogenic organisms. This protocol involves the tissue cyst form of the parasite and the extensive use of syringes and needles that pose a significant biohazard risk.

Animal Biosafety

CAUTION: This protocol involves the handling of mice chronically infected with *Toxoplasma gondii* and requires Animal Biosafety Level 2 (ABSL-2) conditions. Protocols involving live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals. Infected mice do not shed parasites in either feces or urine. There is no infection risk from live parasites. Any risk of infection is associated with the handling of infected tissue.

Biosafety considerations

Toxoplasma gondii is a BSL2 pathogen and all handling of live parasite should be done in an approved biosafety cabinet. In addition to lab coats and gloves, the use of disposable vinyl

sleeves and cuffs with the gloves over the cuffs is recommended. The protocol uses syringes and needles which increases the exposure risk for a lab acquired infection. Syringes and needles are used to homogenize the infected brain tissue prior to loading on the Percoll gradients. These steps are done in a small mortar and care must be taken to have ones hands “upstream” of the needles at all time. In addition, when ejecting the brain homogenate ensure careful handling to avoid spraying or splashing of this material is critical. In addition to needles, the dissection tools required to harvest the brains from the infected animals represent sharps that need to be handled with care and disposed of appropriately. Finally, several steps require centrifugation which can generate aerosols, or in the case of a tube failure, the spread of infected material. For this reason it is essential that all centrifugation steps be conducted within enclosed housings using a swinging bucket rotor. The enclosed rotor buckets should be opened in the biosafety cabinet.

Personal Protective Equipment

Laboratory coat

Disposable Latex or Nitrile gloves

Disposable Sleeve protector with knit cuff (VWR 892028-810)

Basic Protocol 1: Preparation of the infected mouse brain homogenate

The brain is the primary location for the establishment of *T. gondii* tissue cysts (Dubey et al., 2016; Ferguson et al., 1994; Nath & Sinai, 2003). While other organs (Dubey, 1997) and skeletal muscle (Ferreira-da-Silva Mda, Takacs, Barbosa, Gross, & Luder, 2009; Guimaraes, de Carvalho, & Barbosa, 2008; Swierzy et al., 2014) also contain tissue cysts, their relative numbers are low and recovery is complicated by difficulties associated with tissue homogenization. In contrast the brain is readily accessible and easy to homogenize in a manner that maintains the integrity of the resident tissue cysts. This, together with the brain containing the highest concentration of tissue cysts makes it the ideal organ from which to purify cysts for further study.

Materials

Spray bottle with 70% Ethanol

10× Filter sterilized Phosphate Buffered Saline (PBS)

Sterile 1× Phosphate Buffered Saline

Sterile 1× PBS with 1% Tween-80 (Sigma P4780)

Note: Make 1×PBS by diluting out the 10×PBS and then add Tween-80 to a concentration of 1%

4 Liter Plastic beaker with 2 liters of 10% bleach to be placed in the biosafety cabinet

Large low profile ice bucket/pan (Corning/Fisher 432093)

Dissection surface (styrofoam lid covered with aluminum foil)

Dissection tools (sterilized in an autoclave pouch) (Figure 1A,B)

Sharp fine point scissors –straight and curved

Forceps rounded tip

Stainless steel microspatula

Small porcelain mortar and pestle (Fisher FB961B sterilized in an autoclave pouch) (Figure 1A,B)

Sterile small-head cell scraper (Corning #3010)

5ml Luer-Lok Syringes (Becton Dickerson #309646)

Needles (Becton Dickerson):	16 Gauge (Purple-cat #305198)
	18 Gauge (Pink-cat#305196)
	20 Gauge (Yellow-cat#305179)
	23 Gauge (Green-cat#305194)

Preparation of the brain homogenate—Spray down the surface of the Biosafety cabinet with 70% ethanol. Place a 4 liter plastic beaker filled with 2 liters of 10% bleach, small biohazard bags for the animal carcasses and an ice pan with ice. Prepare the dissection platform and dissection tools while also placing the mortar and pestle on ice (Figure 1A,B). Also pre-chill the 1×PBS-1%Tween80 and 1×PBS on ice. All solutions should be pre-chilled prior to harvesting the infected mouse brains.

Harvesting the infected mouse brains

1. Euthanize the mice using an IACUC approved protocol such as CO₂ asphyxiation followed by cervical dislocation.
2. Place the euthanized mouse, immediately following death, on the dissection platform in the biosafety cabinet and spray down with 70% ethanol to surface sterilize (Figure 1C).
3. Using the rounded forceps lift the skin behind the mouse's ears and cut using the curved scissors exposing the skull (Figure 1D).
4. Insert the forceps into the eye sockets to secure the head while making a shallow cut on the back of the skull. Cut the skull cap from back to front on each side of the head and remove or fold the skull cap forward exposing the brain (Figure 1E).
5. Glide the stainless steel microspatula under the brain along the base of the skull severing the spinal cord (Figure 1E). Lift the intact brain on the microspatula and transfer into the prechilled mortar (Figure 2A).

Note: Should a portion of the brain need to be used for histological analyses, harvest the desired portion for fixation and transfer the remainder into the mortar.

6. Drop all dissection tools into the beaker within the biosafety cabinet containing 10% bleach, avoiding splashing, to rapidly disinfect.

Preparation of the Brain Homogenate

1. The tissue cyst purification has been optimized for CBA/J mice (The Jackson Laboratory JAX000656) infected with the Type II *Toxoplasma gondii* strain ME49 (ATCC50611). The maximal average tissue cyst yield is obtained using 2 brains per Percoll gradient. Average tissue cyst yield drops dramatically if using more than 2 mouse brains per Percoll gradient as the gradients get overloaded with brain material reducing the recovery of cysts.
2. Collect 2 brains in the chilled mortar (Figure 2A) and grind using the pestle into a paste with the consistency of toothpaste (Figures 2B,C). Do not add any buffer during this initial homogenization.
3. To the brain homogenate paste add 3ml of PBS with 1% Tween80 (Figure 2D). Gently mix the homogenate and PBS+1% Tween80 with a 5ml pipette, returning the suspension to the mortar. Discard the contaminated pipette into the plastic beaker containing the bleach, while drawing up bleach to decontaminate it. Using the pestle, incorporate the brain homogenate paste into the PBS +1% Tween80 until a uniform consistency is achieved while keeping the mortar on ice (Figure 2E). Small fragments of tissue can be scraped off the pestle and mortar walls using the small head cell scraper (Figure 2E). Discard the pestle and cell scraper into the plastic beaker with bleach. The homogenate will be non-uniform with small flakes of brain tissue (Figure 2F). Further homogenization will be achieved using progressive syringe passages using needles of increasing gauge.

Syringe Passaging the Brain Homogenate (Significant Biohazard Danger)

1. Syringe passaging of the infected brain homogenate involves drawing the homogenate through needles of increasing gauge (decreasing bore) to effectively shred the brain tissue while leaving the tissue cysts intact. This step, which involves syringes, needles and *Toxoplasma* infected brain tissue, presents a significant biosafety risk. In addition to the risk of a needle stick, clogging of needle and the generation of back-pressure within the syringe presents a potentially dangerous situation.

For this reason:	a	Use ONLY LUER-LOK syringes.
	b	NEVER recap or reuse needles. Discard as described below.
	c	Do NOT alter the sequence for the gauge of needle being used.
	d	Do NOT proceed with syringe passage until the homogenate made using the mortar and pestle is devoid of any large fragments of brain tissue.

2. Once the brain homogenate to which the 3ml of PBS+1% Tween80 has been added has the appearance seen in Figure 2F assemble a 5ml LuerLok syringe

with a 16Gauge needle (pink). Slowly draw the brain homogenate into the syringe minimizing the incorporation of air (Figure 2G).

3. Gently eject the homogenate back into the mortar on ice, running it slowly along the wall of the mortar. Avoid splashing the homogenate when discharging back into the mortar. Repeat this uptake and ejection cycle step 5 times (a passage) returning the homogenate to the mortar. Given the large bore of the needle these passages should not require much force. Should the needle at this or any subsequent stage become clogged, discard and use a new syringe and needle. Should there be considerable material within the barrel of the syringe use a hemostat or firm forceps to grip the needle while rotating the syringe off the needle. Return the material within the syringe to the mortar. DO NOT attempt to force a blocked needle using the backpressure of the syringe as this may result in the spraying of your sample and present a dangerous situation.
4. When the 5th passage is completed return the homogenate to the mortar. Draw bleach from the waste bucket into the syringe and eject back into the waste bucket. Discard the syringe and needle (as one unit) into an appropriate sharps container.
5. Repeat this process with 5 passages through the 18 Gauge (Purple) needle. You will notice somewhat higher back pressure is needed to eject the homogenate. At the end of the 5 passages decontaminate and discard your syringe and needle as above.
6. Repeat this process (5 passages each) with the 20 Gauge (Yellow) needle and finally the 23 Gauge (Green) needle using the same precautions. Passage through the narrower bore needles will also cause some degree of foaming (Figure 2H) which can be controlled somewhat by ejecting the homogenate slowly along the wall of the mortar.
7. Once the entire set of syringe passages is completed, add 6ml of 1× sterile PBS (NOT PBS+1%Tween80) and make a uniform suspension by mixing within the mortar using a 10ml pipette. Return this suspension which is ready to load onto the Percoll gradient, to the mortar. The formation and running of the Percoll gradients is discussed in detail in Basic Protocol 2.

Basic Protocol 2: Establishing and Running of Percoll Gradients

Percoll is a density gradient medium that has broad applications for the separation of cells, organelles, viruses and other subcellular particles. Chemically, Percoll is a plastic (polyvinylpyrrolidone (PVP) coated silica that is at physiological ionic strength and pH and is non membrane permeant. Its overall low viscosity and absence of toxicity coupled with the capacity to establish self-forming gradients following centrifugation with moderate g-forces makes it ideal for cell separation based on density. Percoll is also easily removed allowing for the purification of the target cell by centrifugation following dilution. The inert nature of Percoll exhibits minimal interference with downstream assays on the purified target cell population. Percoll gradients fractionate the biological sample on the basis of

density. As the gradient forms during centrifugation, buoyant membrane bound entities (in this case, tissue cysts) enter the Percoll flowing along the centrifugal force. When the tissue cysts arrive at a region of the Percoll gradient matching their own hydrodynamic density, they remain in that region as they lose buoyancy. At first approximation the density of the tissue cysts is governed by the relative ratio of membrane and non-membrane components. This “equilibrium density” at which the tissue cysts are recovered is a reflection of their relative physical composition rather than size, which plays a much more limited role in this system. Shifts in the distribution of tissue cysts over the progression of the infection *in vivo* (Watts et al., 2015) reflect fundamental changes in the physical properties of the cysts governed by changes in their composition. Such information can be very useful in comparing tissue cysts between, for instance, a wild type and mutant parasite. The fact that the Percoll purification can be used as an analytical tool necessitates calibration of the gradients that can be readily achieved using specific density calibration beads as discussed below.

The conditions for the Percoll purification represent a modification of the original protocol described by Cornelissen et al. (Cornelissen et al., 1981). We found that by altering the composition and assembly of the gradient we were able to better resolve tissue cysts based on their density while separating them from the prominent erythrocyte band derived from the brain homogenate (Watts et al., 2015). Separating tissue cysts from the host RBC becomes important if the cysts are to be used for proteomic applications where contaminating RBC can present significant avoidable noise. Users of this protocol should use these gradient conditions as a starting point and feel free to modify the composition of the gradients to suit their specific experimental design.

Materials

10× PBS

Percoll (Sigma P1644-100ml)

Density Calibration beads (Cospheric- www.cospheric.com)

Sterile 50ml Conical Tubes (Falcon 352070)

Sterile 14ml Round Bottom Tubes (Falcon 352059)

Table top centrifuge (Beckman Allegra 6R) with swinging bucket rotor (GH3.8)

Sealable BSL2 certified centrifuge buckets

Peristaltic pump (Rainin Dynamax)

Peristaltic pump tubing (FisherBrand #15-190-510, 1.52mm bore, 16inch)

Glass Capillary tubes (SMI, Model C, P5070-902)

Eppendorf tubes to collect gradient fractions

Digital timer

96-well flat bottom microtiter plate (Corning #3596)

Establishing the step gradients and layering of samples

1. Prepare 50ml of the Percoll stock solutions in a sterile 50ml conical tube.
90% Percoll in PBS (5ml filter sterilized 10×PBS + 45ml Percoll)
40% Percoll in PBS (5ml 10×PBS + 20ml Percoll + 25ml sterile ddH₂O)
20% Percoll in PBS (5ml 10×PBS + 10ml Percoll + 35ml sterile ddH₂O)
Note: Do not make your stocks solutions by using 1×PBS as the diluent as this will result in the stocks that are not iso-osmotic.

Prepare 2 gradients at a time in 50 ml conical tubes:

1. Transfer 9ml of 90% Percoll into each of the tubes. This serves as a density cushion on which the RBC later will rest.
2. Set up a 14ml polypropylene tube in a wire rack and transfer 4.5ml of 40% Percoll to the tube using a pipette. Do not use a syringe and needle to measure this volume as they are not accurate
3. Drop a 5ml syringe with a 16 gauge needle into the tube (Figure 3A) draw up the entire volume into the syringe. Using a syringe to layer the gradient allows for much better control of the flow rate to ensure that the 40% Percoll layer rests on the 90% cushion.
4. Slowly discharge the 40% Percoll onto the 90% Percoll cushion along the wall of the 50ml tube which should be held at an angle (Figure 3B). Minimize splashing at the interface of the 2 layers. Repeat this with the second gradient. Return the 50ml tubes now containing the distinct 90% and 40% Percoll layers to an appropriate rack keeping them upright.
5. Set up a 14ml polypropylene tube in a wire rack and transfer 4.5ml of 20% Percoll to the tube using a pipette. Draw this volume into a 5ml syringe as above and layer onto the 40% Percoll layer. Be particularly careful about doing this slowly and gently as too much force will result in the mixing of the 40% and 20% layers impacting the resolving capacity of the gradient. The gradients can be used immediately or can be prepared hours in advance and kept upright in the refrigerator until use.

This modification of the original 1 step gradient (90%/30%) (Cornelissen et al., 1981) provides much more sensitive resolution of tissue cysts based on their density.

Loading and Running the Percoll Gradients

1. Draw half the brain homogenate generated in Basic Protocol 1 (roughly 4.5ml) into a 5ml syringe with a 16 gauge needle from the pestle. Alternatively you can transfer the entire volume a 14ml polypropylene tube and draw the brain homogenate into a syringe from the tube.

2. Gently layer the brain homogenate on the 20% Percoll later as described above for making the step gradients. Minimize mixing between the 20% Percoll and homogenate layers. Add the remaining brain homogenate (roughly 4.5ml) on the existing layer to establish the gradient that is now ready for centrifugation (Figure 3C, D).

Calibration of Percoll Gradients

1. Calibration of the gradients should be done on a periodic basis using the second prepared gradient. Mix the distinctly colored calibration beads (50–100µl of each density (color)) into 8ml of 1×PBS and bring the total volume up to 9ml. Layer the bead mixture onto the 20% Percoll layer using a syringe as above for the brain homogenate. The beads will begin to settle into the Percoll even without centrifugation (Figure 3E).
2. Gently transfer the gradients into the appropriate sealable centrifuge buckets and transfer to the rotor in a pre-cooled (4°C) tabletop centrifuge. Centrifuge at 2200rpm for 18 minutes. Begin timing once speed is reached. Manually abort the spin at 18 minutes with full brake setting.
3. While the centrifuge is running decontaminate the mortar and pestle and other materials by dropping into the bucket of 10% bleach. Remove the bucket from the hood and wash out with running water in the sink. Leaving the surgical tools in bleach for an extended period will result in their getting dulled and corroded. Replace the ice in the ice pan and prepare numbered 1.5ml microcentrifuge tubes in a rack to be used for the collection of the gradient fractions (1–20). Using different colored tubes can be useful to distinguish between gradients. Transfer the calibrated peristaltic pump (see below) into the biosafety cabinet.
4. At the end of the centrifugation run, return the centrifuge buckets to the biosafety cabinet and carefully remove the gradients with minimal shaking and transfer to a suitable rack. The brain homogenate loaded gradient will be marked by the presence of a distinct RBC later at the 90%/40% boundary. The tissue cysts are concentrated in the layer between the brain homogenate and RBC layer (Figure 3C,F). The calibration gradient exhibits the redistribution of the colored density beads to their respective equilibrium densities (Figure 3F).

Harvesting the gradients and establishing the number and distribution of tissue cysts—*Calibration of the peristaltic pump.*

Prior to harvesting the gradient it is necessary to calibrate the peristaltic pump. Insert the tubing between the rollers of the pump head and attach a glass capillary tube to each end by inserting it gently into the silicone tubing. Measure 10ml of water or PBS into a tube and insert the intake end of the tubing with the capillary at the bottom of the tube. Pump out the contents of the tube while timing the flow rate. A flow rate of 2ml/ minute (1ml every 30 seconds) is optimal.

Harvesting the Gradient

1. Once the peristaltic pump has been calibrated, place 20 numerically numbered 1.5ml microcentrifuge tubes in a rack. Position the gradient adjacent to the peristaltic pump (Figure 4A) and gently place the glass capillary attached to the silicone tubing to the bottom of the gradient. Be certain that the glass capillary is on the bottom of the gradient and not floating above it.
2. Turn on the peristaltic pump. For the sake of illustration the gradient containing the density calibration beads is shown. The light blue (1.13g/ml beads which settle on the bottom of the tube (Figure 3F) now enter the tubing (Figure 4B, red arrow) emerging on the opposite side (Figure 4C, arrow).
3. Place the output capillary over the first microcentrifuge tube in the rack and start an upward timer when the first drop falls (Figure 4D). The beads collect within the tube (Figure 4E, red arrow). At the 30 second time point (roughly 1ml based on the calibration of the pump), move the capillary to the next tube, repeating this step every 30 seconds. A total of 19 fractions are typically collected as fraction 20 is in the brain homogenate. As fractions are collected transfer the tubes to the ice pan. The typical distribution of the density beads by fractions is depicted in Figure 4F.
4. Wash out the tubing by running 10ml of 70% ethanol followed by 50ml of PBS through the tubing. This can be done at the maximum speed. Be sure to reset the flow rate prior to harvesting the second gradient.
5. Repeat the process with the second gradient (in this illustration the gradient containing the brain homogenate). It is useful to use microcentrifuge tubes of a different color to prevent mixing up gradients (Figure 4G).

Counting the purified tissue cysts

1. In order to establish the number and distribution of tissue cysts a direct count of an aliquot is performed. In order to facilitate the counting transfer 100µl of PBS into 2 rows of a flat bottom 96 well plate per gradient (Figure 4H).
2. Gently rock each fraction to mix its contents and transfer 10µl into a well of the 96 well plate going in order from left to right.
3. Spin the plate in the tabletop centrifuge for 8 minutes at 2000 rpm in an appropriate plate holder.
4. Use an inverted microscope with a 10× or 20× objective to scan each well for cysts. Here it is useful to go first to fraction 9 or 10, where the RBC's are concentrated which will allow you focus on the optical plane where the cysts will be found.
5. Count the cysts in each well, being sure to check for the corners of the wells as well as the center. Scan the entire well to establish the number of cysts. Tissue cysts can be quite variable with regard to their size even in the same gradient fraction (Figure 4I, arrows). Since the 10ml counted represents 1/100 of the total

volume of each fraction (1ml) the number of cysts per fraction can be readily established. The distribution of tissue cysts in the gradients varies as a function of the length of infection as seen for data from 99 independent gradients (Watts et al., 2015).

Removal of Percoll

1. Fractions containing tissue cysts can be pooled as needed. In order to remove the Percoll transfer the pooled positive fractions into a suitable tube to allow for a minimum 5 fold dilution with PBS (3ml of fractions + 12ml of PBS in a 15ml conical tube).
2. Centrifuge at 2000rpm for 10–15 minutes in a swinging bucket rotor. This ensures the pellet is at the bottom of the tube. Note that the pellet may be very small depending of the yield. Aspirate the supernatant and freeze (–80°C) or otherwise process the pellet as needed for the specific experiment.

Basic Protocol 3: Imaging of Purified Tissue Cysts

By combining the ability to purify tissue cysts from infected mouse brains efficiently and reproducibly with imaging approaches new insights into the organization and progression of the chronic phase of *Toxoplasma* infection have been derived (Watts et al., 2015). This protocol describes an efficient approach to deposit purified tissue cysts onto slides where they can be stained with fluorescent lectins (as described below), antibodies or vital dyes to establish spatial relationships within tissue cysts. Such relationships when applied to cysts harvested at different time points can yield insights into the changes associated with the organization and function of bradyzoites within tissue cysts during the progression of the chronic phase of infection. In addition, the conditions for tissue cyst deposition can be used and modified to assess the physical durability of tissue cysts that could be relevant when examining the structural stability of mutants.

Materials

Shandon Cytospin 4 Centrifuge
Cytospin funnels (TPX Sample Chamber) (Thermo #A78710018)
Cytoclip stainless steel slide holders (Thermo Fisher #59-910-052)
Cytospin filter cards (Fisher #22-030-410)
Frosted Glass Slides (Fisher #12-544-2)
Cardboard slide holder (Fisher #12-587-10)
Wax pencil (Fisher #NC9072020)
Copin jars with screw on cap (Fisher 08-816)
70% Ethanol for decontamination
Absolute Methanol (at –20°C)

Paraformaldehyde stock (EMS #RT15710)

Triton X-100 (0.2% in PBS) (Sigma #X100)

Carbo-Free Blocking Solution (Vector Laboratories #SP5040)

Dolichos biflorus Lectin (FITC-conjugated) (Vector Laboratories #FL-1031)

Concanavalin A Lectin (Rhodamine conjugated) Vector Laboratories #FL-1001)

Hoescht Dye (10mg/ml) (ThermoFisher #H3569)

Preparation of Slides for Imaging

1. Aspirate the diluted Percoll as above (Basic Protocol 2) and resuspend the pellet in 500 μ l of PBS using a 1ml micropipettor. Adjust the volume of the suspension to have 200–300 cysts per 500 μ l of PBS.
2. Place a pre-labeled (in pencil) glass slide and a filter card into a stainless steel clip (Figure 5A). Slide in the cytospin funnel (Figure 5B) and secure the wire clips to hold the funnel in place (Figure 5C). Place these assembled units into the rotor of the Cytospin centrifuge (Figure 5D).
3. Using a 1ml micropipettor, gently resuspend the tissue cyst pellet (which may not be visible) and load into the opening of the funnel within the centrifuge (Figure 5E). Fill all the funnel assemblies in the centrifuge as quickly as possible to avoid significant wicking of the buffer into the filter pad.

4. Run the centrifuge at 750rpm for 5 minutes at medium acceleration.

These conditions were found to be optimal to maintain the integrity of the deposited tissue cysts. Lower speeds (250–500rpm) resulted in damage due to shearing as cysts appear to get dragged on the slide surface while higher speeds (1000rpm and higher) disrupted cysts as they impacted the glass surface.

5. Disassemble the slide clamp and discard the filter card after lifting it without dragging it across the slide. Be sure to be wearing gloves at this stage as these are still unfixed and infectious tissue cysts. Place the steel clips and funnels in a beaker with 70% ethanol to decontaminate.
6. Place 2 slides back to back and fix in a Coplin jar with cold methanol (-20°C) or 3% paraformaldehyde in PBS (PFA).

PFA fixed slides should not be stored for more than a week as the level of background increases. Methanol fixed slides can be kept in the freezer for months without consequence.

Labeling fixed slides with fluorescence conjugated lectins

1. Transfer methanol fixed slides to be stained back to back to a Coplin jar containing PBS. Rinse slides 3 times with PBS within the Coplin jar. If using PFA fixed slides, permeabilize the sample with 0.2% TritonX100 in PBS for 5 minutes at room temperature prior to returning to PBS. Prepare a cardboard slide

holder by placing a Cytospin filter card within each slide slot. Place the slide within a slide slot.

The location of the deposited tissue cysts will be at punched hole in the filter card.

2. Using a Kimwipe, gently dry a circle on the slide roughly 1cm away from the edge of the hole punch on the filter card under the slide. Keep the sample on the slide moist. Using a wax pencil draw a circle along the perimeter of dried portion of the slide to form a wax dam around the sample.
3. Prepare the lectin labeling solution in 1× Carbo-Free Blocking solution in PBS using a 1:2000 dilution each of FITC conjugated Dolichos Biflorus Agglutinin (DBA) and Rhodamine conjugated Concanavalin A (ConA). In addition, add 1:5000 dilution of Hoescht dye to allow for the detection of bradyzoite nuclei within the tissue cyst.
4. Place 200–300µl of the labeling solution directly onto the deposited tissue cyst sample on the slide allowing it to spread to the wax pencil dam. Close the slide holder, placing a filter card at the edge to keep the lid from touching the droplet of the staining solution. Incubate for 45 minutes to an hour at room temperature.

Note: This labeling procedure can be used with antibodies for which one would typically need to add a step for the secondary antibody. When using antibodies avoid antisera raised in mice unless they are directly conjugated to a fluorophore as anti-mouse secondary reagents robustly stain brain derived tissue cysts.

5. Following labeling, aspirate the labeling mixture off the slide and wash 3 times in a Coplin jar with PBS. Return the slides to the slide holder and gently wipe away the wax dam with a Kimwipe as the wax will interfere with the even spreading of the mounting medium. Mount a coverslip over the stained tissue cyst spot on the slide using a suitable mounting medium. Once set, the slides are ready to view.
6. Tissue cysts can be readily detected using a 20× or higher objective. Based on the number of cysts deposited on the slide it is best to find regions with cysts under low power before examining them under high power. An example of a tissue cyst labeled with DBA and ConA is shown in Figure 5.

Further details regarding the visualization of tissue cysts and the acquisition of images are described elsewhere (Watts et al., 2015).

Commentary

Background Information

The protozoan parasite *Toxoplasma gondii* establishes life-long chronic infections by encysting within muscles and cells within the central nervous system. These tissue cysts are orally transmissible (Jones & Dubey, 2012) and are believed to persist for the life of the infected host (Dubey & Jones, 2008; Knoll, Tomita, & Weiss, 2014), although a recent opinion suggests this may not be entirely true (Rougier, Montoya, & Peyron, 2016). The

clinical relevance of tissue cysts is manifested in their potential to reactivate from an effectively hibernatory state to initiate a new round of active infection (Dubey & Jones, 2008; Knoll et al., 2014). While such reactivation is generally asymptomatic in immune competent individuals, reactivated toxoplasmosis is life threatening in the context of immune suppression due to active HIV-AIDS and in the context of organ transplantation and cancer chemotherapy (Derouin & Pelloux, 2008; Dubey & Jones, 2008; Nath & Sinai, 2003). Although tissue cysts can form in all organs and tissues, the predilection of the parasite for the central nervous system establishes the brain as a primary site for the establishment of tissue cysts *in vivo* (Dubey & Jones, 2008). Recent work, suggests that the establishment of chronic toxoplasmosis mediated by tissue cysts within the brain can induce changes in the neuronal architecture (David et al., 2016; Parlog, Schluter, & Dunay, 2015), neurochemistry (David et al., 2016; Prandovszky et al., 2011) and even behavior (Afonso, Paixao, & Costa, 2012; Vyas, 2015). Other studies examining human infection have suggested a potential involvement of chronic *Toxoplasma* infections in diverse neurological and psychological conditions (reviewed in (Sugden et al., 2016)). More relevant in the context of the chronic infections is the potential of tissue cysts and the bradyzoites they house reactivating within the host resulting in life threatening toxoplasmosis typically associated with severe immune suppression (Nath & Sinai, 2003; Pereira-Chiocola, Vidal, & Su, 2009). Despite their central role in the parasite life cycle and the pathogenesis of disease, little is known about the biology of tissue cysts and the bradyzoites they house. The optimization of the protocol to purify tissue cysts from the brains of infected mice facilitates studies on this life cycle stage of the parasite. Purification of tissue cysts with minimal host contamination is particularly timely given the access it provides for state of the art cell biological, biochemical and physiological analyses. Rapid isolation of highly purified tissue cysts allows for increasingly accessible high throughput transcriptomic and proteomic studies on this critical though understudied form of the parasite.

The presence of a slow growing “dormant” infection for extended periods of time within the immune competent host is interspersed with periods of reactivation and acute disease that is rapidly resolved driving a new round of encystation (Chew, Wah, Ambu, & Segarra, 2012; Takashima et al., 2008). However, reactivation in the context of immune suppression, most notably with the loss of T cell immunity as is the case with HIV-AIDS results in uncontrolled parasite replication that can be life threatening (Israelski, Chmiel, Poggensee, Phair, & Remington, 1993). Given that the CNS is a primary site of encystation, the manifestation of reactivated disease during immune suppression is toxoplasmic encephalitis which is lethal if left untreated (Nath & Sinai, 2003). Given the high seroprevalence of *Toxoplasma* infections in the general human population that approaches 30% worldwide (Tenter, Heckeroth, & Weiss, 2000) and the persistence of the agent within tissue cysts presents this poorly understood life cycle form as the primary source of symptomatic toxoplasmosis in the HIV-AIDS populations (Nath & Sinai, 2003).

Despite their importance in the development of symptomatic disease in the immune compromised, we know surprisingly little about the biology of tissue cysts *in vivo*. Much of what we know comes from morphological studies (Dubey et al., 1998; Ferguson, 2004; Ferguson et al., 1991; Ferguson & Hutchison, 1987; Fortier, Coignard-Chatain, Soete, & Dubremetz, 1996; Sims, Hay, & Talbot, 1990) as well as cell culture based systems which

have focused more on the transition of tachyzoites to encysted forms rather than the chronic phase of infection or reactivation. (Dzierszinski, Nishi, Ouko, & Roos, 2004; Singh, Brewer, & Boothroyd, 2002; White, Radke, & Radke, 2014). A true understanding of the chronic phase of *Toxoplasma* infections necessitates examination of *in vivo* derived tissue cysts as well as studies on how this phase progresses. This approach was the focus of our recent work that established that contrary to the prevailing wisdom, tissue cysts were not uniform entities, exhibiting considerable heterogeneity in their organization and structure (Watts et al., 2015). Arguably the biggest surprise was that bradyzoites within cysts retain considerable replicative potential while exhibiting patterns of replication suggestive of higher order regulatory control (Watts et al., 2015). These new findings warrant further investigation, which the increased availability of “omic” approaches can rapidly address patterns of mRNA and protein expression in tissue cyst populations. Furthermore, insights into tissue cyst and bradyzoite biology provides the first functional foothold into understanding why these life cycle stages remain largely refractory to existing drugs while revealing new potential drug targets.

These important studies have been hindered by the inherent difficulties associated with working with *in vivo* derived tissue cysts. Our own experience, using a single parasite strain (serially passaged from animal to animal) and a specific inbred mouse strain (all female) revealed a tremendous heterogeneity in tissue cyst yield within any given time point of harvest (Watts et al., 2015). There is considerable variation with regard to tissue cyst quality and yield governed by parasite strain and mouse genetic background reported in the literature (reviewed in (Sinai et al., 2016)). What is also lacking is a standardization of methods to count tissue cysts. Most often this is done by direct enumeration in dilutions of brain homogenates which can become quite inaccurate when cyst yields are low. We therefore focused on optimizing the only published protocol for the purification of tissue cysts using Percoll gradients (Cornelissen et al., 1981), with an emphasis on reproducibility, efficiency and safety. By altering the set-up of the gradients we were able to greatly improve the resolving capacity and for the first time establish the distribution of the physical properties of the cysts represented by their hydrodynamic properties (Watts et al., 2015). This provides an analytical parameter that is driven by the physical and chemical composition of the tissue cyst. This parameter could potentially be altered following drug treatment or represent a previously unrecognized property of a mutant parasite. The standardization of the tissue cyst recovery from the gradients facilitated by the collection of fractions at fixed time intervals for a given flow rate allows for comparisons between gradients across a significant time span (Watts et al., 2015). Importantly, this approach is focused on safety. The original protocol (Cornelissen et al., 1981) recommended the collection of the tissue cyst containing region by puncturing the tube with a syringe and either drawing the material from the side of the tube or the bottom. Finally, the fractionation of the tissue cysts and the counting of the cysts in aliquots dispensed into the wells of a 96 well plate allows for rapid direct enumeration compensating for the time it takes to purify the cysts relative to the doing physical counts of fixed volumes of brain homogenates. In conclusion, users of this protocol should view it as the starting point that can be specifically adapted to their respective experimental needs. Purified tissue cysts can be readily incorporated into morphological, biochemical and physiological studies that will provide

new insights into what is a changing picture of the chronic phase of *Toxoplasma* infection (Sinai et al., 2016).

Critical Parameters and Troubleshooting

1. The efficiency of this purification system in the presented configuration is optimal when using 2 mouse brains per gradient. Increasing the material loaded onto the gradient results in a significant loss of purified cysts within the gradient as material appears to get trapped in the brain homogenate layer.
2. The yield of tissue cysts is directly related to the number of cysts in the mouse brain which can vary significantly. Importantly, cyst containing fractions can be pooled to concentrate the cysts during the removal of the Percoll.

Other points where common errors may be an issue are highlighted throughout the protocol section. As noted above, this specific protocol and configuration should be viewed as a starting point subject to further optimization based on the specifics of the experiment being performed.

Anticipated Results

The anticipated results are evident in the compiled data from 99 independent gradients run across 5 distinct time points of infection over a 2 year period (Watts et al., 2015). In principle the level of detection of purified cysts is under 100 cysts/brain as the detection of a single cyst in any give fraction is representative of 100 cysts within that fraction. This protocol is designed for typical, “normal” tissue cysts which may not be the case for mutant cysts that are structurally fragile (Tomita et al., 2013; Tomita et al., 2016) and may not survive the purification or enter the gradient. In such instances, a quantitative PCR based approach to determine the parasite burden may be required to establish the presence of parasites in samples containing defective tissue cyst populations. This may necessitate modification of the homogenization protocol to capture potentially fragile cysts.

Time considerations

Once the mouse brains are harvested, the protocol has to be run to completion. The homogenization of the brains including all the syringe passages takes roughly 45 minutes. The pouring of 2 gradients from the stock Percoll solutions (which can be done in advance) takes roughly 10 minutes. Running the gradients takes roughly 20 minutes while harvesting the gradient takes 15 minutes each. Centrifugation of the tissue cysts in the 96 well plate for counting takes 8–10 minutes. Finally counting the tissue cysts in each gradient will take 10–15 minutes. Overall 2 gradients can be run from the time of harvesting the brains to completing the counts in 2.5 to 3 hours.

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Figure 1. Harvesting the brain from infected mice

A. Sterilization of mortar and pestle and dissection instruments. **B.** Mortar and pestle and the dissection instruments including: small curves scissors, small straight scissors, firm rounded tip forceps and microspatula. **C.** Surface sterilization of euthanized mouse using 70% ethanol on a dissection surface in the biosafety cabinet. Note the 4 liter plastic beaker with bleach in the corner (red asterisk). **D.** Exposure of the skull by an incision through the skin between the ears. This is followed by the cutting of the skull from the back to the front. **E.** Lifting the skull cap to the front (skull) exposing the brain. Note that the forceps are used to secure the head holding the skull in the eye sockets. A microspatula is inserted under the skull severing the spinal cord. **F.** The intact brain is removed using the microspatula.

Figure 2. Preparation of the brain homogenate

A. 2 intact mouse brains are placed on a prechilled mortar. **B and C.** The brains are ground into a paste with the consistency of toothpaste. **D.** 3ml of PBS with 1% Tween80 is added to the mortar. Note the sleeve covers on the arms. **E.** The pestle is used to incorporate the brain paste into the added buffer with a small head cell scraper being used to scrape chunks off the walls of the mortar and the pestle head. **F.** Appearance of the brain homogenate. **G.** Syringe passage with a large bore (16G) needle. **H.** Final round of syringe passage using a fine bore (23G) needle. Note that the brain homogenate is somewhat foamy. **I.** Addition of 6ml PBS to the brain homogenate and mixing by aspirating up and down using a pipette to generate the material that will be loaded onto the gradient.

Figure 3. Establishing and running the Percoll Gradient

A. Loading a 5ml syringe with a large bore needle in a tube with the premeasured amount of Percoll. **B.** Layering the first Percoll step onto the 90% Percoll cushion by slowly ejecting the contents of the syringe along the wall of the tube. The needle should just above the material in the tube and the syringe drawn upward as the Percoll is discharged. The brain homogenate is added at the top of the gradient. **C.** Schematic for the organization of the Percoll gradient before and after centrifugation. Insertion of the capillary connected to the tubing of the peristaltic pump at the bottom of the gradient is indicated. **D.** Appearance of a

gradient with the brain homogenate prior to centrifugation. **E.** Appearance of a gradient with calibration beads prior to centrifugation. **F.** Appearance of gradients following centrifugation. The gradient to the left with the brain homogenate reveals the band of RBC, the region containing the concentrated tissue cysts and the brain homogenate. The calibration gradient is has separated the density beads into distinct layers within the gradient.

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Figure 4. Harvesting the Percoll Gradients

A. Arrangement of the materials needed to harvest the gradients. From Left to Right: ice pan with ice, rack with the resolved gradients, peristaltic pump with tubing to which the glass capillaries have been added. The direction of the pump flow is indicated with the red arrow. A rack with prelabeled microcentrifuge tubes is in the foreground. **B.** Pumping out the gradient from the bottom up. The blue beads from the bottom of the gradient are seen in the tubing (red arrow). **C.** The blue beads emerge at the output end of the pump head (red arrow). **D.** As the first drop falls into the first tube (red arrow) the count-up timer is started. **E.** Midway through the collection of the first fraction the blue beads are evident within the glass capillary. **F.** A representative calibration curve indicating the fractions where specific beads are recovered as well as where in the brain homogenate gradients the RBC band and the brain homogenate layer are present. **G.** Setting up to harvest the brain homogenate loaded gradient. Note the use of different colored tubes to collect each gradient. **H.** Setting up wells in a 96 well plate to count the cysts in each fraction of the gradient. **I.** A representative 20× field from a single fraction demonstrate the size differences of 3 cysts (arrowheads) that all share the same average density.

Figure 5. Imaging of purified tissue cysts

A–C Assembly of the Cytospin holders to pellet the purified tissue cysts onto the slide surface. **A.** A glass slide and hole punched filter card are placed into the stainless steel clip holder. **B.** A sample funnel is placed into the holder such that the base of funnel aligns with the lower hole punch on the filter card. **C.** Assembled Cytospin holders. **D–E.** Cytospin holders are placed in the centrifuge and the sample added to the funnels using a micropipettor. **F.** Following centrifugation and the disassembly of the holder the slides are placed in a Coplin jar with fixative. **G–I** Imaging of a tissue cyst with Dolichos biflorus lectin (DBA-green) (**G**) and Concanavalin A (ConA-Red) (**H**) acquired on a Zeiss Axiophot stand using a 100× 1.4NA objective. The merged image (**I**) clearly shows the differential distribution of the glycans recognized by these lectins within the tissue cyst.