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Dear Dr. D'Souza,

Dr. Dutta and I have edited our manuscript "Studying *Cryptosporidium* infection in 3D tissue derived human organoid culture systems by microinjection" in accordance with your suggestions. Please let us know if there is anything other information we can provide or any further corrections that need to be made.

Regards,

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TITLE:

Studying *Cryptosporidium* Infection in 3D Tissue-Derived Human Organoid Culture Systems by Microinjection

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KEYWORDS:
Cryptosporidium, organoids, microinjection, host-microbe, intestine, lung, cryptosporidiosis, sporozoites, oocysts

SUMMARY:
We describe protocols to prepare oocysts and purify sporozoites for studying infection of human intestinal and airway organoids by *Cryptosporidium parvum.* We demonstrate the procedures for microinjection of parasites into the intestinal organoid lumen and immunostaining of organoids. Finally, we describe the isolation of generated oocysts from the organoids.

ABSTRACT:
Cryptosporidium parvum is one of the major causes of human diarrheal disease. To understand the pathology of the parasite and develop efficient drugs, an *in vitro* culture system that recapitulates the conditions in the host is needed. Organoids, which closely resemble the tissues of their origin, are ideal for studying host-parasite interactions. Organoids are three-dimensional (3D) tissue-derived structures which are derived from adult stem cells and grow in culture for extended periods of time without undergoing any genetic aberration or transformation. They have well defined polarity with both apical and basolateral surfaces. Organoids have various applications in drug testing, bio banking, and disease modeling and host-microbe interaction studies. Here we present a step-by-step protocol of how to prepare the oocysts and sporozoites of *Cryptosporidium* for infecting human intestinal and airway organoids. We then demonstrate how microinjection can be used to inject the microbes into the organoid lumen. There are three major methods by which organoids can be used for host-microbe interaction studies—microinjection, mechanical shearing and plating, and by making monolayers. Microinjection

enables maintenance of the 3D structure and allows for precise control of parasite volumes and direct apical side contact for the microbes. We provide details for optimal growth of organoids for either imaging or oocyst production. Finally, we also demonstrate how the newly generated oocysts can be isolated from the organoid for further downstream processing and analysis.

INTRODUCTION:

Development of drugs or vaccines for treatment and prevention of *Cryptosporidium* infection has been hindered by the lack of *in vitro* systems that precisely mimic the *in vivo* situation in humans[1,2]. Many of the currently available systems either only allow short term of infection (<5 days) or do not support the complete life cycle of the parasite[3,4]. Other systems which enable the complete development of the parasite are based on immortalized cell lines or cancer cell lines which do not faithfully recapitulate the physiological situation in humans[5-7]. Organoids or 'mini-organs' are 3D tissue derived structures which are grown in an extracellular matrix supplemented with various tissue specific growth factors. Organoids have been developed from various organs and tissues. They are genetically stable and recapitulate most functions of the organs of their origin, and can be maintained in culture for extended periods of time. We have developed a method for infecting human intestinal and lung organoids with *Cryptosporidium* that provides an accurate in vitro model for the study of host-parasite interactions relevant to intestinal and respiratory cryptosporidiosis[8-13]. In contrast to other published culture models, the organoid system is representative of real-life host parasite interactions, allows for completion of the life cycle so that all stages of the parasite life cycle can be studied, and maintains parasite propagation for up to 28 days[10].

Cryptosporidium parvum is an apicomplexan parasite that infects the epithelium of the respiratory and intestinal tracts, causing prolonged diarrheal disease. The resistant environmental stage is the oocyst, found in contaminated food and water[14]. Once ingested or inhaled, the oocyst excysts and releases four sporozoites that attach to epithelial cells. Sporozoites glide on hosts cells and engage host cell receptors, but the parasite does not fully invade the cell, and appears to induce the host cell to engulf it[15]. The parasite, which is internalized within an intracellular but extracytoplasmic compartment, remains at the apical surface of the cell, replicating within a parasitophorous vacuole. It undergoes two rounds of asexual reproduction—a process called merogony. During merogony, type I meronts develop which contain eight merozoites that are released to invade new cells. These merozoites invade new cells to develop into type II meronts containing four merozoites. These merozoites, when released, infect cells and develop into macrogamonts and microgamonts. Microgametes are released and fertilize the macrogametes producing zygotes that mature into oocysts. Mature oocysts are subsequently released into the lumen. Oocysts are either thin-walled which immediately excyst to reinfect the epithelium, or thick walled which are released into the environment to infect the next host[14]. All stages of the *Cryptosporidium* life cycle have been identified in the organoid culture system previously developed by our group[10].

Since human organoids faithfully replicate human tissues[9,11,13], and support all replicative stages of *Cryptosporidium*[10], they are the ideal tissue culture system to study *Cryptosporidium* biology and host-parasite interactions. Here we describe the procedures for infecting organoids with both *Cryptosporidium* oocysts and excysted sporozoites, and isolating the new oocysts produced in this tissue culture system.

PROTOCOL:

All tissue handling and resection was performed under Institutional Review Board (IRB) approved protocols with patient consent.

1. Preparation of *C. parvum* oocysts for injection:

NOTE: *Cryptosporidium* oocysts were purchased from a commercial source (see the **Table of Materials**). These oocysts are produced in calves and are stored in phosphate-buffered saline (PBS) with antibiotics. They can be stored for about 3 months at 4 °C and should never be frozen. We normally use oocysts within one month. Organoids can be infected with either intact oocysts, or sporozoites may be isolated from excysted oocysts and used to infect organoids if it is important not to have oocysts carryover from the original inoculum.

1.1. Prepare *Cryptosporidium* oocysts for infecting cells (**Figure 1A**).

1.1.1 Keep oocysts on ice throughout all manipulations until they are added to the organoids.

1.1.2. Calculate the number of oocysts needed for a full six-well plate of organoids (usually about 5×10^5–2.5×10^5 for the plate). Count the numbers of oocysts in a hemocytometer to verify the quantity and transfer to a centrifuge tube.

NOTE: To aid in visualization, oocysts may be mixed 1:1 with an oocyst-specific fluorescent antibody (see the **Table of Materials**) before being loaded onto the hemocytometer. The fluorophore-labeled oocysts can then be easily visualized and enumerated using a fluorescence microscope. We suggest injecting about 100–1000 oocysts/organoid. In general, 1000–2000 organoids can be grown in a six-well plate.

1.1.3. Bring the volume of the oocysts suspension up to 900 µL with PBS. Add 100 µL of sodium hypochlorite (e.g., Clorox) bleach (at 4 °C). Incubate for 10 min on ice.

1.1.4. Centrifuge for 3 min in a microcentrifuge at 8000 x *g* at 4 °C. Orient the tubes in the centrifuge with the cap opening facing inward. The pellet can be hard to see so knowing where the parasites have pelleted in the tube is essential.

1.1.5. Remove the supernatant with a pipette being careful to avoid the pellet. Add 1 mL of Dulbecco's modified Eagle's medium (DMEM) and vortex to mix.

1.1.6. Centrifuge for 3 min in a microcentrifuge at 8000 x *g* at 4 °C.

1.1.7. Repeat washes with DMEM two more times.

1.1.8. Prepare expansion medium (OME) or differentiation organoid medium (OMD) to which taurocholate has been added to a final concentration of 0.5% (w/v) (See **Table of Materials**). Taurocholate should always be prepared and added fresh.

NOTE: We have successfully used 0.5% taurocholate in our infection assays where the inoculum is intact oocysts, and saw improved rates of infection without deleterious effects on the host cells. However, taurocholate may have unanticipated effects on cells, and lower concentrations have been used successfully in infection assays[16].

1.1.9. Resuspend oocysts in 100 µL of organoid culture medium supplemented with 0.5% (w/v) sodium taurocholate. Count oocysts again as described in step 1.1.2.

1.1.10. Add Fast Green dye to the suspension in order to visualize injection.

1.1.11. Fill micro-loader tips (see the **Table of Materials**) with the oocyst suspension and use it to fill pulled capillaries.

CAUTION: The whole procedure should be done in a tissue culture hood with level-2 safety protocols. Use of masks is recommended as *Cryptosporidium* oocysts can also be infectious when airborne.

2. In vitro purification of sporozoites from *C. parvum* oocysts

2.1. Purify sporozoites from *C. parvu*m oocysts after bleaching and washing out the bleach as described above.

2.1.1. Transfer the oocysts to a 15 mL tube. Resuspend oocysts in room temperature excystation medium (0.75% w/v sodium taurocholate in DMEM) to obtain 1 x 10^7 oocysts/mL. The addition of taurocholate improves the excystation rate of the oocysts, improving sporozoite yield.

2.1.2. Incubate oocyst suspension at 37 °C for 1–1.5 h.

2.1.3. Check the sample microscopically for extent of excystation; 60–80% excystation is reasonable for good recovery of sporozoites. If the level of excystation is low, incubate longer (another 30 min to 1 h).

2.1.4. Determine the percent excystation relative to the number of starting oocysts. Excystation is calculated as:

% excystation = [1 – (number of intact oocysts/number of oocysts at start)] x 100

2.1.5. Wash cells to remove excystation reagents by adding 14 mL of PBS or medium, mixing, and recovering cells (intact oocysts, oocyst shells, and sporozoites) by centrifugation at 3400 x *g* for 20 min to recover sporozoites. Aspirate carefully to avoid losing cells.

2.1.6. Resuspend the sporozoite pellet in 1–2 mL of DMEM to obtain 3 x 10^7 oocysts/mL (based on the number of starring oocysts).

2.1.7. To remove remaining oocysts and shells, filter the suspension through a 3 µm filter (**Figure 1B**). Use a 47 mm filter holder apparatus fitted with polycarbonate filter (3 µm pore size) attached to a 10 mL syringe barrel. Place the filter holder apparatus on top of a 15 mL tube. Place the assembly in an ice bucket or in cold room.

2.1.8. Add 7.5 mL of the sporozoite suspension to the filter assembly and allow to filter through by gravity. Wash through with another 7.5 mL of DMEM.

NOTE: To ensure success in sporozoite isolation fresh oocysts and good excystation are critical. If there are too many unexcysted oocysts, the suspension will not flow through by gravity. Applying pressure on the syringe can force unexcysted oocysts through. Microinjection of sporozoites is more challenging than that of oocysts because sporozoites may clump and block the capillary. To avoid this, we recommend making a wider capillary tip when injecting organoids with sporozoites. To achieve sufficient levels of infection, 2–4 times the number of sporozoites need to be injected into each organoid as compared to organoids infected with oocysts.

2.1.9. Centrifuge the filtered sporozoite suspension at 3400 x *g* using a swinging bucket rotor for 20 min to pellet sporozoites.

2.1.10. Resuspend in 50–100 µL of OME or OMD organoid culture medium (see the **Table of Materials**) supplemented with 0.05% (w/v) Fast Green dye and L-glutathione, betaine, L-cysteine, linoleic acid and taurine-containing reducing buffer[5] (see the **Table of Materials**).

NOTE: Incubating oocysts for too long may result in the lysis of sporozoites and poor recovery and therefore should be avoided.

3. In vitro culture of human intestinal and lung organoids for microinjection

3.1. Culture intestinal organoids under expansion and differentiation media conditions.

NOTE: The details of intestinal and lung organoid propagation have been previously described in other articles[8,13] (see **Table of Materials** for media recipes). Here, we briefly describe the organoid culture method with specific reference to optimization for *Cryptosporidium* injection and growth. We have found that for imaging of parasites in organoids, organoids grown in expansion medium are preferable to those in grown differentiation media as there is less debris accumulation than that seen in organoids grown in differentiation medium. However, if the goal is to isolate oocysts, organoids grown in differentiation media produce far higher numbers of oocysts.

3.1.1. Maintain organoids in 3D cultures in extracellular matrix (see the **Table of Materials**) at 37

°C. Add OME (expansion media) on top and refresh every day.

NOTE: For lung organoids, we do not have separate expansion and differentiation media.

3.1.2. To split and plate organoids for microinjection, remove media from the 6-well plate containing human organoids and add F12+++ (See the **Table of Materials**) to the well and break up the matrix by pipetting with a 1 mL pipette tip several times. Collect cells into a 15 mL tube (2 mL of F12+++ per tube is enough for further procedures).

3.1.3. Add 10–12 mL of F12+++ into another 15 mL tube and place a fire-polished glass pipet into the medium, pipette up and down 3 times to break up the human intestinal and lung organoids.

NOTE: Use a long glass pipet (20–30 cm) and fire-polish it briefly. Do not make the opening (1 mm diameter) very small because organoids can be damaged. Make the tip of the pipet smooth by briefly fire-polishing it. Break organoids into smaller pieces of ~50 µm. Lung organoids have a thicker outer membrane and therefore require stronger shearing with the glass pipette as compared to intestinal organoids. Moreover, they have a slower growth rate than intestinal organoids (up to 14 days between each passaging).

3.1.4. Add F12+++ up to 5–7 mL and centrifuge at 350 x *g* for 5 min.

NOTE: The centrifugation speed in this step is higher than normal in order to make a good cell pellet that is well separated from the extracellular matrix (see the **Table of Materials**). We have observed that compared to mouse small intestine organoids, human small intestine organoids are harder to disrupt.

3.1.5. Remove as much medium as possible without disturbing the cells, then resuspend the pellet with matrix maintained at 4 °C; 200–300 µL of matrix per well of a six-well plate is required. Organoids should be split one in three to maintain a fairly high cell density.

3.1.6. Plate the organoids in matrix droplets of about 5–10 µL each in the well of a six-well plate. Incubate for 20–30 min at 37 °C and then add expansion medium (OME) on top.

3.1.7. Change the medium every 2–3 days.

NOTE: In about 5–7 days, organoids growing in EM reach a size of 100–200 µm and are ready for injection.

3.1.8. To differentiate the organoids, after 5–6 days in EM, change the media to differentiation media (DM) conditions and keep for 5–6 additional days before injecting the parasites.

NOTE: For expansion of organoids, it is recommended to plate the organoids densely. For microinjection, use of a six-well plate is recommended with organoids plated at a lesser density. For example, plate organoids from three wells of a six-well plate into a full six-well plate for microinjections. Matrix should be maintained at -20 °C for long term storage and thawed at 4 °C or on ice before use. Expansion of lung organoids is done is a similar manner but using lung organoid specific media components (**Table 1**)[8] .

4. Microinjection of oocysts/sporozoites into the organoid lumen

4.1. Microinject parasites into the apical side of the 3D organoid (**Figure 2**).

4.1.1. Prepare glass capillaries of 1 mm diameter using a micropipette puller.

NOTE: Settings used on the micropipette puller (See **Table of Materials**) are: Heat = 663, Pull = 100, Velocity = 200, Time = 40 ms. Settings will need to be adjusted according to the user instructions for a particular machine.

4.1.2. Cut the tip of the capillary with forceps. The size/diameter of the capillary end measures about 9–12 µm; this enables easy flow of oocysts (4–5 µm size).

4.1.3. Fill capillaries with oocyst or sporozoite suspension using micro-loader tips.

4.1.4. Load the oocyst-filled capillary onto a microinjector.

4.1.5. Microinject 100–200 nL suspension into each organoid under an inverted microscope at 5x magnification, keeping the pressure constant. After microinjection, refresh media with OME or OMD every day and maintain the plate at 37 °C.

NOTE: We do not use a micromanipulator for microinjection. Use of the same capillary is recommended for the entire experiment to ensure equal injection in every sample.

5. Immunofluorescence staining of organoids

5.1.1. Collect organoids (1–2 x 24 wells) with a P1000 pipette into a 15 mL tube containing cold F12+++.

5.1.2. Pellet organoids at 300 x *g* for 2 min, remove the supernatant without disrupting the pellet, and gently pipet the pellet loose into the remaining volume.

5.1.3. Add 5 mL of 2% paraformaldehyde in PBS. To prevent the organoids from sticking to the wall do not invert the tube. Allow the organoids to settle to the bottom of the tube and fix at 4 °C overnight or 1 h at room temperature.

5.1.4. Remove the fixative and add 10 mL of permeabilization buffer (0.2% Triton in PBS).

5.1.5. Rotate the tube at room temperature for 20 min (this ensures that all the organoids remain in suspension).

5.1.6. Pellet the organoids at 300 x *g* for 2 min, and then discard the supernatant.

5.1.7. Resuspend the organoids gently in 500 µL of blocking solution (See the **Table of Materials**) and transfer to a 2 mL microcentrifuge tube.

5.1.8. Incubate for 20 min at room temperature on a shaker. Allow organoids to settle to the bottom of the tube by gravity. Replace the blocking solution with primary antibody solution (See **Table of Materials**) and incubate for 1–2 h at room temperature or overnight at 4 °C.

5.1.9. Wash 3x with PBS containing 0.1% Tween. Let the organoids settle every time and remove the supernatant.

5.1.10. Add secondary antibody solution (See the **Table of Materials**) and incubate for 2 h at room temperature.

5.1.11. Wash 3x with PBS containing 0.1% Tween. Leave 50 µL of PBS after the third wash.

5.1.12. Mount on the slide by pipetting the organoids suspended in 50 µL of PBS on the slide. Remove excess PBS, add a drop of mounting agent (See the **Table of Materials**) and add the coverslip on top. Seal the sides with nail polish and let it dry.

6. Isolation of oocysts from organoids

6.1. Isolate newly formed oocysts from the organoid lumen.

NOTE: Oocysts are isolated from the organoids by immunomagnetic separation using an oocyst isolation kit (see the **Table of Materials**) with the modifications described below. The isolated oocysts can then be analyzed by immunofluorescence and electron microscopy.

6.1.1. Start with differentiated organoids that have been maintained in OMD for 5–7 days and that are uninfected, infected for 1 day and infected for 5 days. Use the first two as negative controls.

NOTE: We have found that differentiated organoids produce more oocysts than lung or expanding intestinal organoids[10].

6.1.2. Collect organoids into 15 mL centrifuge tubes. Centrifuge the organoids for 20 min at 3000 x *g* and 10 °C.

NOTE: This high speed is needed to make sure no oocysts are lost out of any organoids that may be broken.

6.1.3. Remove the organoid media and replace it with 5 mL of water.

6.1.4. Disrupt the organoids by repeated vigorous pipetting with a fire-polished glass Pasteur pipette.

6.1.5. If clumps are visible, transfer the organoid suspension to a glass dounce homogenizer, and homogenize until organoids are well disrupted. The dounce homogenizer will not affect the oocysts.

6.1.6. Once there are no visible clumps, add 5 mL of buffer A from the oocyst isolation kit. Mix and then add 120 µL of the magnetic beads coated with anti-oocyst IgM.

6.1.7. Incubate the cell suspension and magnetic beads for 2 h at room temperature with continuous mixing on a rocker platform.

6.1.8. At the end of the incubation, place the tubes containing cells and beads on a magnetic separation rack designed for 15 mL tubes.

6.1.9. Rotate the tubes in magnetic separation rack manually for 3 min. The beads will adhere to the side of the tube next to the magnet.

6.1.10. Carefully, with a 10 mL pipette, remove the supernatant from the beads. Resuspend the beads in 450 µL of Buffer B and transfer to a 1.5 mL microcentrifuge tube.

NOTE: Keep the supernatant until the isolation of the oocysts is confirmed.

6.1.11. To collect any remaining beads and oocysts, wash the 15 mL tube with 450 µL of Buffer B and add this wash to the magnetic beads in the microcentrifuge tube.

6.1.12. Repeat step 6.1.11 one more time. All beads and captured oocysts should now be transferred to the microcentrifuge tube.

6.1.13. Place the microcentrifuge tube on a magnetic separation rack designed for holding microcentrifuge tubes.

6.1.14. Rotate the tube in the magnetic separation rack by hand for 3 min.

6.1.15. Carefully remove the supernatant with a pipette into a new tube.

NOTE: Keep the supernatant until the isolation of the oocysts is confirmed.

6.1.16. Remove the microcentrifuge tube containing magnetic beads and oocysts from the magnetic separation rack.

6.1.17. Add 100 µL of 0.1 N HCl to magnetic beads to elute the oocysts off the beads. Vortex for

30 s.

NOTE: Vortexer should be set to slightly less than maximum speed.

6.1.18. Incubate the beads in 0.1 N HCl for 10 min at room temperature.

6.1.19. Vortex again. Then place the tube back on magnetic separation rack. Wait for beads to adhere to the side of the tube and then transfer the supernatant to a new microcentrifuge tube.

6.1.20. Repeat steps 6.1.17 through 6.1.19 and combine the second eluate with the first elution.

6.1.21. Neutralize the eluate with 20 µL of 1 N NaOH, or another neutralizing buffer such as 1 M Tris, pH 8.

6.1.22. To count oocysts, take 10 µL of the eluate, combine it with 10 µL of oocyst-specific antibody (See **Table of Materials**) and count fluorescent oocysts on a hemocytometer.

NOTE: Isolated oocysts can be stored at 4 °C or used immediately for immunofluorescence or electron microscopy imaging.

REPRESENTATIVE RESULTS:

The protocols presented here results in the efficient purification of oocysts and sporozoites (**Figure 1A**) ready for microinjection. The excystation protocol results in the release of sporozoites from approximately 70–80% of the oocysts, therefore it is essential to filter out the remaining oocysts and shells through a 3 µm filter. Filtration results in almost 100% sporozoite purification (**Figure 1B**). Furthermore, addition of a green dye helps ensure injection of all organoids and allows visualization of injected organoids for at least for 24 h after injection (**Figure 2B**).

These protocols for preparation of oocysts and sporozoites are straightforward and have been used for many years, so it is expected that the treated oocysts and purified sporozoites will be viable and infectious. However, in our studies, we used scanning electron microscopy to ensure that the excystation process did not damage the sporozoites or oocysts (**Figure 2A**)[10]. Injection of equal amounts of oocysts into the organoid lumen can be visually confirmed by simple microscopic imaging (**Figure 2C**). A portion of infected organoids should be set up to verify parasite propagation by quantitative PCR as we have described[10].

Progress through the parasite life cycle can be visualized by collection of infected organoids at different time points post infection and analysis by transmission electron microscopy or by immunofluorescence combined with 4′,6-diamidino-2-phenylindole (DAPI) staining of parasite nuclei[10]. For example, antibodies to merozoite surface antigens, such as gp40 and gp15[17] can be used to identify meront stages; type I meronts will have 8 nuclei and type II meronts, 4 nuclei[10]. Recently, a panel of monoclonal antibodies specific to trophozoites, merozoites, type I versus II meronts, and macrogamonts has become available[18]. These antibodies would also be very effective in marking progress of the parasite through its various life cycle stages in the organoids.

Immunofluorescence assays can also be used to explore which cell types are infected by *Cryptosporidium*. This was especially important to look at in the airway organoids as very little is known about respiratory cryptosporidiosis, and the exact host cell for the parasite was not known. We conducted immunofluorescence assays on *Cryptosporidium*-infected organoids, co-localizing CC10, a marker for club cells and found that *Cryptosporidium* infected both CC10-negative and positive cells (**Figure 3**). These results were corroborated by TEMs in which we observed *Cryptosporidium* infecting secretory and non-secretory cells in the airway organoids[10].

After differentiated organoids have been infected for five days, there should be significant numbers of oocysts being produced. In our hands, infection of organoids from one six-well plate yielded about 4000 oocysts, which could be easily identified and counted on a hemocytometer by labeling with an oocyst-specific antibody. The presence of four sporozoites in the oocysts could be confirmed by drying down a portion of the oocysts onto an adhesive slide fixing with methanol and combining DAPI staining with oocyst specific antibody (**Figure 4**). Verification of production of thick walled oocysts could be done by TEM analysis[10].

FIGURE & TABLE LEGENDS:

Figure 1: Preparation and purification of *Cryptosporidium* oocysts and sporozoites. (**A**) Schematic representation of the method used for oocyst and sporozoite preparation for infection. (**B)** Image showing in vitro excystation of oocysts. Filtration of unexcysted oocyts and shells gives a purified solution of sporozoites. Scale bar equals 10 µm.

Figure 2: Microinjection of oocysts into the organoid lumen. This figure has been modified from Heo et al.[10]. (**A**) scanning electron microscopy (SEM) images of oocysts and sporozoites. (**B**) Image showing oocyst-injected organoids. The green dye helps visualize the injection of each organoid and persists over at least 24 h. (**C**) Image of an organoid injected with oocysts.

Figure 3: Immunofluorescence image of *Cryptosporidium*-infected airway organoid. Mucin is labeled with anti-mucin 5 antibody (red) in the lumen of the organoid, club cells are labeled with anti-CC10 (yellow), *Cryptosporidium* is detected with oocyst-specific antibody (green), and cell nuclei are stained with DAPI (blue). Panel B is an enlargement of the area indicated in the square in panel A.

Figure 4: Immunofluorescence image of oocyst isolated from differentiated intestinal organoids. Oocyst wall is labeled with oocyst-specific antibody in green and the four sporozoite nuclei are visualized with DAPI (blue)

DISCUSSION:

Culture of *Cryptosporidium* parasites in intestinal and airway organoids provides an accurate model to study host-parasite interactions[10] but also has many other applications. For example, current methods of selecting and propagating genetically modified *Cryptosporidium* parasites require passage in mice[19] which does not allow isolation of parasites that have modifications essential for in vivo infection. Organoid culture of *Cryptosporidium* provides an alternative to this procedure. However, we have noted that electroporated sporozoites clump together and block the micropipette. For the purpose of selecting genetically modified parasites, organoids can be grown on collagen coated transwells in a two-dimensional format under differentiation conditions to allow infection with transfected sporozoites and consequently the selection of the genetically modified oocysts. The transwells allow access to both the apical and basolateral surfaces and are stable for extended periods of time.

Currently, we culture organoids in a two-dimensional format for high throughput screening of drugs for cancer tissue-derived organoids (unpublished data). This method of organoid culture can also be adapted for testing of anti-*Cryptosporidium* drugs using the genetically-modified luciferase tagged *Cryptosporidium* strains[19]. Moreover, even though the infection is not tightly synchronized, infection of organoids with sporozoites provides sufficient synchronization of the life cycle that drugs can be tested for their efficacy against specific life cycle stages.

Organoid co-culture systems are now being developed taking into account some other aspects of the host system such as microbiota and immune cells[20]. Thus, the ability to dissect interactions between the parasite and host cells, immune cells and microbiota will soon be possible in vitro. Genetic manipulation of *Cryptosporidium* is also now possible[19], and the combination of fluorescent reporter strains of *Cryptosporidium* and organoid culture will provide the tools for single cell sequencing of infected cells, and even more specifically single cell sequencing of cells infected with specific stages of the parasite.

The success of the experiments described here is highly dependent on the viability and infectivity of the oocysts. Different batches of *Cryptosporidium* oocysts can vary widely in excystation rates and ability to infect host cells. Sufficient yields of sporozoites is dependent on good excystation rates and the excystation rate is not always correlated to infectivity. If low levels of infection or poor excystation are observed with a particular batch of oocysts, time and effort may be saved by obtaining a new lot of oocysts rather than attempting to increase oocyst numbers, or lengthening incubation times.

Organoid culture media should be refreshed every alternate day. Use of earlier passages of organoid cultures is advisable. It is important to thaw a new vial of organoids if organoids start to differentiate in later passages as health of organoid cultures vastly determine viability of the parasite. After infection, organoid media should be refreshed every day to avoid accumulation of toxic substances in the media.

Organoid culture of *Cryptosporidium* is limited in that the parasite cannot be propagated indefinitely, and the infection peters out after three passages over 28 days[10]. Microinjection of sufficient organoids for mouse experiments such as we have described can be time-consuming and physically taxing. Nevertheless, to date, no other method enables the complete life cycle in an in vitro system completely representative of human infection, nor has any culture system been described that allows exploration of the host-pathogen interactions important for respiratory infection. Organoid culture of *Cryptosporidium* provides a powerful new tool that opens up avenues of exploration into host-parasite interactions not previously possible for *Cryptosporidium*.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1 Checkley, W., et al. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. *The Lancet Infectious Diseases.* **15** (1), 85-94, doi:10.1016/S1473-3099(14)70772-8, (2015).
2 Bones, A. J., et al. Past and future trends of *Cryptosporidium* in vitro research. *Experimental Parasitology.* **196** 28-37, doi:10.1016/j.exppara.2018.12.001, (2018).
3 Muller, J., Hemphill, A. In vitro culture systems for the study of apicomplexan parasites in farm animals. *International Journal for Parasitology.* **43** (2), 115-124, doi:10.1016/j.ijpara.2012.08.004, (2013).
4 Karanis, P., Aldeyarbi, H. M. Evolution of *Cryptosporidium* in vitro culture. *International Journal for Parasitology.* **41** (12), 1231-1242, doi:10.1016/j.ijpara.2011.08.001, (2011).
5 Morada, M., et al. Continuous culture of *Cryptosporidium parvum* using hollow fiber technology. *International Journal for Parasitology.* **46** (1), 21-29, doi:10.1016/j.ijpara.2015.07.006, (2016).
6 DeCicco RePass, M. A., et al. Novel Bioengineered Three-Dimensional Human Intestinal Model for Long-Term Infection of *Cryptosporidium parvum*. *Infection and Immunity.* **85** (3), doi:10.1128/IAI.00731-16, (2017).
7 Miller, C. N., et al. A cell culture platform for *Cryptosporidium* that enables long-term cultivation and new tools for the systematic investigation of its biology. *International Journal for Parasitology.* **48** (3-4), 197-201, doi:10.1016/j.ijpara.2017.10.001, (2018).
8 Sachs, N., et al. Long-term expanding human airway organoids for disease modelling. *bioRxiv.* **https://www.biorxiv.org/content/early/2018/05/09/318444** (2018).

9 Dutta, D., Clevers, H. Organoid culture systems to study host-pathogen interactions. *Current Opinion in Immunology.* **48** 15-22, doi:10.1016/j.coi.2017.07.012, (2017).

10 Heo, I., et al. Modelling *Cryptosporidium* infection in human small intestinal and lung organoids. *Nature Microbiology.* **3** (7), 814-823, doi:10.1038/s41564-018-0177-8, (2018).

11 Dutta, D., Heo, I., Clevers, H. Disease Modeling in Stem Cell-Derived 3D Organoid Systems. *Trends in Molecular Medicine.* **23** (5), 393-410, doi:10.1016/j.molmed.2017.02.007, (2017).

12 Clevers, H. Modeling Development and Disease with Organoids. *Cell.* **165** (7), 1586-1597, doi:10.1016/j.cell.2016.05.082, (2016).

13 Sato, T., et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology.* **141** (5), 1762-1772, doi:10.1053/j.gastro.2011.07.050, (2011).

14 O'Hara, S. P., Chen, X. M. The cell biology of *Cryptosporidium* infection. *Microbes and Infection.* **13** (8-9), 721-730, doi:10.1016/j.micinf.2011.03.008, (2011).

15 Lendner, M., Daugschies, A. *Cryptosporidium* infections: molecular advances. *Parasitology.* **141** (11), 1511-1532, doi:10.1017/S0031182014000237, (2014).

16 Feng, H., Nie, W., Sheoran, A., Zhang, Q., Tzipori, S. Bile acids enhance invasiveness of *Cryptosporidium* spp. into cultured cells. *Infection and Immunity.* **74** (6), 3342-3346, doi:10.1128/IAI.00169-06, (2006).

17 O'Connor, R. M., Kim, K., Khan, F., Ward, H. Expression of *Cpgp40/15* in *Toxoplasma gondii*: a surrogate system for the study of *Cryptosporidium* glycoprotein antigens. *Infection and Immunity.* **71** 6027-6034 (2003).

18 Wilke, G. et al. Monoclonal Antibodies to Intracellular Stages of *Cryptosporidium parvum* Define Life Cycle Progression In Vitro. *mSphere.* **3** (3), doi:10.1128/mSphere.00124-18, (2018).

19 Vinayak, S., et al. Genetic modification of the diarrhoeal pathogen *Cryptosporidium parvum*. *Nature.* **523** (7561), 477-480, doi:10.1038/nature14651, (2015).

20 Dijkstra, K. K., et al. Generation of Tumor-Reactive T Cells by Co-culture of Peripheral Blood Lymphocytes and Tumor Organoids. *Cell.* **174** (6), 1586-1598 e1512, doi:10.1016/j.cell.2018.07.009, (2018).

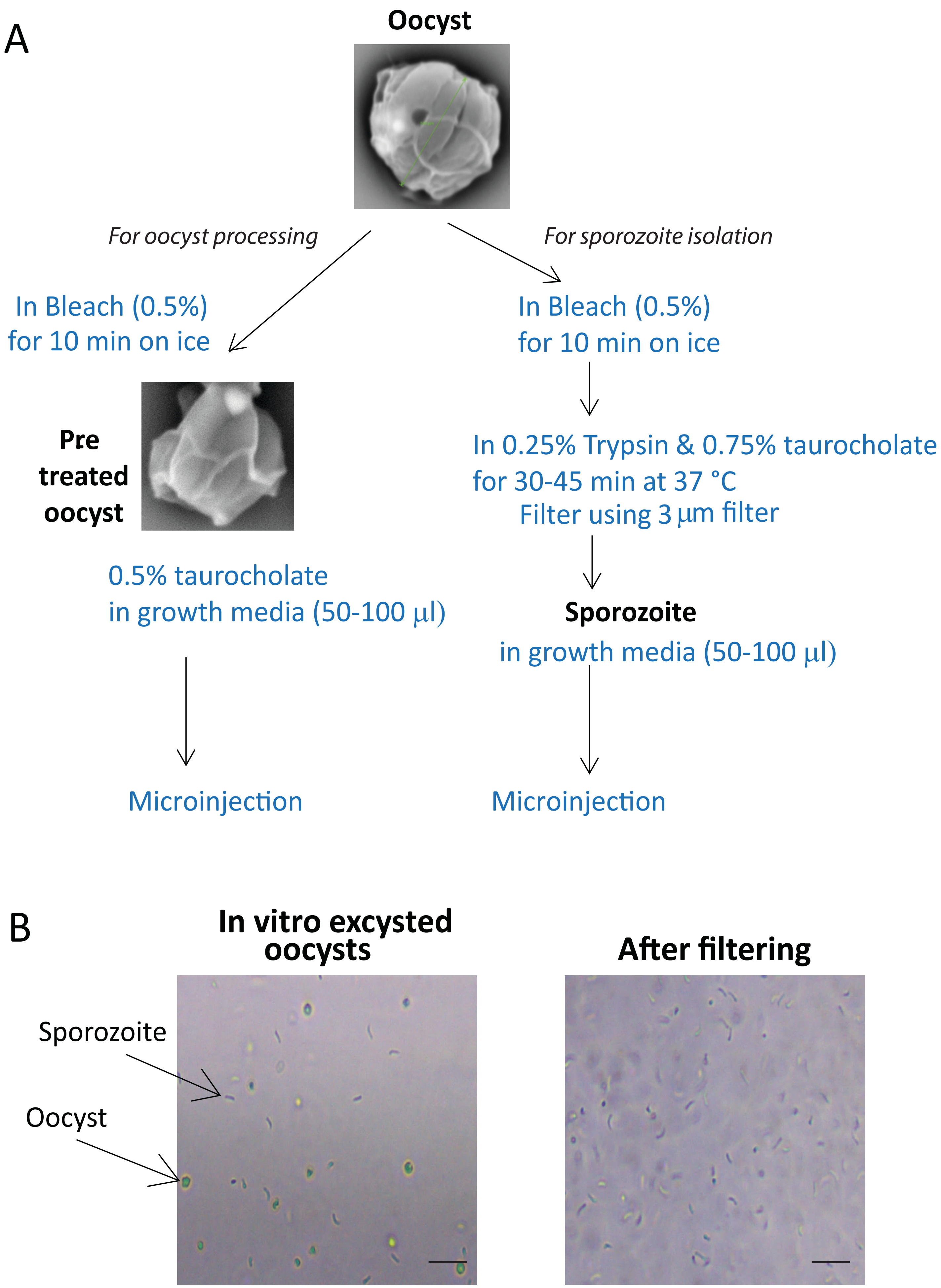
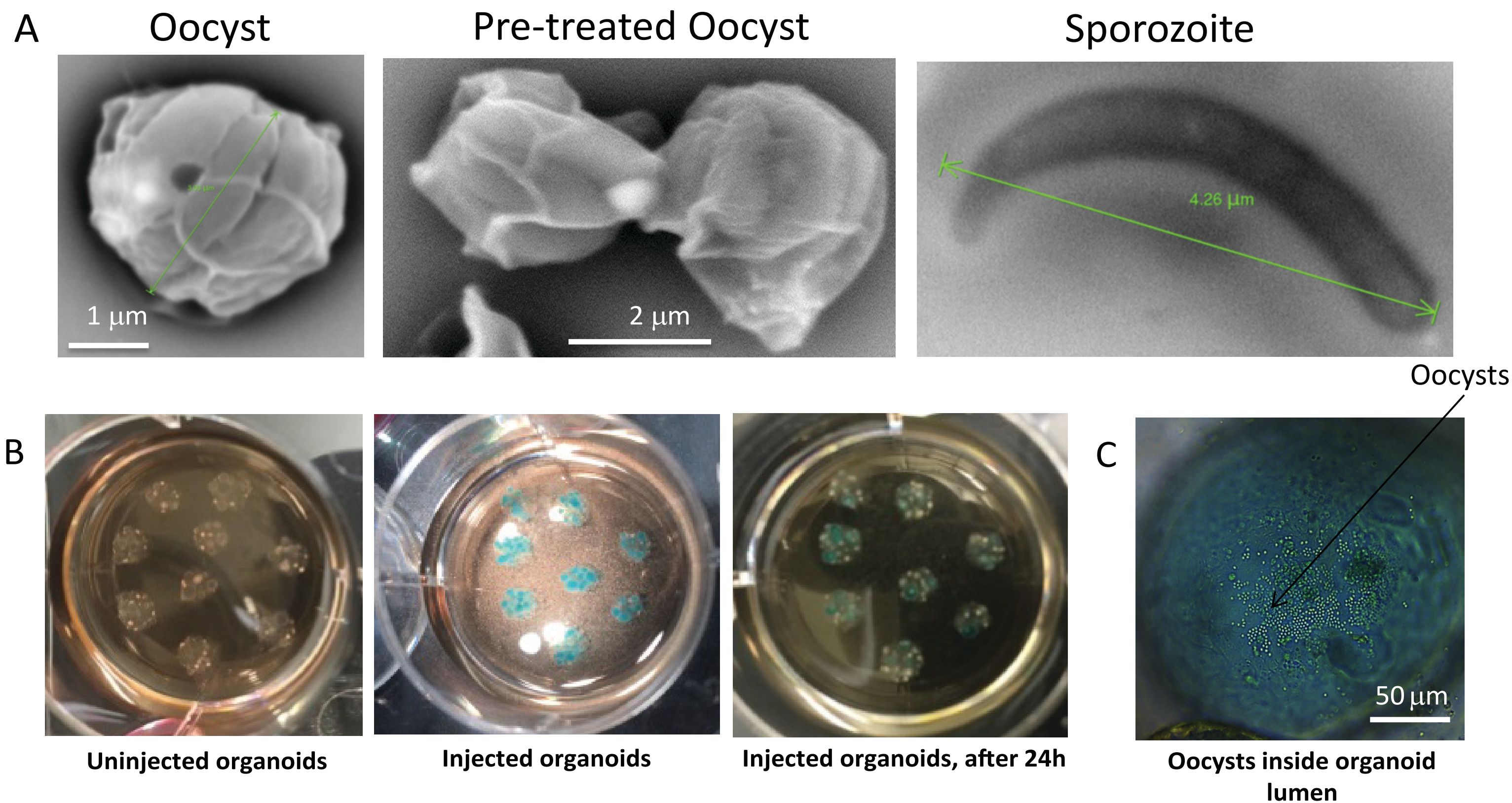

A
Oocyst
For oocyst processing
For sporozoite isolation
In Bleach (0.5%)
for 10 min on ice
In Bleach (0.5%)
for 10 min on ice
Pre treated oocyst
In 0.25% Trypsin & 0.75% taurocholate
for 30-45 min at 37 °C
Filter using 3 μm filter
0.5% taurocholate
in growth media (50-100 μl)
Sporozoite
in growth media (50-100 μl)
Microinjection
Microinjection
B
In vitro excysted oocysts
After filtering
Sporozoite
Oocyst

Figure 1


A
Oocyst
Pre-treated Oocyst
Sporozoite
1 µm
2 µm
4.26 µm
B
Uninjected organoids
Injected organoids
Injected organoids, after 24h
C
Oocysts
50 µm
Oocysts inside organoid lumen

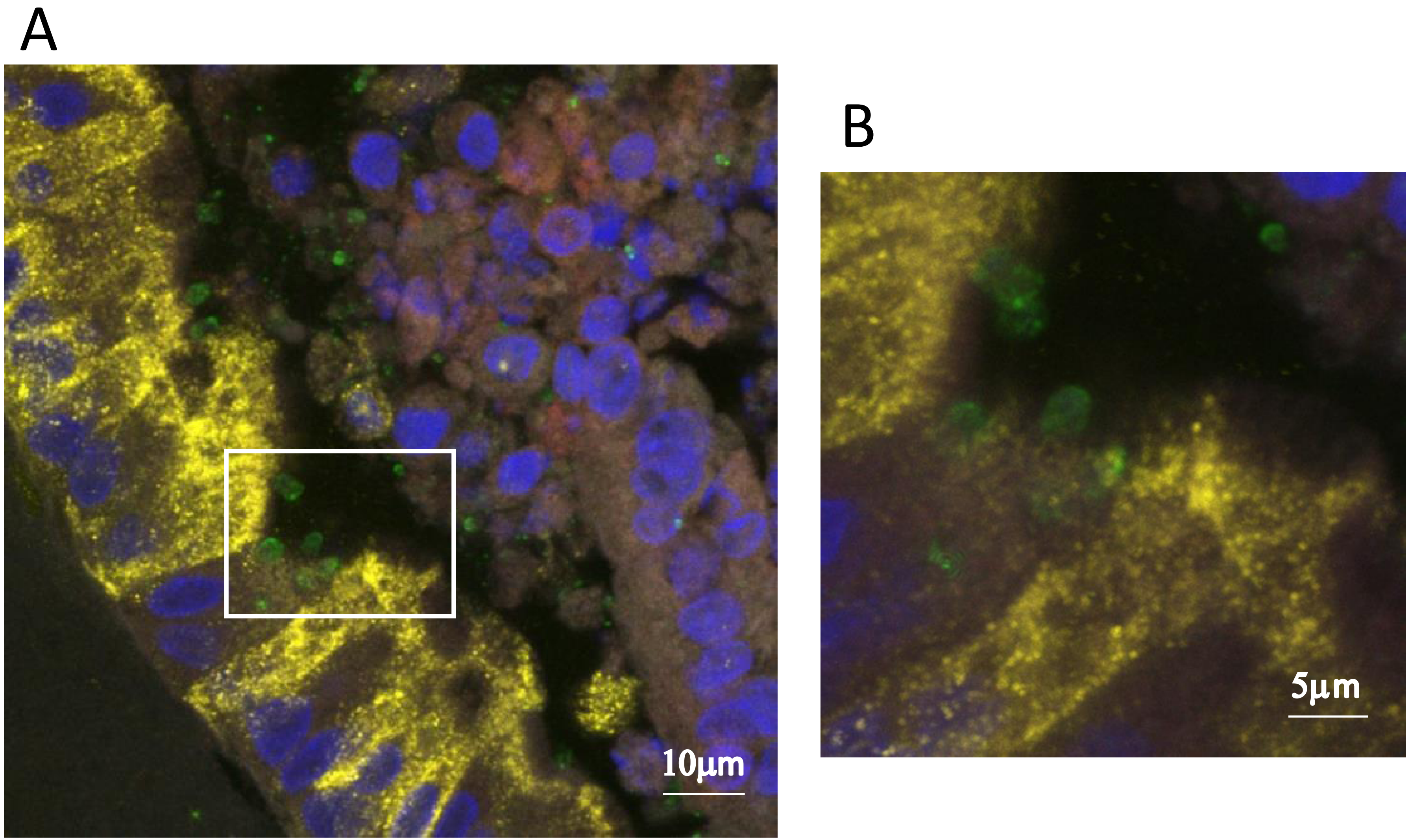
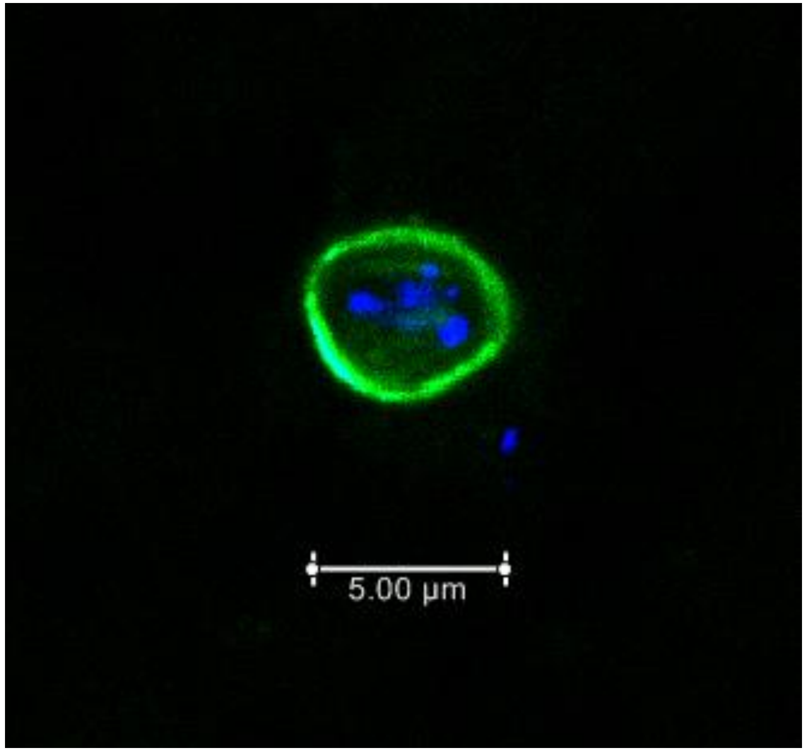

A
B
10µm
5µm

Figure 4



Name of Material/ Equipment	Company
Basement membrane extract (extracellular matrix)	amsbio
Crypt-a-Glo antibody (Oocyst specific antibody)	Waterborne, Inc
Crypto-Grab IgM coated Magnetic beads	Waterborne, Inc
Dynamag 15 rack	Thermofisher Scientific
Dynamag 2 rack	Thermofisher Scientific
EMD Millipore Isopore Polycarbonate Membrane Filters- 3µm	EMD-Millipore
Fast green dye	SIGMA
Femtojet 4i Microinjector	Eppendorf
Glass capillaries of 1 mm diameter	WPI
Matrigel (extracellular matrix)	Corning
Microfuge tube 1.5ml	Eppendorf
Micro-loader tips	Eppendorf
Micropipette puller P-97	Shutter instrument
Normal donkey Serum	Bio-Rad
Penstrep	Gibco
Sodium hypoclorite (use 5%)	Clorox
Super stick slides	Waterborne, Inc
Swinnex-25 47mm Polycarbonate filter holder	EMD-Millipore
Taurocholic acid sodium salt hydrate	SIGMA
Tween-20	Merck
Vectashield mounting agent	Vector Labs
Vortex Genie 2	Scientific industries, Inc

Adv+++ (DMEM+Penstrep+Glutamax+Hepes)	Company
DMEM	Invitrogen
Penstrep	Gibco
Glutamax	Gibco
Hepes	Gibco

INTESTINAL ORGANOID MEDIA-OME (Expansion media)	Company
A83-01	Tocris
Adv+++	
B27	Invitrogen
EGF	Peprotech
Gastrin	Tocris
NAC	Sigma
NIC	Sigma
Noggin CM	In house*
P38 inhibitor (SB202190)	Sigma
PGE2	Tocris
Primocin	InvivoGen
RSpoI CM	In house*
Wnt3a CM	In house*
In house* - cell lines will be provided upon request	

INTESTINAL ORGANOID MEDIA-OMD (Differentiation media)

LUNG ORGANOID MEDIA- LOM (Differentiation media)	Company
Adv+++	
ALK-I A83-01	Tocris

B27	Invitrogen
FGF-10	Peprotech
FGF-7	Peprotech
N-Acetylcysteine	Sigma
Nicotinamide	Sigma
Noggin UPE	U-Protein Express
p38 MAPK-I	Sigma
Primocin	InvivoGen
RhoKI Y-27632	Abmole Bioscience
Rspo UPE	U-Protein Express

Reducing buffer (for resuspension of oocysts and sporozoites for injection)	**Company**
L-Glutathione reduced	Sigma
Betaine	Sigma
L-Cysteine	Sigma
Linoleic acid	Sigma
Taurine	Sigma

Blocking buffer (for immunoflourescence staining)	**Company**
Donkey/Goat serum	Bio-Rad
PBS	Thermo-Fisher
Tween 20	Merck

List of Antibodies used	**Company**
Alexa 568 goat anti-rabbit	Invitrogen
Crypt-a-Glo Comprehensive Kit- Fluorescein-labeled antibody Crypto-Glo	Waterborne, Inc
Crypta-Grab IMS Beads- Magnetic beads coated in monoclonal antibody reactive	Waterborne, Inc

DAPI	Thermo Fisher Scientific
Phalloidin-Alexa 674	Invitrogen
Rabbit anti-gp15 antibody generated by R. M. O'Connor (co-author).	Upon request
Sporo-Glo	Waterborne, Inc

Catalog Number	Comments
3533-010-02	
A400FLR-1X	Final Concentration = Use 2-3 drops/slide
IMS400-20	
12301D	
12321D	
TSTP02500	
F7252-5G	
5252000013	
TW100F-4	
356237	
T9661-1000EA	
612-7933	
P-97	
C06SB	
15140-122	
50371478	
S100-3	
SX0002500	
T4009-5G	
8221840500	
H-1000	
SI0236	

Catalog Number	Final amount
12634-010	500ml
15140-122	5ml of stock in 500ml DMEM
35050038	5ml of stock in 500ml DMEM
15630056	5ml of stock in 500ml DMEM

Catalog Number	Final concentration
2939-50mg	0.5µM
	make upto 100 ml
17504044	1X
AF-100-15	50ng/mL
3006-1mg	10 nM
A9125-25G	1.25mM
N0636-100G	10mM
	10%
S7076-25 mg	10µM
2296/10	10 nM
ant-pm-1	1ml/500ml media
	20%
	50%

To differentiate organoids, expanding small intestinal organoids were grown in a Wnt-rich medium for six to seven days after splitting, and then grown in a differentiation medium (withdrawal of Wnt, nicotinamide, SB202190, in a differentiation medium (withdrawal of Wnt, nicotinamide, SB202190, prostaglandin E2 from a Wnt-rich medium or OME)

Catalog Number	Final concentration
	make upto 100 ml
2939-50mg	500nM

17504044	0.076388889
100-26	100ng/ml
100-19	25ng/ml
A9125-25G	1.25mM
N0636-100G	5mM
Contact company directly	10%
S7076-25 mg	1µM
ant-pm-1	1:500
M1817_100 mg	2.5µm
Contact company directly	10%

Catalog Number	**Final concentration**
G4251-10MG	0.5 µg/µl of OME/OMD/LOM
61962	0.5 µg/µl of OME/OMD/LOM
168149-2.5G	0.5 µg/µl of OME/OMD/LOM
L1376-10MG	6.8 µg/ml of OME/OMD /LOM
T0625-10MG	0.5 µg/µl of OME/OMD/LOM

Catalog Number	**Final concentration**
C06SB	2%
70011044	Make upto 100ml
P1379	0.1%

Catalog Number		
A-11011	Dilution-1:500; RRID: AB_143157	
A400FLK	Dilution- 1:200	
IMS400-20	Dilution-1:500	

D1306	Dilution-1:1000; RRID : AB_2629482	
A22287	Dilution-1:1000; RRID: AB_2620155	
Upon request	Dilution-1:500	
A600FLR-1X	Dilution- 1:200	

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Response: We have now added details of puller settings to 4.1.1

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Response: We have structured the discussion to include all the above mentioned points.

• **Figures:**
1) Fig 1B, 3A, 3B: Add scale bars,

Response: We have added scale bars to all figures now.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Eppendorf, falcon, Matrigel, WPI, Crypto-Grab, Crypt-a-Glo.1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Response: We have removed all commercial words and replaced them with more generic terms.

• **Table of Materials:**
1) Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as antibodies (with RRIDs and concentrations), magnetic beads
2) Please use the attached template and order items in alphabetical order in the "Name" column.

Response: We have ordered all entries alphabetically and also added details about magnetic beads.

Response to reviewers:

Reviewer #1:

Manuscript Summary:
The manuscript by Dutta et al represents essentially a follow-up of a previous study published by the same authors introducing for the first time an efficient system that enables the completion of the full biological cycle of Cryptosporidium parvum using tissue derived organoids. In the current study the authors provide essential experimental details that are fundamental for an efficient culture of Cryptosporidium in organoids. The procedure for maintaining the organoids in culture as well as for microinjecting them with the parasites are rate limiting steps that have a major effect on the yield of this method.

Major Concerns:
The authors should provide additional experimental details to help the scientific community take full benefit of this method. For instance, the authors do not clearly indicate how many sporozoites are microinjected in which amount of organoids. The authors should discuss the difference, if any exists, between using oocyst as opposed to sporozoites for microinjection since they have tried both approaches.

Response: We thank the reviewer for the valuable advice. We have now added more details about the number and difference between oocyst and sporozoite injection (Lines 201-208).

Minor Concerns:
The authors should be more careful with their statements and avoid making excessive conclusions. For instance, claiming that organoids reflect in vivo conditions is an unnecessary exaggeration. The organoids should be valued for what they are, better than cell culture, but they certainly do not reflect in vivo conditions.

Response: We have modified the language of the manuscript and have tried to avoid exaggeration of claims as suggested by the reviewer.

Overall, although the current work does not represent a major innovation compared to the previous work by the same authors it brings important technical details that are essential for vulgarizing the use of organoids in general and in the Crytosporidium field more specifically.

Reviewer #2:

Manuscript Summary:
Cryptosporidium is one of the major causes of human diarrheal disease. To understand pathology of the parasite and develop efficient drugs, an in vitro culture system that recapitulates the conditions in humans is needed. This report provides protocols to prepare oocysts and purify sporozoites for studying infection of human intestinal and airway organoids by Cryptosporidium parvum. It also describes in details the microinjection procedure for injection of the microbes into the intestinal organoid lumen, resulting in full development of all stages of the parasite life cycle. All the steps listed in the procedure are clearly explained and all the critical steps are highlighted. All the materials and equipment needed are listed in the tables and the potential applications for the method/protocol are fully discussed. This is an excellent description of the

methodology that should significantly advance the research field.

Minor Concerns:

1). A brief description of the human organoids in the introduction section may be helpful to the readers.

Response: We thank the reviewer for the valuable advice. We have now added a description of human organoids in the introduction (Line 69-73).

2). A bar value for Figure 3 will be appreciated.

Response: We have now added a scale bar to Figure 3.

Reviewer #3:

Manuscript Summary:
The manuscript describes stringent methodology utilized for Cryptosporidium infection of intact 3D human intestinal and airway organoids via microinjection of sporozoites. Visualized demonstration of the protocol will be extremely useful for researchers working on the mechanisms of cryptosporidiosis. However, demonstration of at least a key parameter of host processes that is altered in Cryptosporidium infection should have added additional strength to the manuscript.

Major Concerns:
None

Minor Concerns:

Few minor concerns outlined below should also be addressed
Line 107: Number of oocysts calculated is for the whole plate or per well?

Response: We have now added details of number of oocyts and organoids per plate to the text (Line 120-128).

Line 111: In general, how many organoids will be there per well in the 6-well plate?

Response: We have now added details of number of oocyts and organoids per plate to the text (Line 120-128).

Line 127: Taurocholate itself at this concentration (0.5%) could alter activities of ion transporters that are known to be modulated by Cryptosporidium infection. Earlier studies described use of lower concentrations [Gold et al, J. parasitol, 87,997, 2001 (0.375%): Kumar et al, Cell Microbiol, 20, e12830, 2018 (0.2%)]. In this manuscript, 2 methods are described for preparing sporozoites for microinjection (Figure 1). In method 1, 0.5% taurocholate remains in the treatment media, whereas in method 2, 0.75% taurocholate is washed out before injection. Distinctive use and rationale of the two methods should be clearly described.

Response: Taurocholate is used in these assays to improve excystation of Cryptosporidium oocysts. We routinely add 0.75% taurocholate to aid in excystation of oocysts for collection of sporozoites (Cevallos et al, Infect Immun. 2000 Sep;68(9):5167-75) after which the taurocholate is removed and the sporozoites used for infection of cells or organoids. We have tested different concentrations of taurocholate in infection assays using Caco2 cells where the inoculum is intact oocysts, and 0.5% improved rates of infection without appearing to affect the cells (unpublished observations). However the reviewer makes a good point and we have added a note (Line 148-151) to the effect that taurocholate may have unanticipated effects on cells, and that lower concentrations have been used successfully in infection assays (Feng et al, Infect Immun. 2006 Jun; 74(6):3342-6.

Line 322: The purpose of using 0.1N HCl should be stated.

Response: **0**.1N HCl elutes the oocysts off the magnetic beads**.** We have now added this to the text (Line 390-391).

Line 385: Sources of Crypt-a-Glo and all other reagents used for immunological detection of the parasite should be stated.

Response: We have added details of sources in the materials.

Figure numbers are missing for all figures.

Response: We have added figure numbers to all figures now.

Reviewer #4:

Manuscript Summary:
In the manuscript entitled "Studying Cryptosporidium infection in 3D tissue derived human organoid culture systems by microinjection" authors explain the protocol to prepare oocysts and purify sporozoites for studying infection by microinjection of human intestinal and airway organoids. This is a significant technical progress for the study of cryptosporidiosis, and the diffusion of the protocol is important. Nevertheless, further revision of the manuscript is needed. In the introduction the parasite life cycle has to be corrected. Some aspects of the protocol need clarification. Figures and legend need to be modified.

Major Concerns:

Introduction:

There are mistakes in the parasite life cycle that have to be corrected.
Lines 75—78: Concerning transmission of Cryptosporidium authors should clarify that the gastrointestinal infection caused by C. parvum and that respiratory cryptosporidiosis in humans has been reported to be uncommon.

Response: Recent studies have shown that respiratory cryptosporidiosis is not as uncommon as previously thought (see Mor, et al Am J Trop Med Hyg. 2018 Apr;98(4):1086-1090.) Thus we would prefer not to make a strong statement about the rarity of the respiratory form of the disease.

Line 78: replace" rupture" by "excystes".

Response: done

Lines 79-80: Authors state that "Cryptosporidium apparently induces the cell to engulf it". This is not true, the sporozoites of Cryptosporidium have a gliding motility and the presence of the apical complex composed of secrete organelles enable the adherence and invasion to the cell inducing the cell membrane to enclose the parasite in the parasithophorous vacuole.

Response: we have modified the sentence in the text (lines 84-85).

Line 80-81: Replace "The parasite then remains on the periphery of the cell, under the cell mebrane but outside the cell cytoplasm" by "the parasite which is internalized within an intracellular but extracytoplasmic compartment remains in the apical surface of the cell".

Response: we have modified the sentence in the text (lines 84-85).

Line 82: Replace "It undergoes 2 round of sexual reproduction" by "It undergoes 2 round of sexual reproduction or merogony. Type I meronts which contain eight merozoites that are released to invade new cells.are formed ".

Response: we have modified the sentence in the text (lines 87-89).

Line 84: replace "Type II merozoites » by « These merozoites when released ..."; replace "macrogametes and microgametes" by "macrogamonts and microgamonts".
Macrogametes and microgametes are formed after macrogamonts and microgamonts. The text has to be modified.

Response: we have modified the sentence in the text (lines 90-92).

Lille 88: Replace "All stages of the Cryptosporidium life cycle were found in the organoid culture system" by All stages of the Cryptosporidium life cycle have been identified in the organoid culture system previously develop by our group".

Response: we have modified the sentence in the text (lines 95-96).

Protocol:
Maybe give a brief introduction to explain that is possible to infect organoids either with oocysts or sporozoites because is not very clear.
Lines 98-101: Specify if these oocysts are commercial, how they were produced (calf or mice passage) and storage solution.

Response: we have added the details to the text (Line 108-114).

Lines 107-109: Specify how oocysts area counted, which technique.

Response: we have added the details to the text (Line 120-125).

Line 138: Is not clear why authors recommend the utilization of masks if the experimentation is performed in a tissue culture hood with level 2 safety protocol. The use of mask is not in the recommendations for handling Cryptosporidium.

Response: we have recommended the use of masks as some of the steps of centrifugation needed exposure outside of the culture hood and as a precautionary measure, we used masks for these steps.

Lines 157-159: It would be important to add a step to check oocysts in the suspension before excystation to verify percentage of intact oocysts.

Response: we have added this to the text (Line 154).

Lines 222-223: Specify at which temperature have to be the matrigel for manipulation.

Response: we have added the details to the text (Line 247-252).

Can authors give more details about the micro injection process. Is microinjection followed by microscopy?

Response: we have added the more details about the microinjection process. Also, as we will be demonstrating this part in the video, we believe that will make the process much more clear for the readers.

Can the protocol for immune staining of organoids be detailed in the protocol section?

Response: We have added a new section (5) detailing the staining protocol (Lines 294-327)

Figures:
Figure 1: In figure 1A add the volumes needed for each case. In figure 1B, is it possible to replace the picture for a new one with a higher magnification? Add a scale to the pictures. In the legend I do not understand when authors state "how filtration purifies sporozoites from non excysted oocysts".

Response: We have added more details to Figure 1.

- We have added scale bars to all pictures.

- We have now modified the legend to make it more descriptive.

Figure 2: The image of the sporozoite does not have a good resolution. It is not clear to me what authors want to show in C.

Response: As 1B is a scanning EM image of a sporozoite using a Phenom table top machine, this was the highest resolution which could be achieved. Figure 2C shows an injected organoid (with oocysts and dye) showing that oocyts can be visualized inside the lumen after injection.

Figure 3: Add scales. In the legend is stated that authors used a Crypt-a-Glo antibody. This antibody produced by Waterborne, Inc is designed to detect the oocyst stage of Cryptosporidium parvum which is extracellular. However, in the picture we can see different intracellular stages of the parasite.

Response: - We have added scale bars to all pictures. The target of the Crypt-a-glo antibody has not been published, but it is likely one of the COWP proteins. The expression of these proteins begins during sexual stage development (macrogamonts) and continues through the development of the oocyst. That is likely why Crypt-a-glo reacts with these intracellular stages in Figure 3.

Minor Concerns:
Summary:
Line 36: replace microbes by "parasites".

Response: done

Introduction
Line 67: Rephrase "replace cancer cell lines and therefore not precisely representative"

Response: done

Line 96: Protocol: Replace "Cryptosporidium parvum" by "C. parvum". Species names must be abbreviated after first time the whole name has been mentioned.

Response: done

Line 105: Add "Keep" at the beginning of the sentence.

Response: done

Line 107: Replace "concentration" by "quantity".

Response: done

Line 120: Delete "carefully"at the beginning of the sentence.

Response: done

Line 127: It is not clear what authors mean by "Make up organoid media".

Response: we have modified the sentence.

Line 130: Replace "Resuspended" by "resuspend"

Response: done

Line 130: Are you sure is the right volume?

Response: yes, the volume is correct.

Line 148: Replace "to give" by "to obtain".

Response: done

Line 157: Misspelling of "excystation".

Response: done

Line 166: Replace "mls" by "ml" and "to give" by "to obtain"

Response: done

Line 177: Rephrase as follows: "to success on sporozoite isolation to use fresh oocysts and to get a good excystation rate are critical".

Response: done

Line 199: Rephrase as follows: "produce by far higher numbers of oocysts".

Response: done

Line 238: Rephrase as follows: Expansion of lung organoids is done in a similar manner but using lung organoid specific media components (Table 1)8 . For lung organoids, we do not have separated expansion and differentiation media.

Response: done

Line 247: replace "microbes" by "parasites".

Response: done

Lines 270-273: Rephrase as follows: "Start with differentiated organoids that are uninfected, infected after 1 day and infected after 5 days. The first two will be used as negative controls. We have found that differentiated organoids produce more oocysts than lung or expanding intestinal organoids10".

Response: done

Line 309: Replace "the" by "a".

Response: done

Line 313: Replace "by hand" by "manually".

Response: done

Line 352: Rephrase as follows: addition of a green dye helps to ensure injection of all organoids and to keep visible.

Response: done

Line 356: Rephrase as follows: have been used for many years.

Response: done

Line 384: Rephrase as follows:" a portion of the oocyst suspension".

Response: done

Line 392: Delete "for".

Response: done

Line 393: Delete "there are".

Response: done

Lines 397-399: Rephrase as follows: "under differentiation conditions to allow infection with transfected sporozoites, and consequently the selection of the genetically modified oocysts. The transwells allow access to both the apical and basolateral surfaces and are stable for extended periods of time".

Response: done

Line 403: "can also be adapted for testing of anti-Cryptosporidium drugs using".

Response: done

Line 409-410: Rephrase as follows: "Organoid systems have now being developed taking into account some aspects of the host system such as microbiota and immunity18".

Response: done

Lines 417-425. Rephrase this paragraph, it is not very clear.

Response: we have modified the paragraph.

Place periods (.) at the end at each sentence. They are not always present.

Response: done

COMMENT [A2] - Add to the table of materials

Response – has been added

COMMENT [A3] - Add to the table of materials

Response – has been added

COMMENT [A4] - Which medium? What is the composition? Add it to the table of materials

Response – details have been added to text and materials

COMMENT [A5] - Which medium? What is the composition? Add it to the table of materials

Response – details have been added to text and materials

COMMENT [A6] - What speed and duration?

Response – details have been added to text

COMMENT [A7] - Unclear which buffer this is.

Response – detail added

COMMENT [A8] – Unclear which medium this is.

Response – detail added

COMMENT [A9] – I have highlighted this for completeness

Response – ok

COMMENT [A10] – Unclear which medium this is.

Response – detail added

COMMENT [A11] – Define the composition and add to the table of materials.

Response – added to materials

COMMENT [A12] – What were the sources? Human? Please mention this clearly. Also, please add an ethics statement at the start of the protocol section to state that all tissue handling and resection was performed under IRB approved protocols with patient consent..

Response – details have been added and ethics statement has been introduced.

COMMENT [A13] – If this is specific only to intestinal organoids please say so clearly.

Response – detail added

COMMENT [A14] – Please add a step before this to briefly mention organoid culture conditions, medium/matrix used etc

Response – Additional step/detail added

COMMENT [A15] – Mention tip size if relevant.

Response – added

COMMENT [A16] – Unclear when the organoids were added. Please mention the source of the organoids?

Response – detail added

COMMENT [A17] – Opening? Please provide an example of ideal opening diameter.

Response – detail added

COMMENT [A18] – Unclear what is meant, please revise the language.

Response – language revised

COMMENT [A19] – Reference?

Response – This is a fact we have noticed by experience and not specifically mentioned in any reference.

COMMENT [A20] – I do not see this in the table of materials.

Response – Both Matrigel and BME have been added

COMMENT [A21] – Reference?

Response – This is a fact we have noticed by experience and not specifically mentioned in any reference.

COMMENT [A22] – Which matrix? Add to the table of materials.

Response – added

COMMENT [A23] – What is the composition? Add to the table of materials.

Response – added

COMMENT [A24] – Add to the table of materials.

Response – added

COMMENT [A25] – Please provide generic settings as well so that they can be applied to other instruments.

Response – The conditions for this specific machine have been mentioned. For other machines, using similar settings, standardization should be done.

COMMENT [A26] – Velocity?

Response – yes, added

COMMENT [A27] – Do you view this under a microscope? Mention magnification required

Response – detail added

COMMENT [A28] – Are organoids kept in the six-well plate as in section 3? If not, please mention how they are to be transferred?

Response – detail added

COMMENT [A29] – Please add a step to mention how the organoids are stored until later steps (section 6). Mention medium and environmental conditions,

Response – detail added

COMMENT [A30] – Table 1?

Response – merged into Material table

COMMENT [A31] – Mention antibodies used and add them to table of materials along with concentrations and RRIDs.

Response – added

COMMENT [A32] – Mention antibodies used and add them to table of materials along with concentrations and RRIDs.

Response – added

COMMENT [A33] – Mention imaging settings including magnification, excitation light wavelength, and emission filter settings.

Response – these settings depend on type of microscope and depend on what range of secondary Ab is used.

COMMENT [A34] – How do you check for differentiation?

Response – this is a standard method and mentioned in Materials. If one wishes to confirm, they could check markers by qPCR.

COMMENT [A35] – Please remove the commercial name from the manuscript and add the item to the table of materials.

Response – Replaced with generic name.

COMMENT [A36] – Eluate?

Response – corrected

COMMENT [A37] – Mention antibodies used and add them to table of materials along with concentrations and RRIDs.

Response – added

COMMENT [A38] – Which antibody is used? Mention concentration as well. Also all it to the table of materials with concentration and RRIDs.

Response – added

COMMENT [A39] – Reference?

Response – reference added

COMMENT [A40] – Reference?

Response – added

COMMENT [A41] – Please remove product names

Response – removed

COMMENT [A42] – Permissions? Please upload the permissions agreement/re-use license as a supplementary file.

Response – attached

COMMENT [A43] – Only one is shown here

Response – corrected

COMMENT [A44] – green

Response – added

COMMENT [A45] – please merge the multiple tables into 1 table. Please remove the commercial names and add all items to procure to the table of materials

Response – both tables have now been merged to one

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Title:	Modelling *Cryptosporidium* infection in human small intestinal and lung organoids
Author:	Inha Heo, Devanjali Dutta, Deborah A. Schaefer, Nino Iakobachvili, Benedetta Artegiani et al.
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Date:	Jun 25, 2018

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