2016

Imaging the Oocysts in the Midgut of the Mosquito

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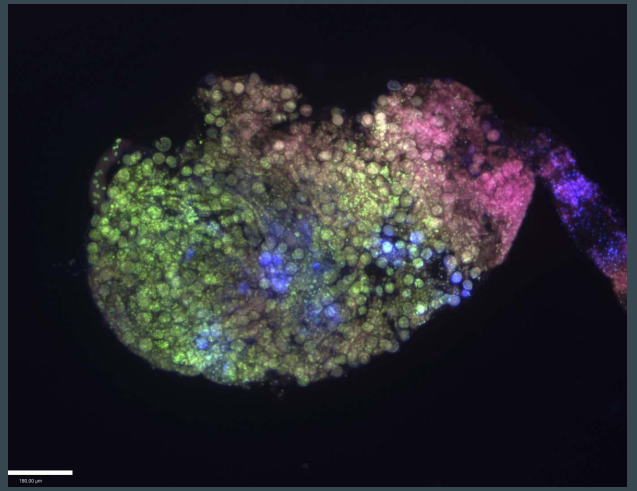
MS Project Portfolio
University of Nebraska
Entomology Summer 2016
Hillary Guzik



Project Outcomes

- Learn how to dissect the midgut from the mosquito
  - Protocol on how to conduct the dissection

- Produce a portfolio of high end microscopy images of the oocysts inside of the midgut
  - Using Leica SP5 Point Scanning Confocal (Analytical Imaging Facility at Einstein College of Medicine)
  - Reconstruct the oocyst in 3D
  - To further be used in Dr. Kami Kim's research

- Develop an image analysis protocol to automatically count each individual oocyst
  - Using Volocity analysis software (PerkinElmer)
  - Gather total counts and area measurements of individual oocysts
  - Written for future use
  - To further be used in Dr. Kami Kim's research

Dissection Protocol

Goal: Learn how to properly dissect the midgut from the mosquito and write up a protocol on how to do so.

Dissection Protocol

1. Vacuum out infected mosquitoes using house air and special tube (with fine mesh fabric over the entry point to house air) and put the collected mosquitoes into a tube.
2. Place tube into freezer for 5 to 10 seconds.
3. Prepare two dishes, one of PBS and the other of 75% ethanol.
4. Plug in your stereoscope and place a glass slide onto the stereoscope.
5. Put all mosquitoes into PBS.
6. Pipette a tiny amount of PBS into the glass plate.
7. Put the mosquitoes you plan to work with into the ethanol dish.
8. Place the selected mosquitoes onto the glass plate into the PBS drop.
9. Cut off the mosquito head.
10. Pull apart the thorax from the abdomen. In this process the eggs, midgut and malpighian tubes should emerge.
11. Cut off the malpighian tubes (leave some intact so that the midgut does not rupture) and the foregut.
12. Place the dissected midgut into a tube or on a slide for oocyst screening.

Staining the Oocyst

With fluorescence protein to highlight the parasites and oocysts

Goal: Produce a portfolio of high end microscopy images of the oocysts inside of the midgut

Project Notes: Staining the oocyst inside the intact midgut proved to be a challenge. Not only the midgut need to be penetrated by both the primary and secondary antibodies, but also the oocyst. The outer part of the oocyst is thought to be mosquito proteins in origin and the inner parasite (CSP) in origin. Many protocols were tested for appropriate staining.

Staining the oocyst

The strains tested included:

- Mercurochrome
- Hoechst 33342
- Syto Green
- Syto Red
- 8 lectin binding: Jacalin, VVA, HPA, DBA, DBA, Con A, s-WGA, SNA-1 , GSL-1
- Anti gfp M and P terminus
- Circumsporozoite protein (CSP) N and C terminus

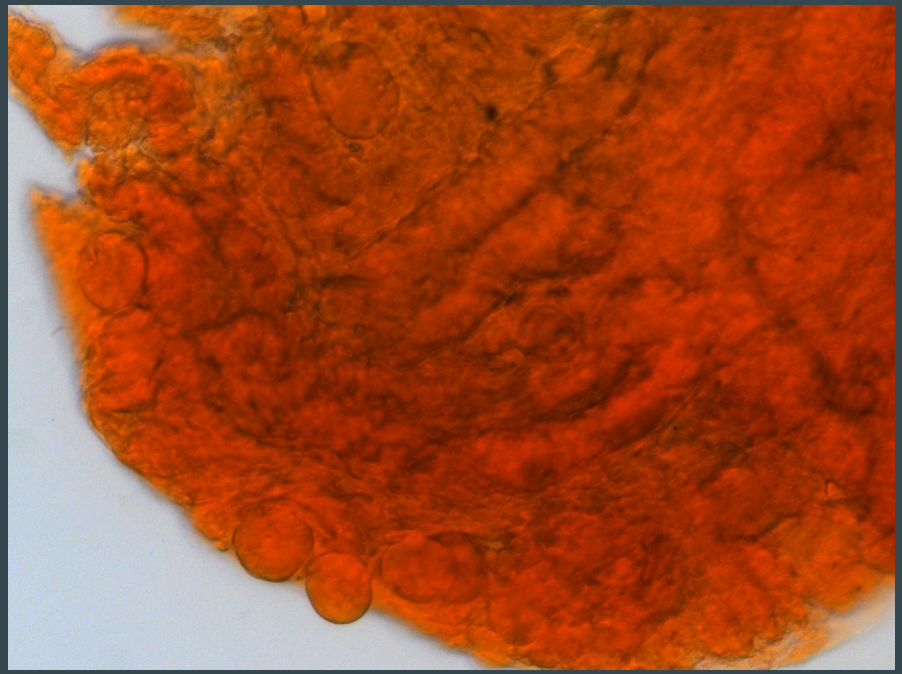
The fluorescence images were acquired on the Leica SP5 point scanning confocal microscope. Each midgut was imaged with the 63X objective with either a 1x or 4x zoom. The parameters for imaging were 512x512, line averaging of 3, Z-step size of 1um.

Transmitted light images were acquired on the Zeiss Observer CLEM or on the lab's EVOS microscope, both at various magnifications.

The conditions of interest for Dr. Kami Kim were R3-HA, R3-KO, WT, and GFP

Mercurochrome Was suppose to stain individual oocyst, but it did not work.

Transmitted light, image Zeiss Observer CLEM, 10x



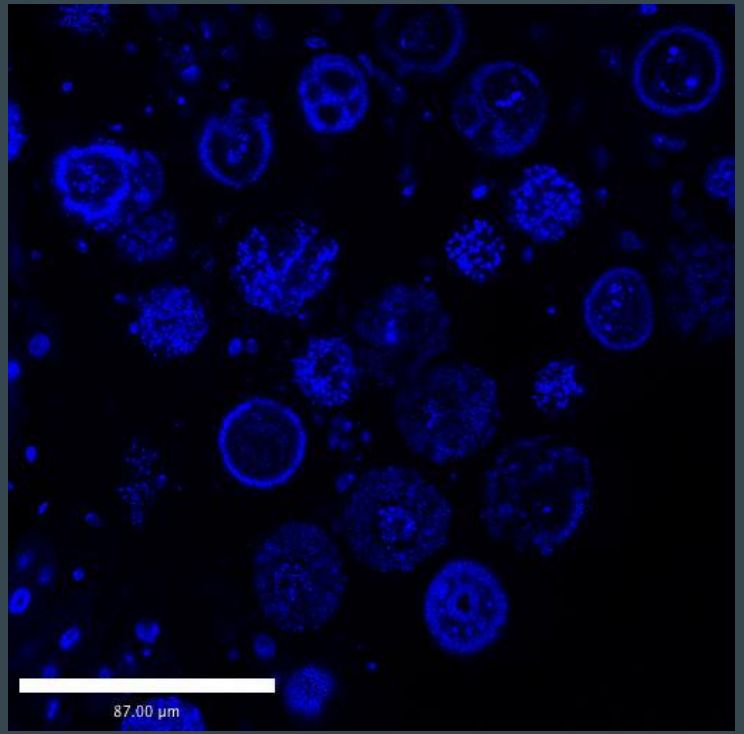
Transmitted light, image Zeiss Observer CLEM, 20x

Mercurochrome Staining protocol

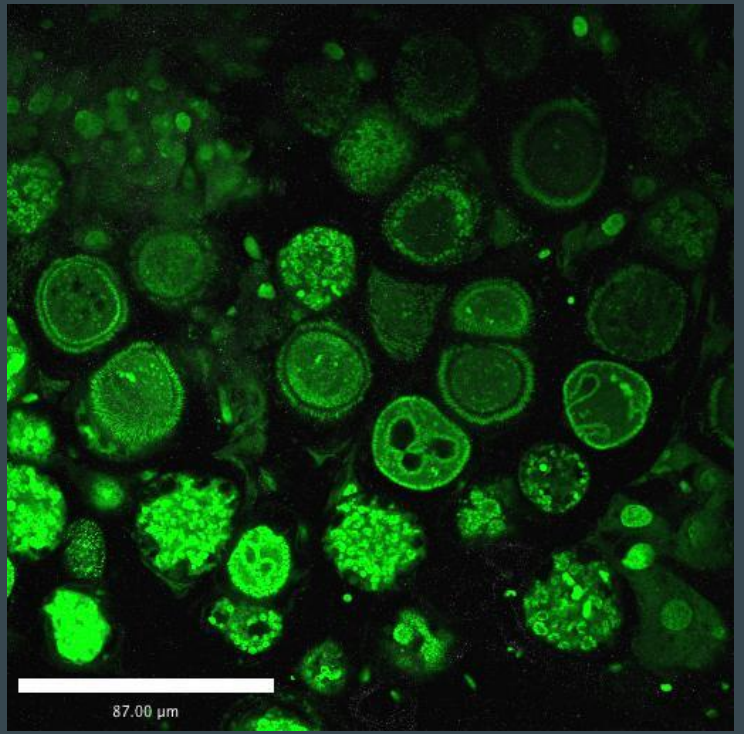
1. Fix midguts 30 min
2. Spin down to form a pellet at bottom of tube (so you don't suck out any midguts)
3. Permeabilized 1% triton rotating for 1 hour at 37℃
4. Block with 3% BSA 0.2% triton overnight at 4℃
5. Wash three times
6. Stained with mercurochrome 1 min (1g in 5ml of 1x PBS)
7. Wash two times 30 sec each
8. Mount fluoromount

Nuclear stains: Hoechst, Syto Green, Syto Red

Hoechst and Syto Green had similar results (show below). Syto Red did not work, but it is considered a “live cell” stain so it made sense that did not work, since after fixation the parasites should be dead.



Hoechst stain oocysts (blue), SP5, 63x, 1x zoom



Syto Green stain oocysts (green), SP5, 63x, 1x zoom

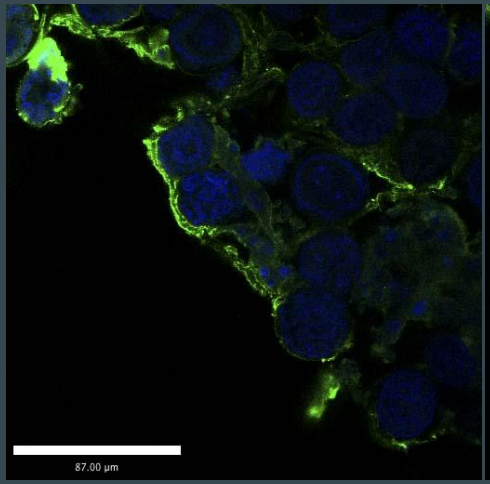
Syto Green and Syto Red Staining protocol

1. Fix midguts 30 min
2. Spin down to form a pellet at bottom of tube (so you don't suck out any midguts)
3. Permeabilized 1% triton rotating for 1 hour at 37℃
4. Block with 3% BSA 0.2% triton overnight at 4℃
5. Wash three times 3 min (non-phosphate buffer)
6. Stain with Syto Green or Syto Red for 30 min
7. Wash three times 3 min each (non-phosphate buffer)
8. Mount fluoromount

Lectin: Eight different lectins were tried: Swga, GSL1, Jacalin, ConA, Wa, Wpa, DBA, SNA1

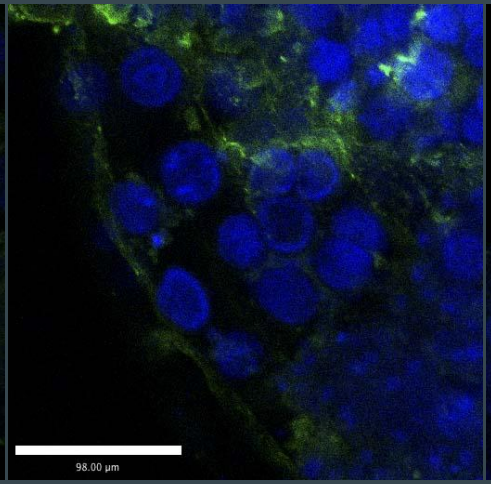
None of the lectins prove to stain the oocyst wall. Swga, GSL1 and Jacalin, (below) were the only ones to have any stain. All others looked like ConA (below) with no staining.

Swga



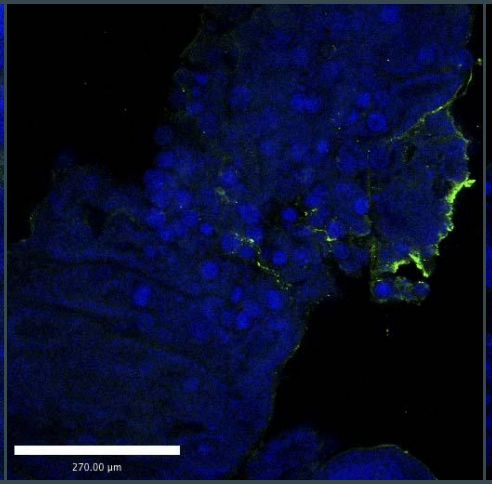
Hoechst (blue), Lectin (green) SP5, 63x 1x zoom

GSL1



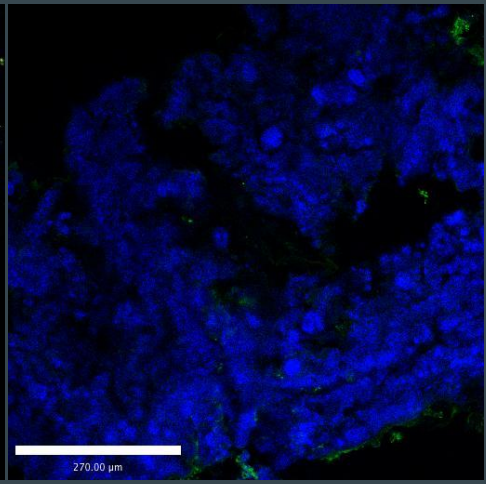
Hoechst (blue), Lectin (green) SP5, 63x 1x zoom

Jacalin



Hoechst stain oocysts (blue), SP5, 20x 1x zoom

ConA



Hoechst stain oocysts (blue), SP5, 20x 1x zoom

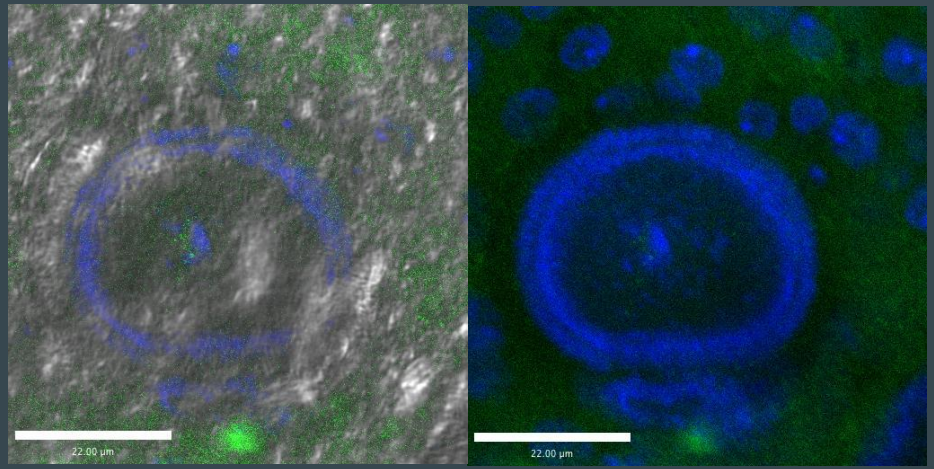
Lectin Staining protocol

1. Fix midguts 30 min
2. Permeabilized with 1% triton
3. Wash PBS
4. Block 3% BSA overnight in cold room
5. Add lectin conjugate 10ug/ml in PBS for 90 min at 37℃
6. Wash with PBS three times
7. Stain with anti-rabbit Alexa Fluor 488 at 1:1000 dilution for 90 minutes at 37°C
8. Wash PBS three times
9. Mount on coverslip with fluoromount

Circumsporozoite Protein (CSP)

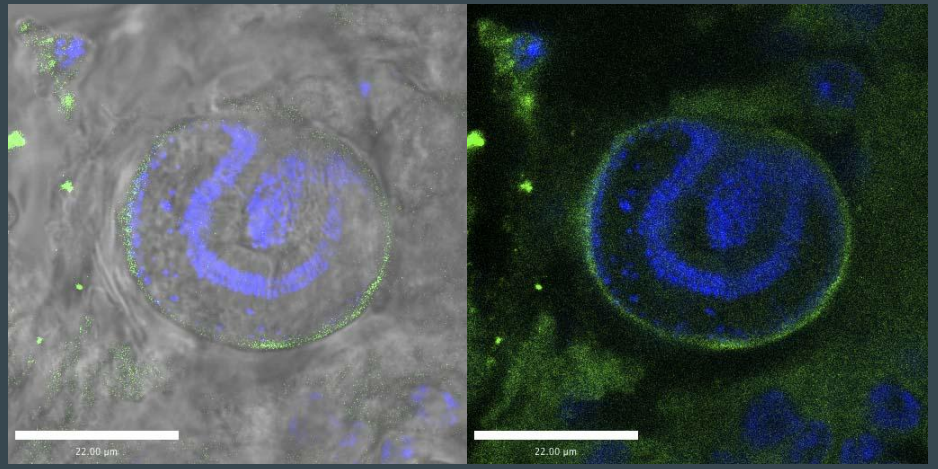
Comparing N terminus and C terminus.

N-terminus did not stain the outer wall of the oocyst, while C-terminus did



CSP-N Terminus

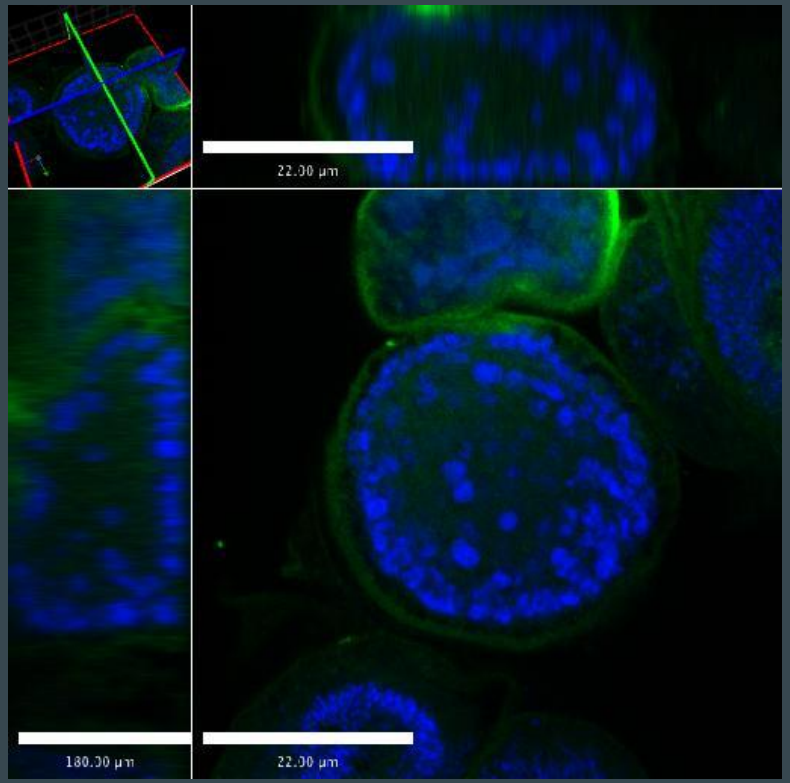
Hoechst stain oocysts (blue), CSP (Green), SP5, 63x, 1x zoom



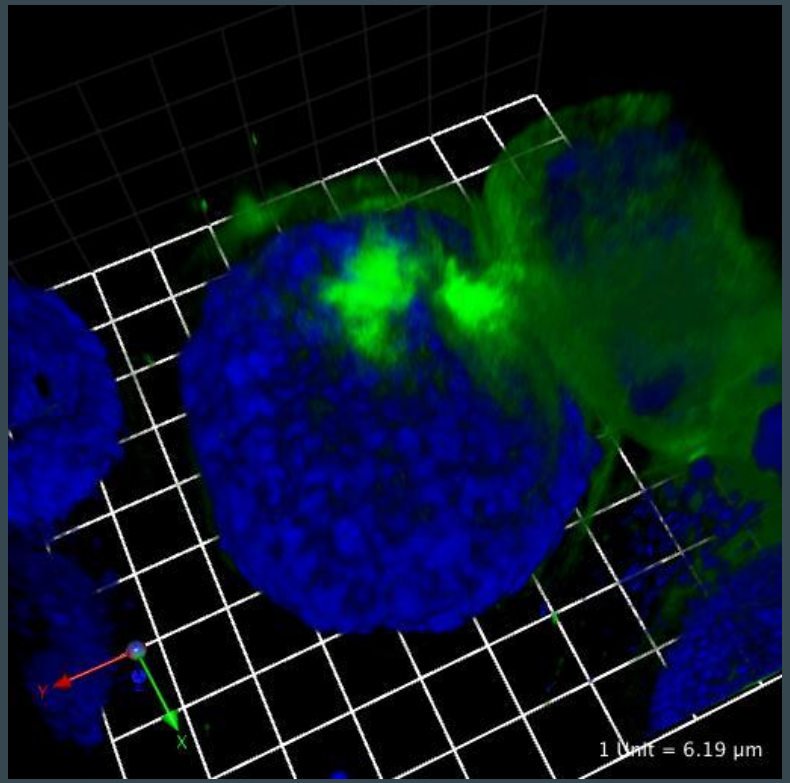
CSP-C Terminus

Hoechst stain oocysts (blue), CSP (green), SP5, 63x, 4x zoom

CSP 3D reconstructions oocysts to illustrate the CSP around the oocyst

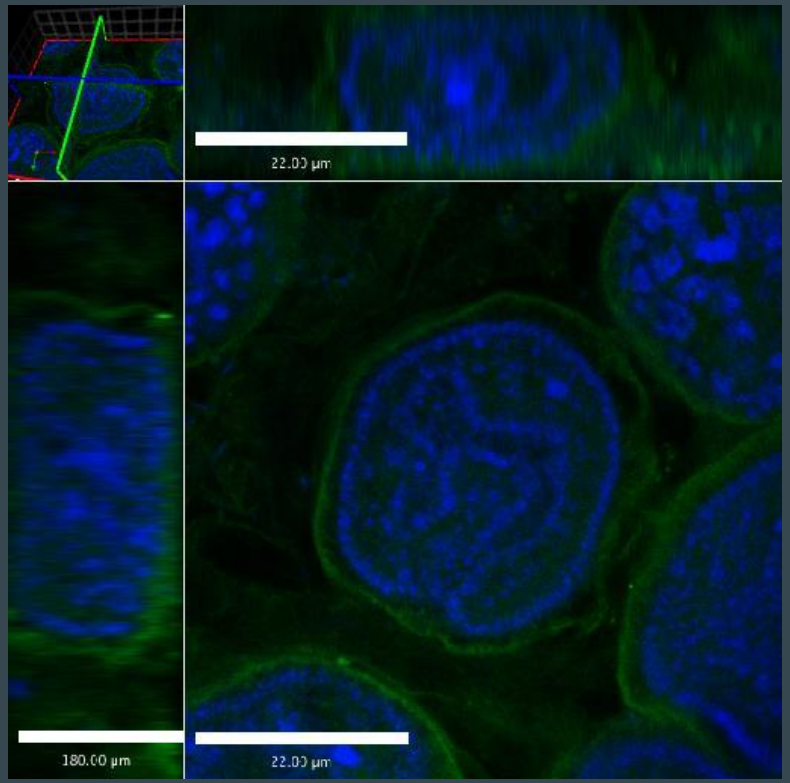


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) and CSP (Green), SP5, 63x 4x zoom, Z-series 1 um slice.

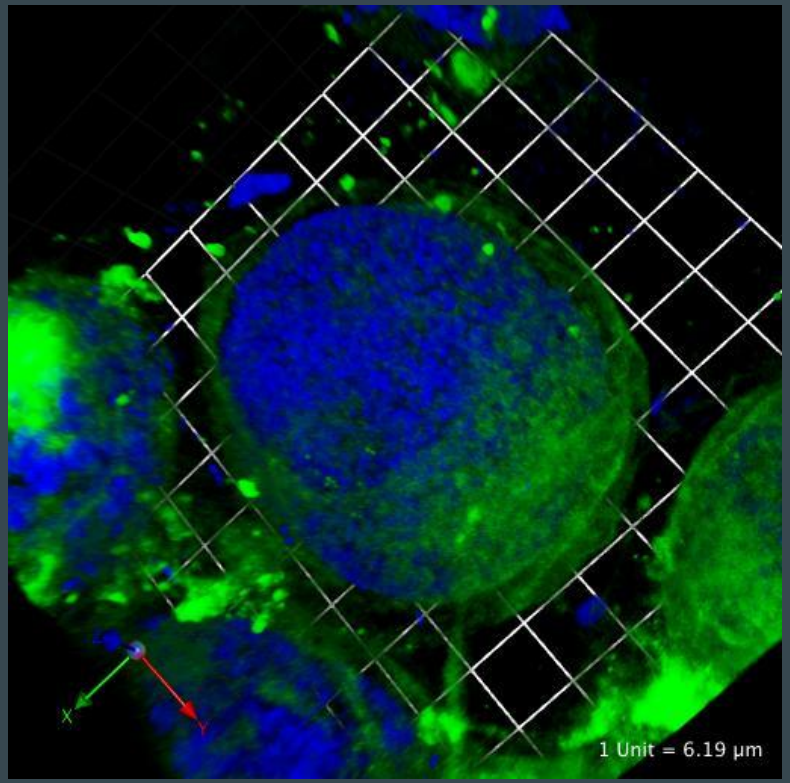


3D reconstruction of oocysts. Hoechst stain (blue) and CSP (Green), SP5, 63x 4x zoom, Z-series 1 um slice.

CSP 3D reconstructions oocysts illustrate the CSP around the oocyst



Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) and CSP (Green), SP5, 63x 4x zoom, Z-series 1 um slice.



3D reconstruction of oocysts. Hoechst stain (blue) and CSP (Green), SP5, 63x 4x zoom, Z-series 1 um slice.

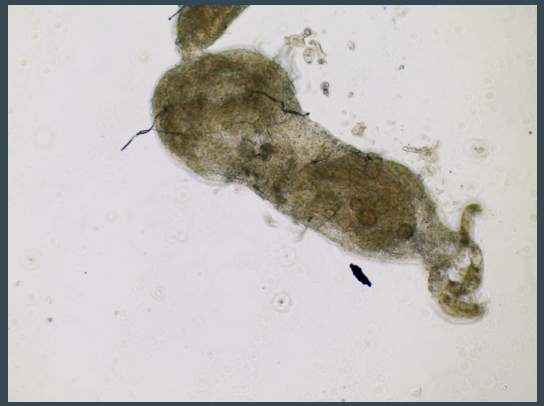
CSP and Hoechst Staining protocol

1. Fix midguts 30 min
2. Spin down to form a pellet at bottom of tube
3. Permeabilized 1% triton rotating for 1 hour at 37℃
4. Wash (non-phosphate buffer)
5. Block with 3% BSA 0.2% triton overnight at 4℃
6. Wash three times at 3 min each (non-phosphate buffer)
7. Stained with primary antibody, anti-CSP C-terminus rabbit 1:500 and Anti-CSP N-terminus 1:500 dilution
8. Wash three times at 3 min each (non-phosphate buffer)
9. Stained with secondary antibody Alexa 488 anti rabbit mixed with Hoechst 1:1000 dilution, for 60 min at room temp
10. Wash three times at 3 min each (non-phosphate buffer)
11. Mount with fluoromount

Conditions of Interest

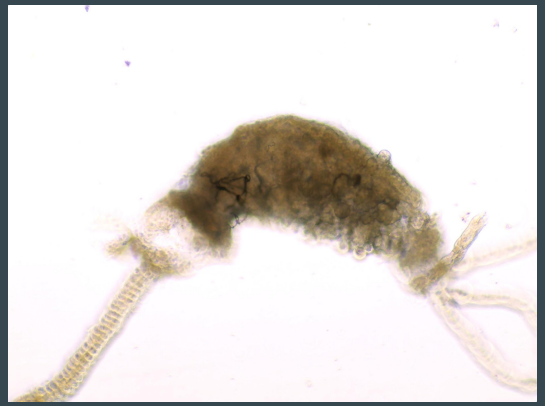
Midguts were stained with Hoechst and Anti CSP-C terminus, however the anti CSP-C did not seem to work in some midguts, but did in others. Below is transmitted light images of each condition that aided in the selection of midguts with at least one oocyst. CSP and Hoest (previous slide) protocol was followed for staining.

- R3-HA
- R3-KO
- WT
- GFP



Transmitted light image of R3-HA midgut on the EVOS desktop system

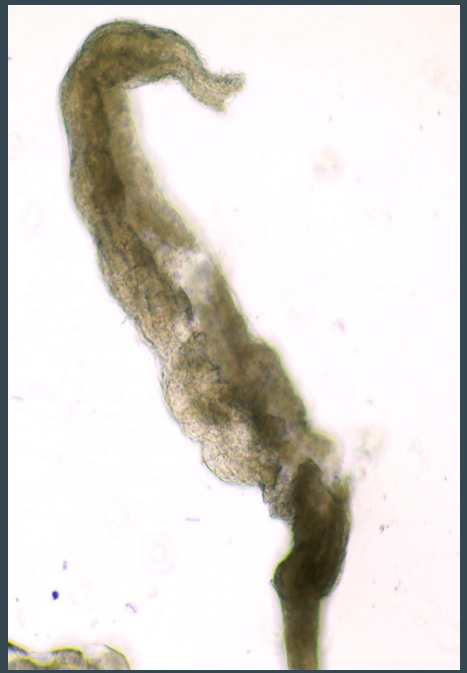
Transmitted light image of R3-KO midgut on the EVOS desktop system



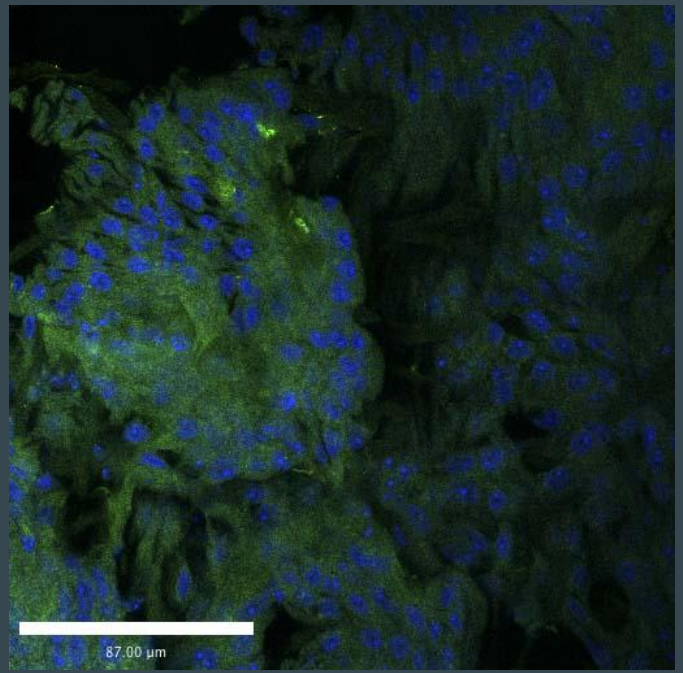
Transmitted light image of WT midgut on the EVOS desktop system

GFP Comparing M terminus and P terminus.

Neither the M or P terminus was proven to work because no oocysts were present. However Li-Ming Ting, the researcher who works on this project, picked most of theses midguts out as having oocysts.

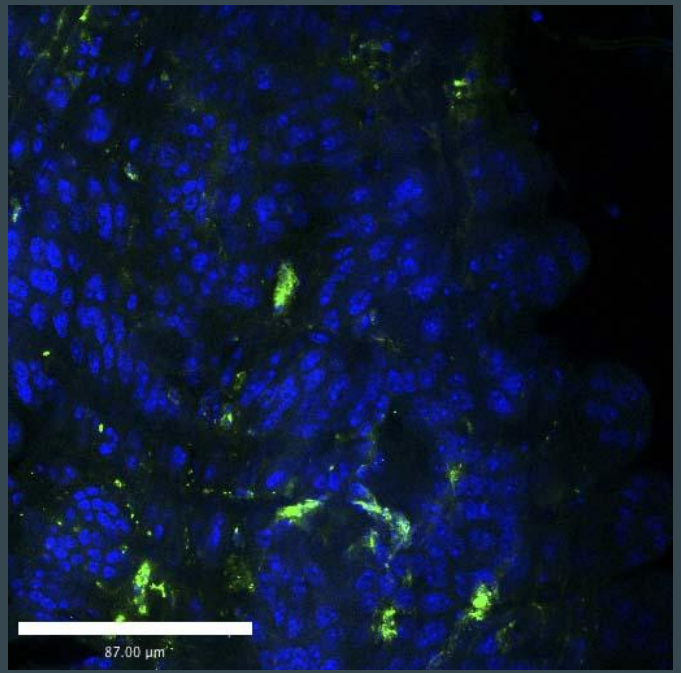


Transmitted light image of midgut on the EVOS desktop system



GFP-M

Hoechst (blue), GFP-M terminus (Green) SP5, 63x, 1x zoom

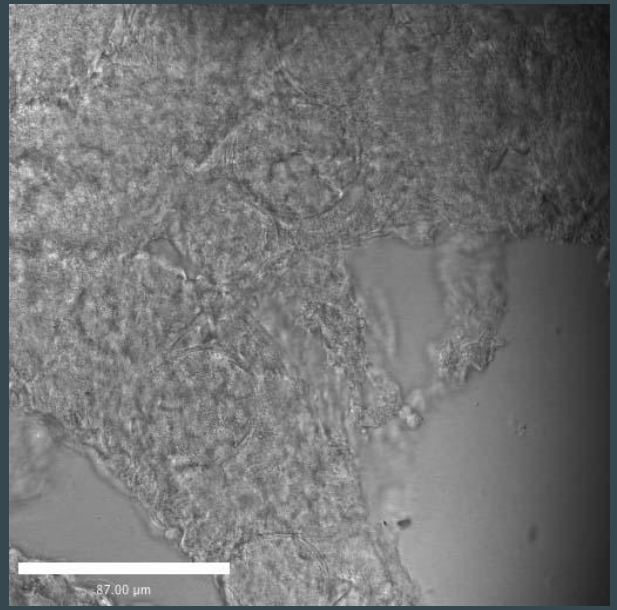


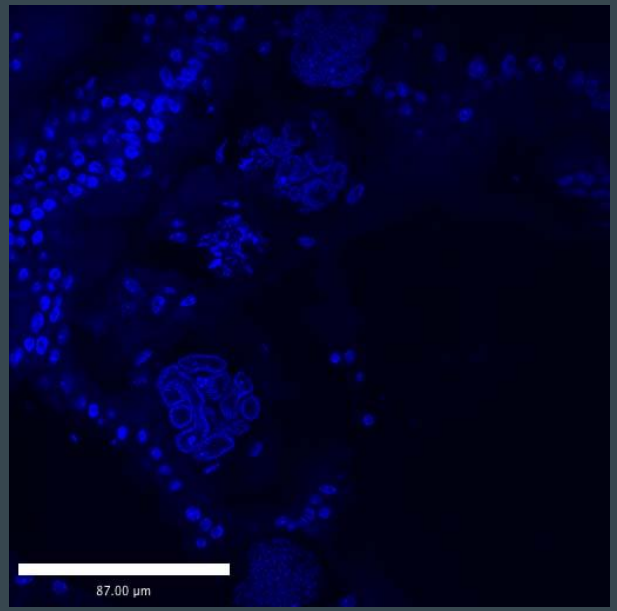
GFP-P

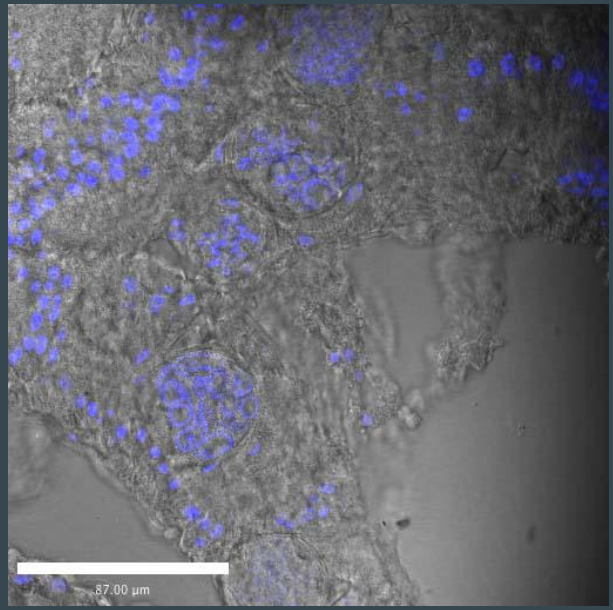
Hoechst (blue), GFP-P terminus (Green) SP5, 63x, 1x zoom

R3-HA A condition of interest

An example of the phase and Hoechst oocysts inside of the midgut







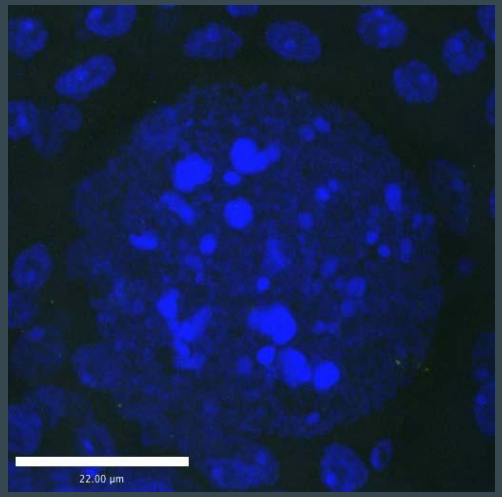
Hoechst stain oocysts (blue) with phase image, SP5, 63x 1x zoom

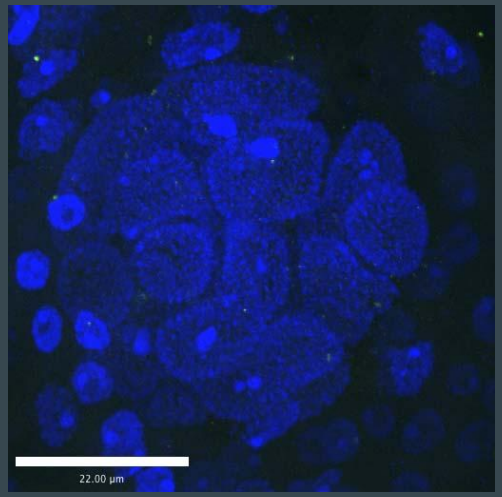
R3-HA

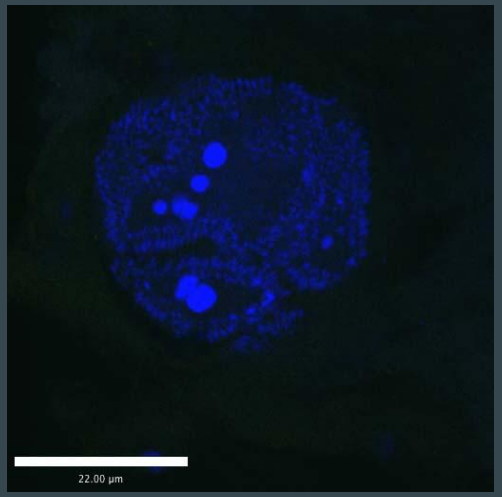
Morphological types of oocysts present.

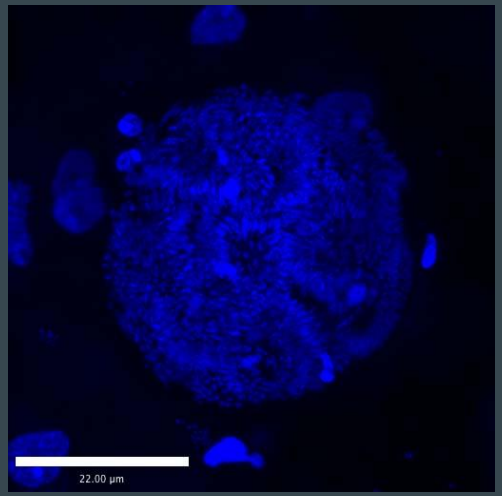
Shown here are many stages of the oocysts development.

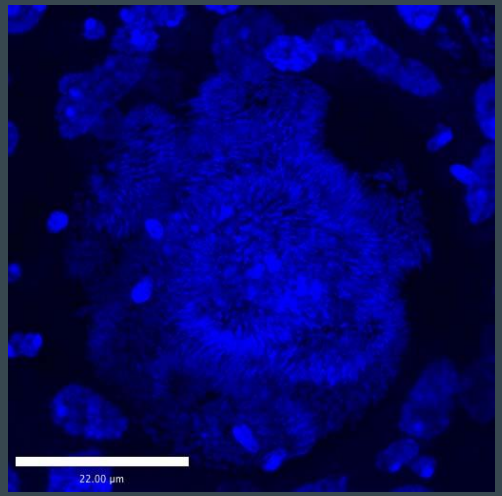
Images projection of z-series. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

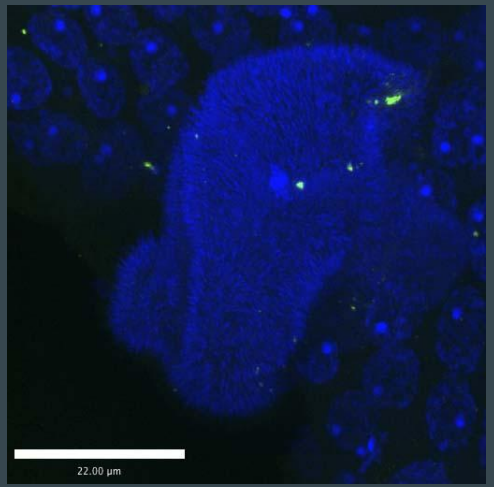




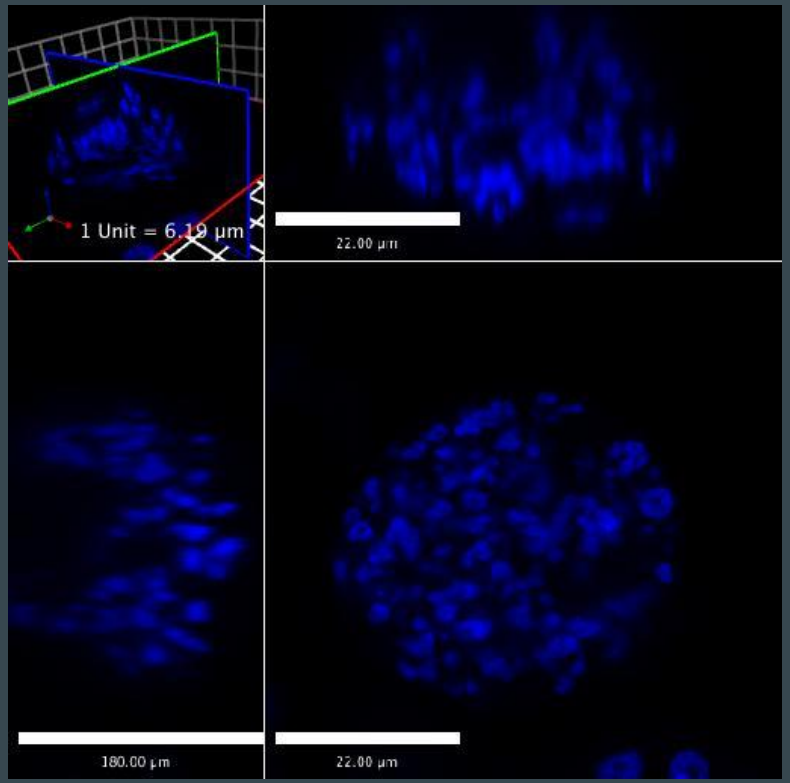




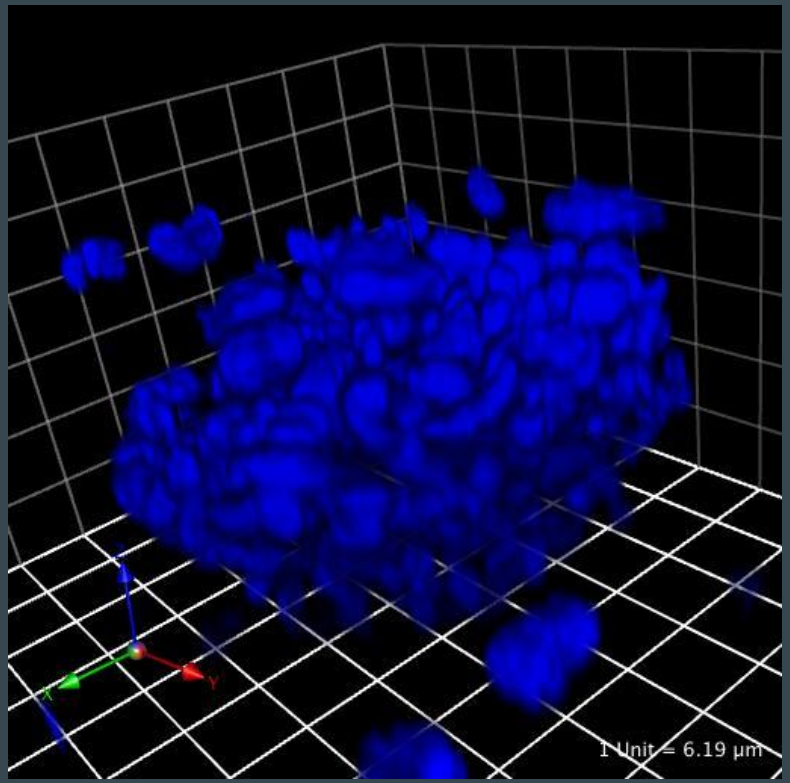




R3-HA 3D reconstructions oocysts

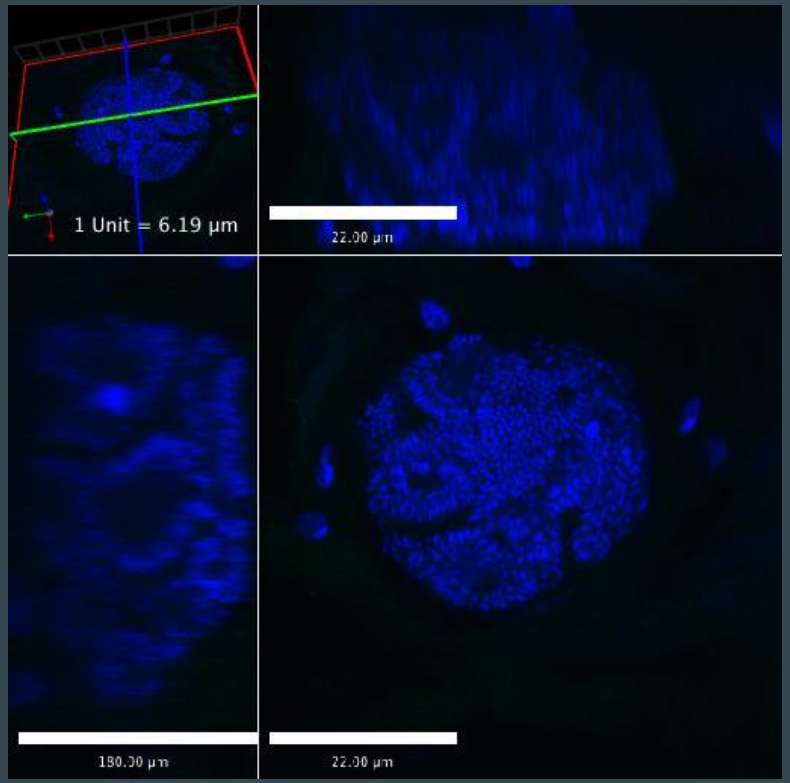


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.



3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

R3-HA 3D reconstructions oocysts



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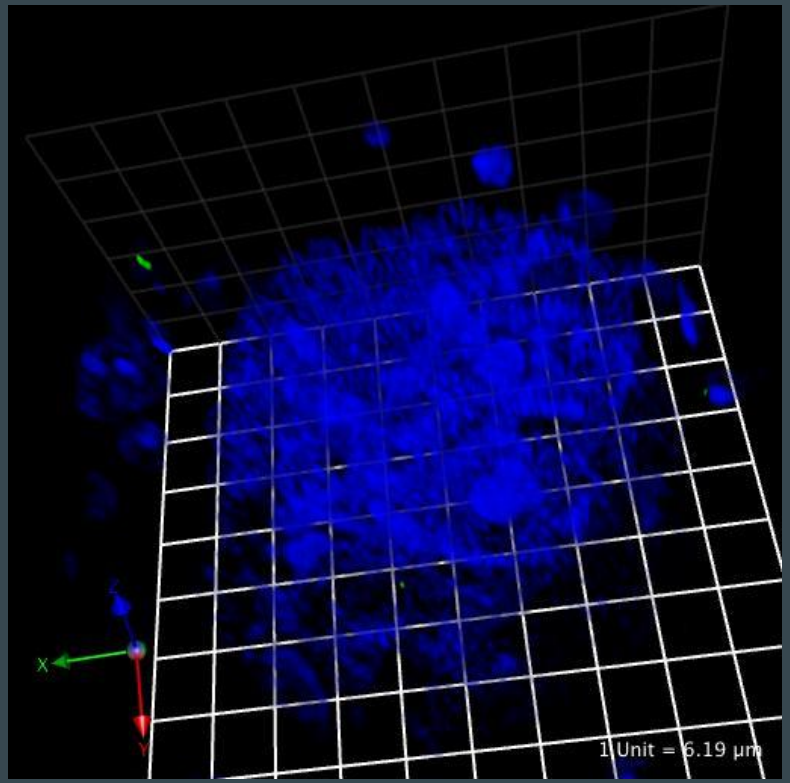
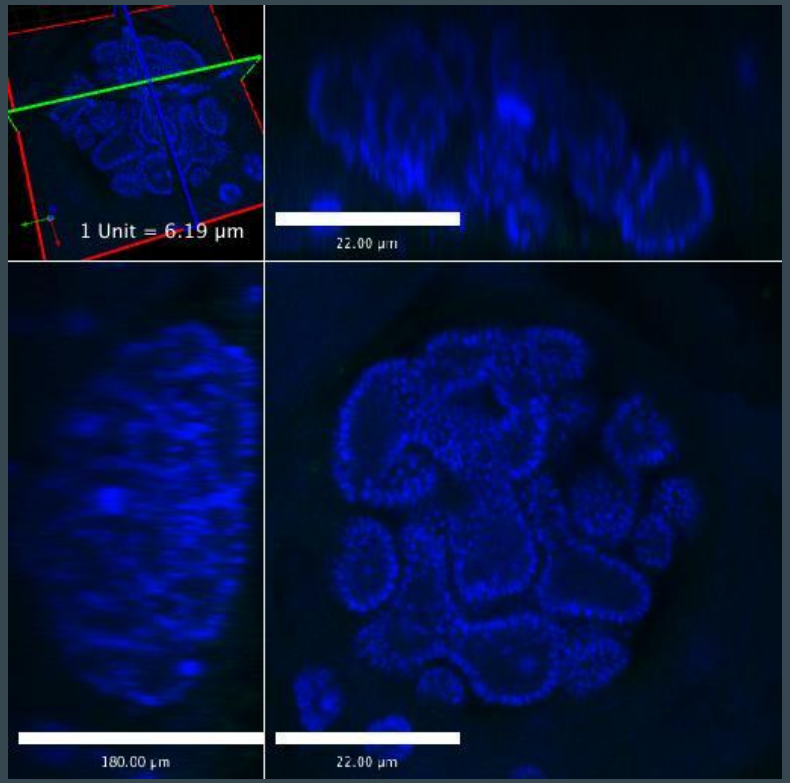


3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

R3-HA 3D reconstructions oocysts



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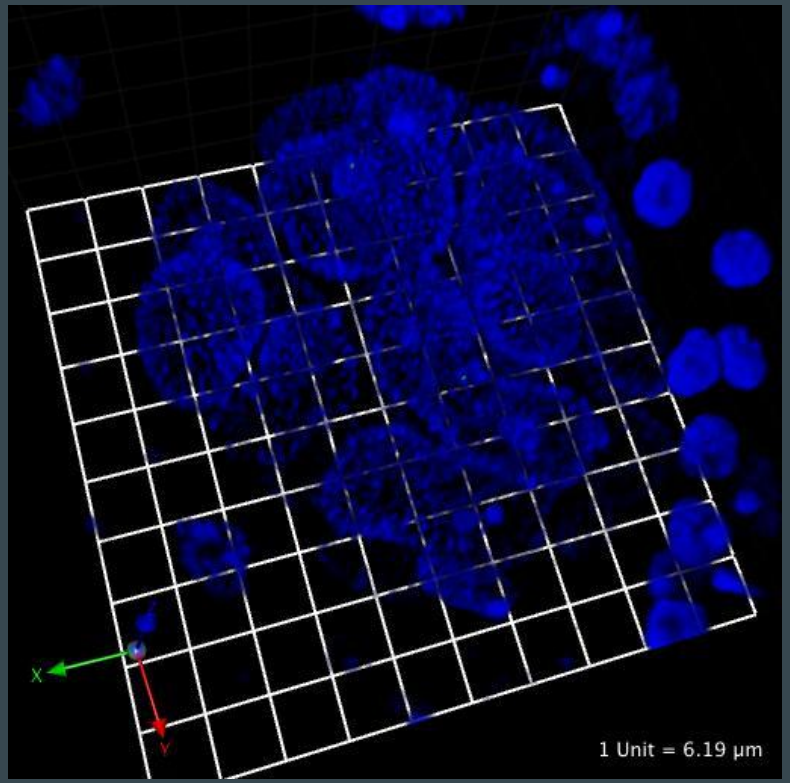
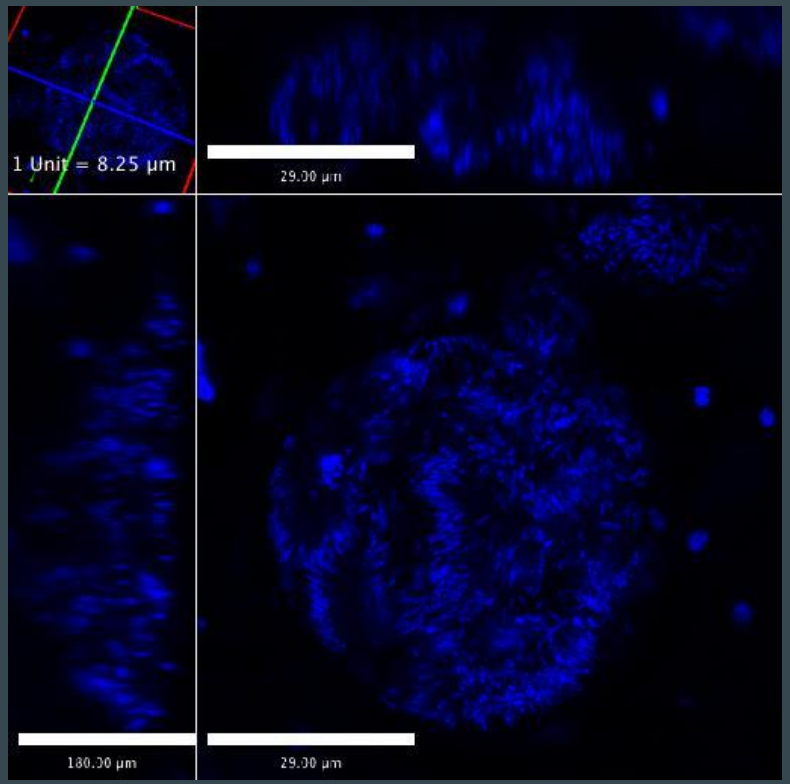
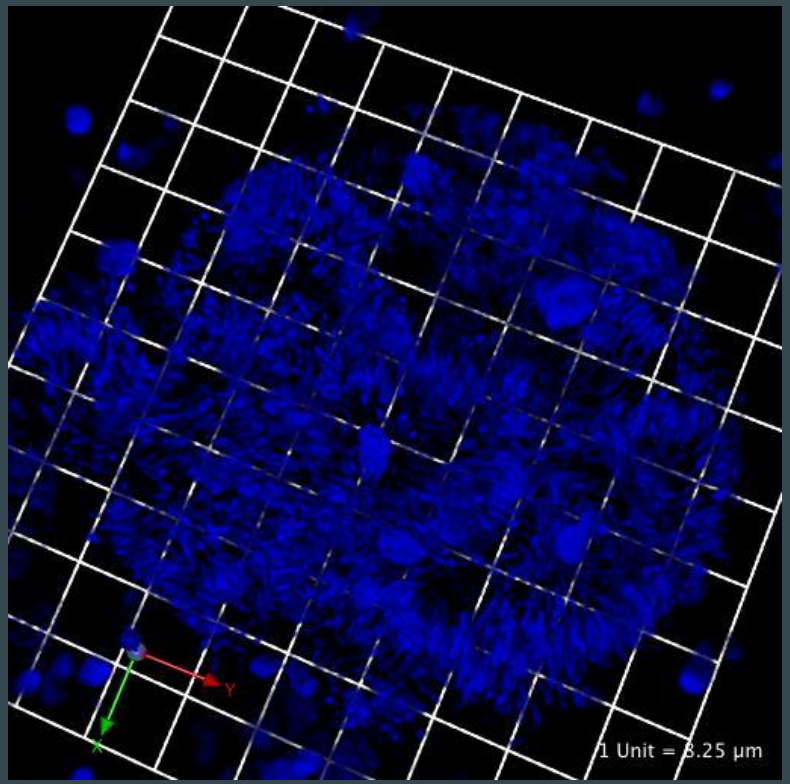


3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

R3-HA 3D reconstructions oocysts

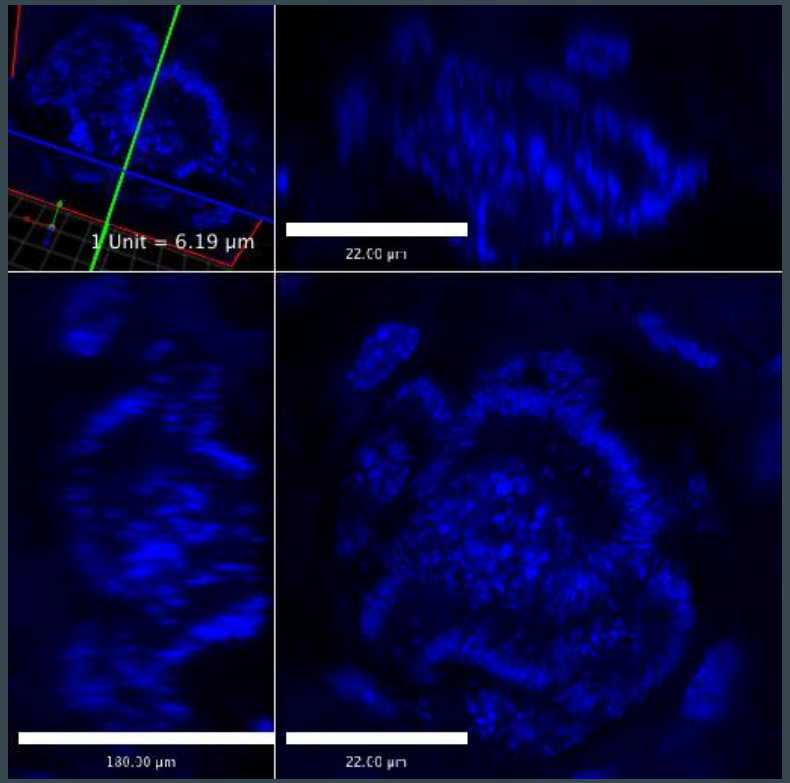


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

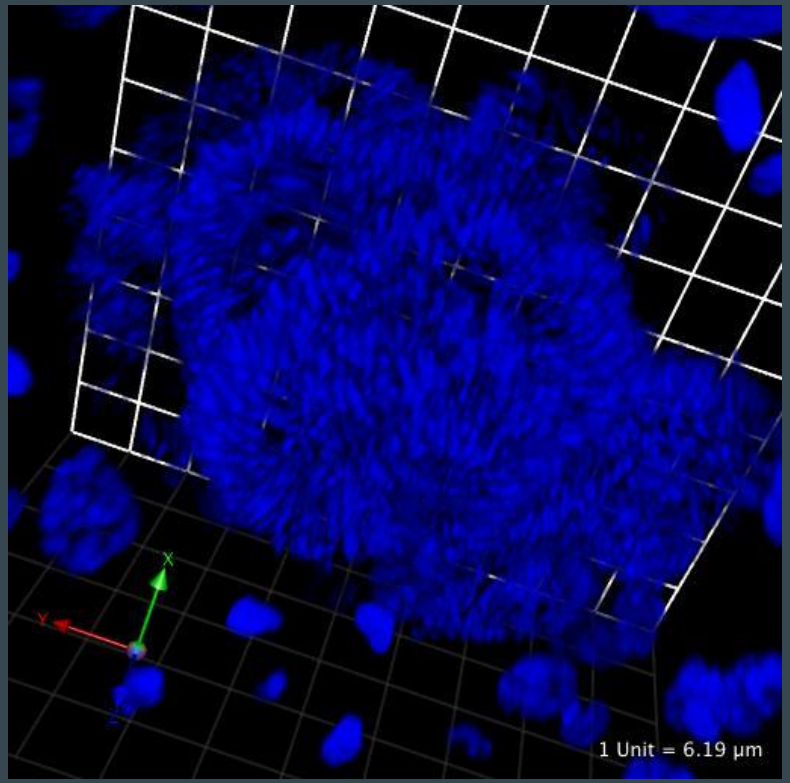


3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

R3-HA 3D reconstructions oocysts



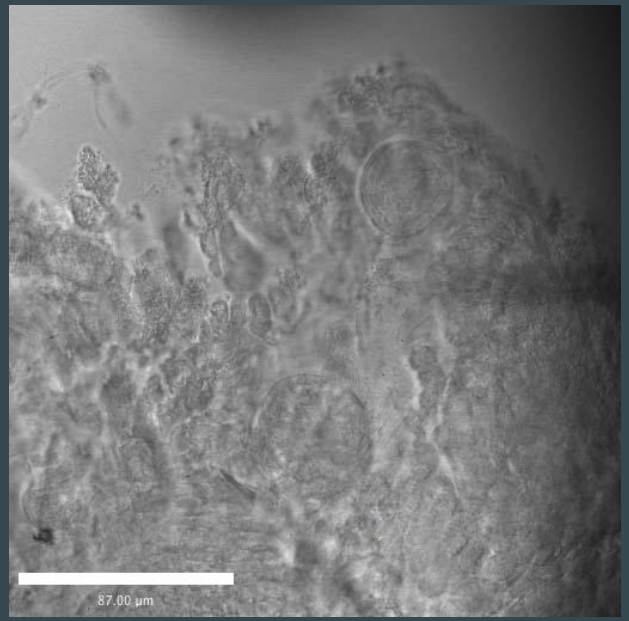
Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

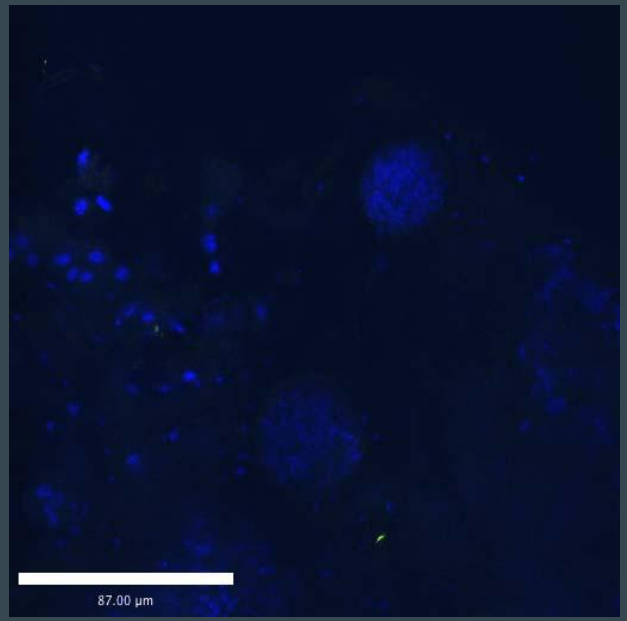


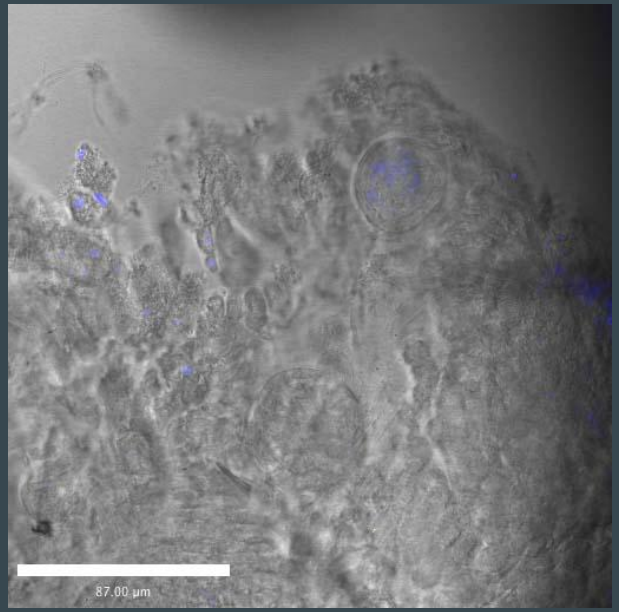
3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

R3-KO A condition of interest

An example of the phase and Hoechst oocysts inside of the midgut







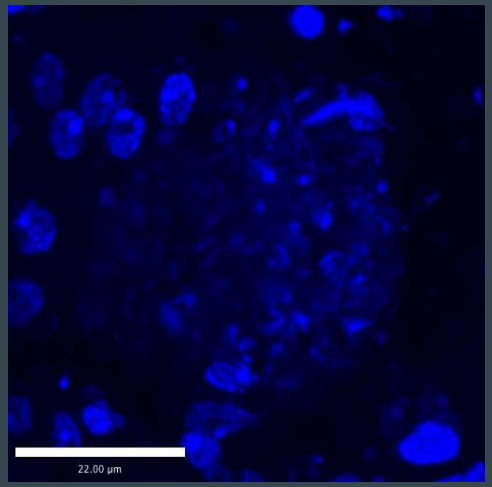
Hoechst stain oocysts (blue) with phase image, SP5, 63x 1x zoom

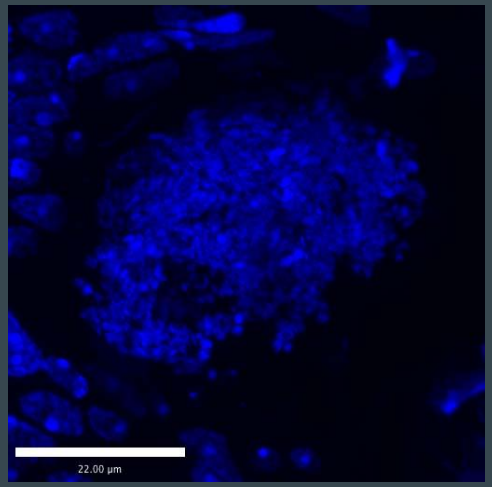
R3-KO

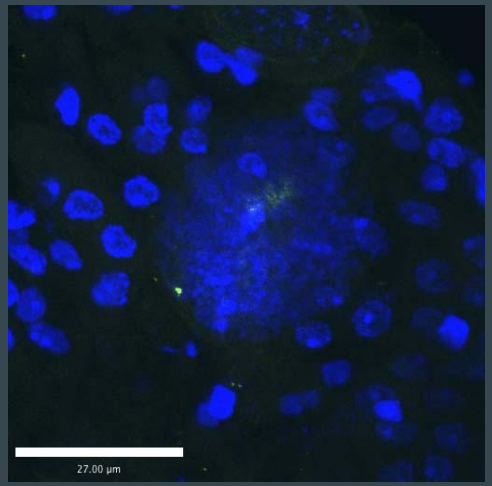
Morphological types of oocysts present.

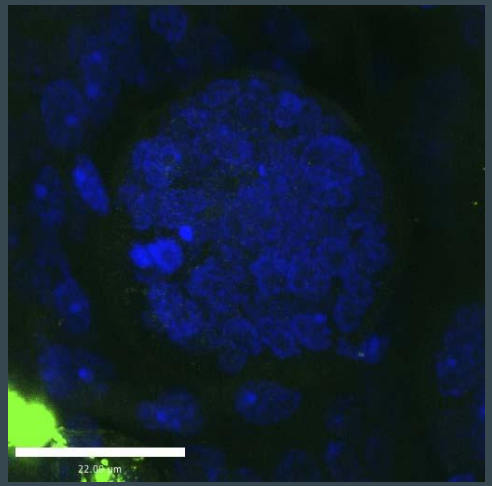
Seen here there is only one stages of the oocysts development.

Images projection of z-series. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

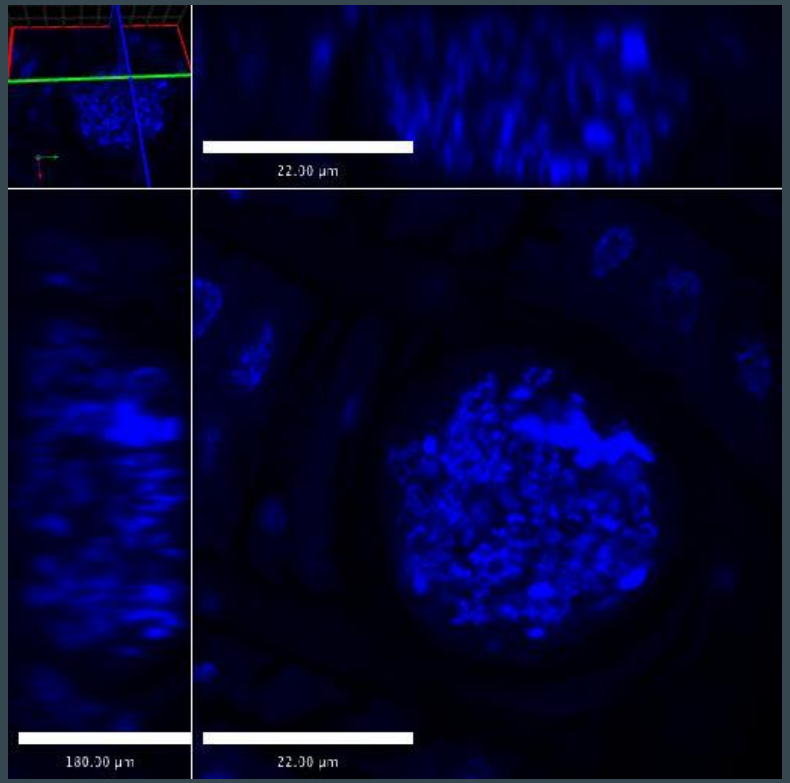




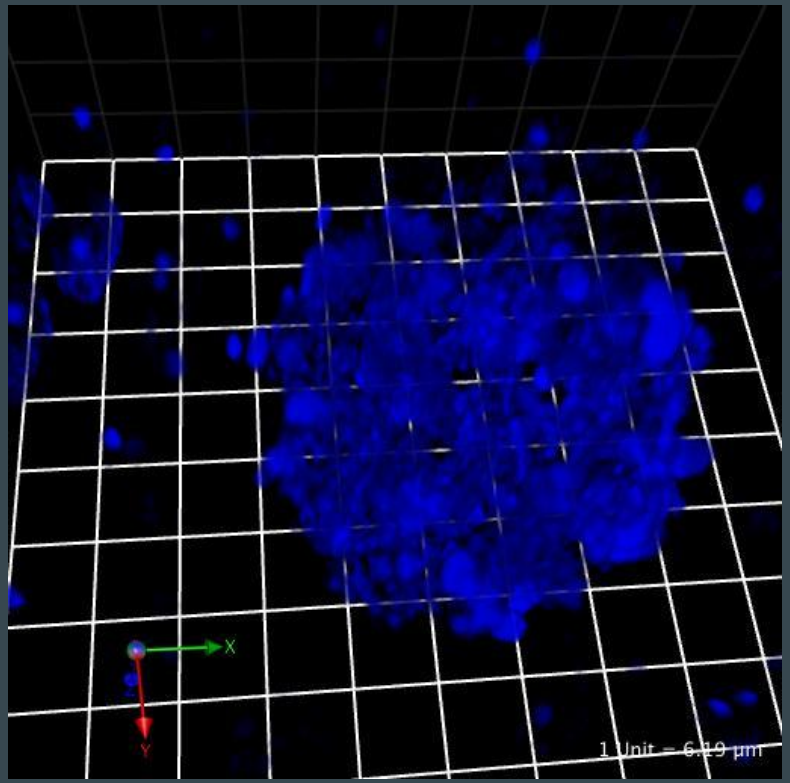




R3-KO 3D reconstructions oocysts

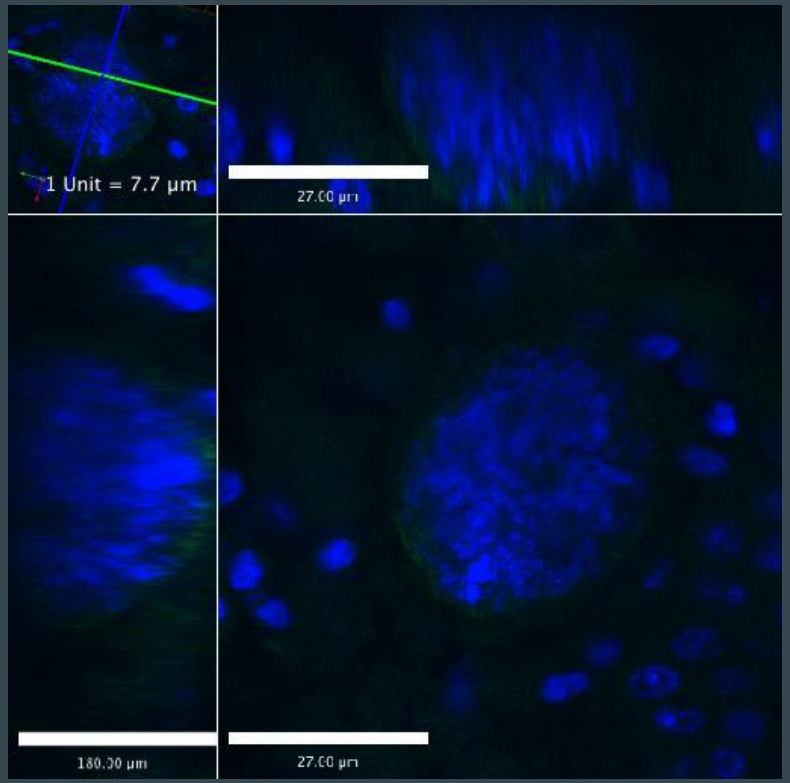


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

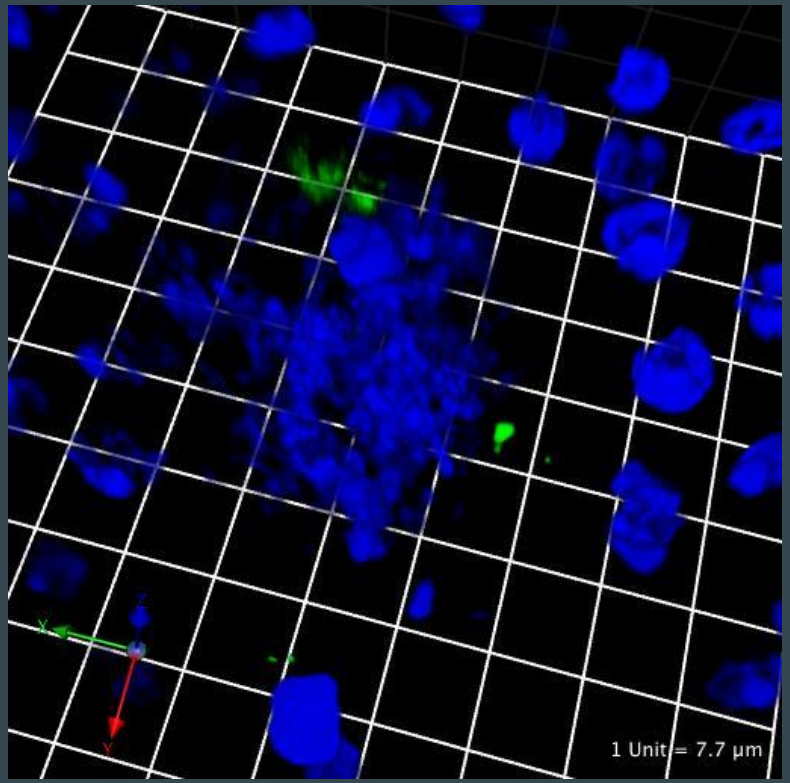


3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

R3-KO 3D reconstructions oocysts

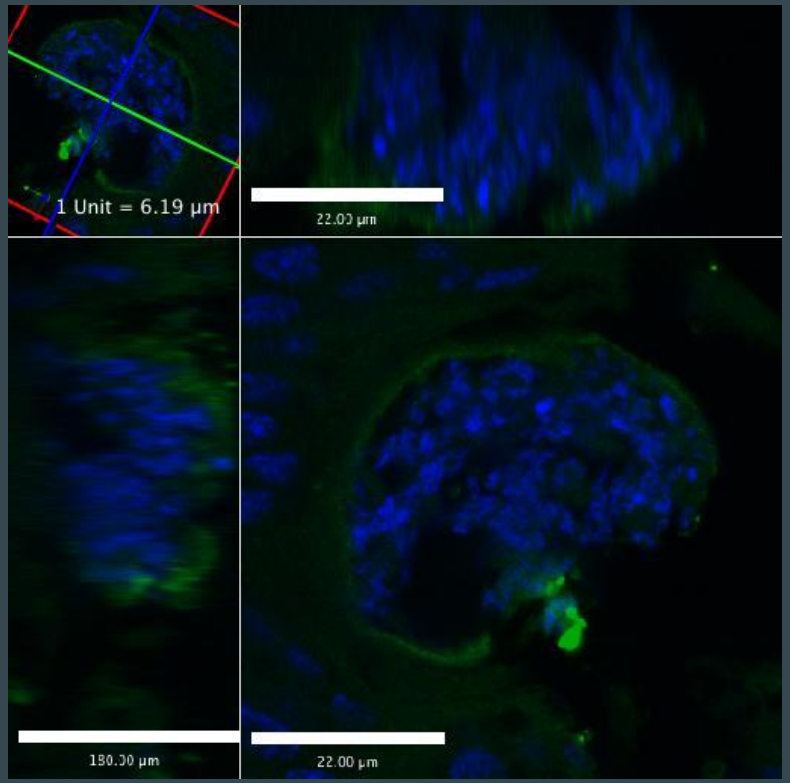


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

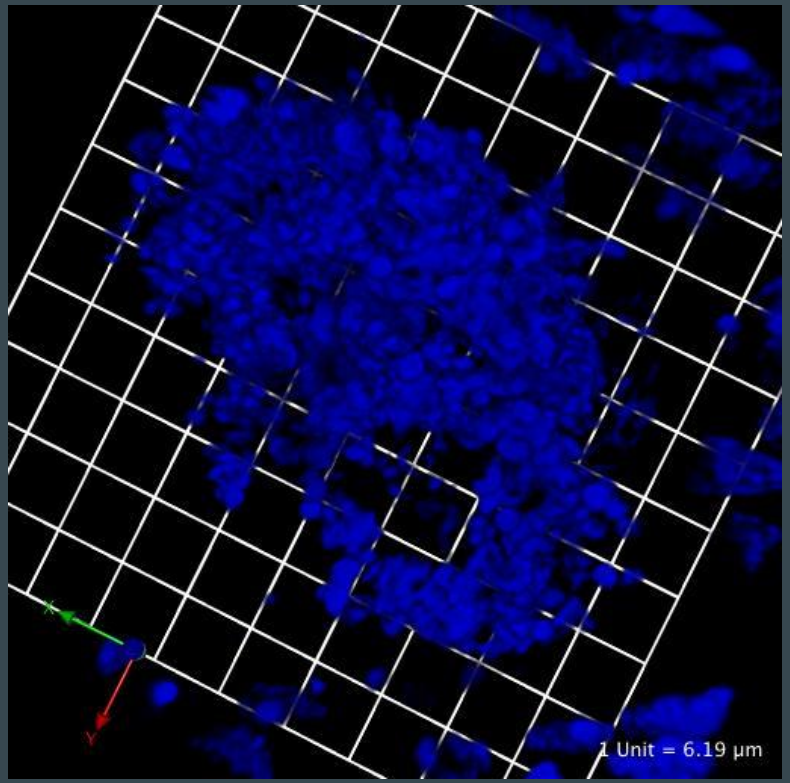


3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

R3-KO 3D reconstructions oocysts

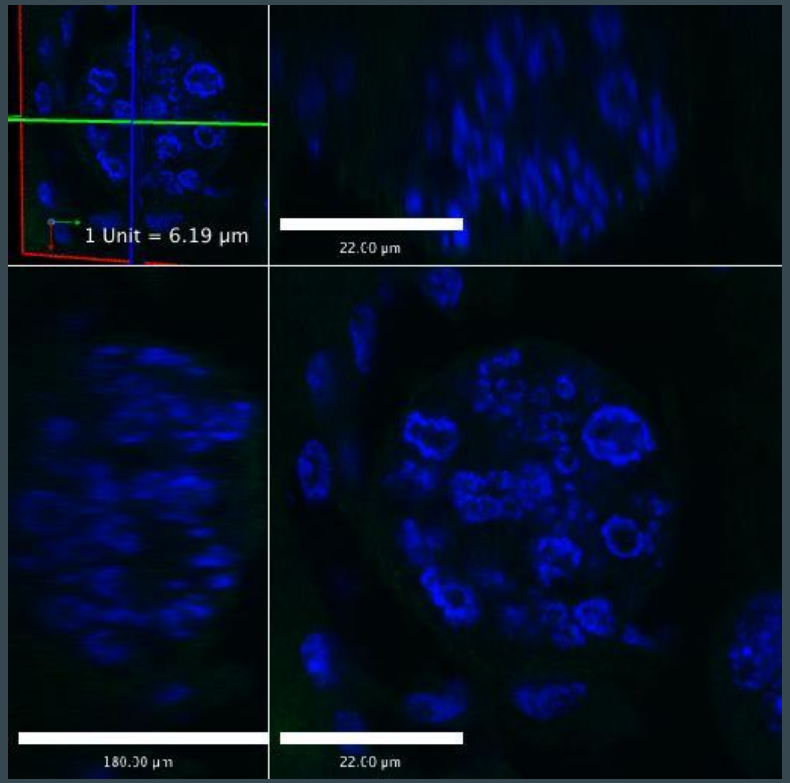


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

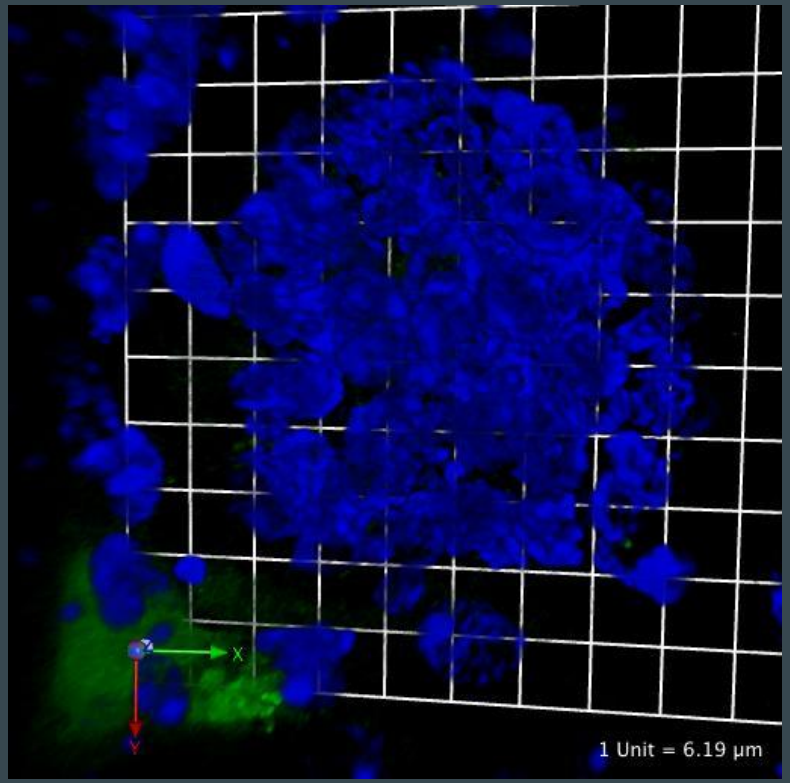


3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

R3-KO 3D reconstructions oocysts



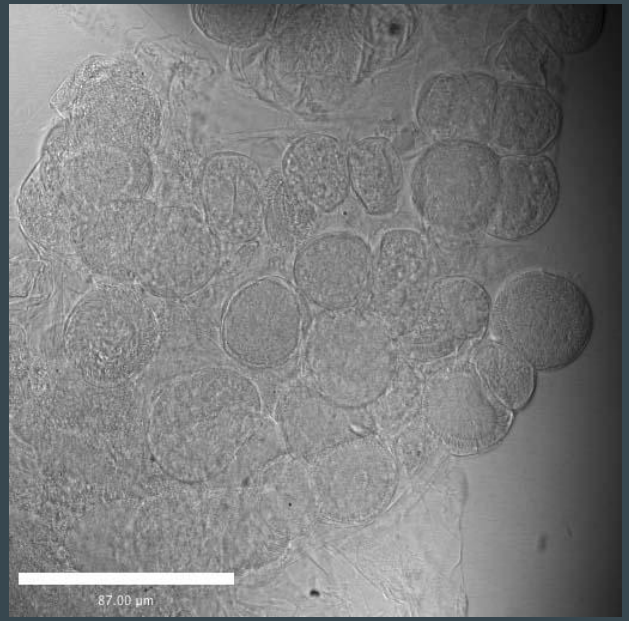
Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

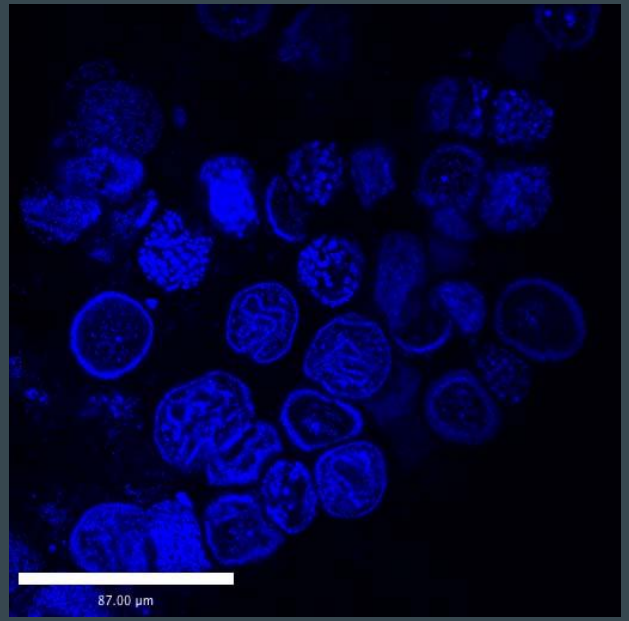


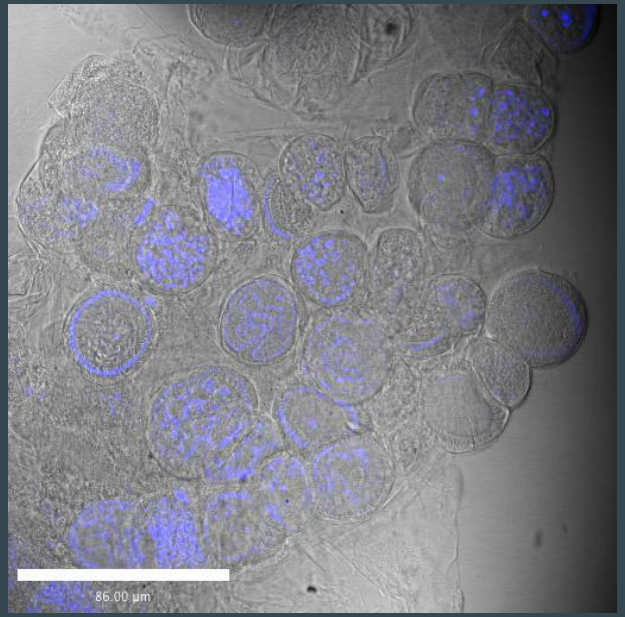
3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

WT A condition of interest

An example of the phase and Hoechst oocysts inside of the midgut







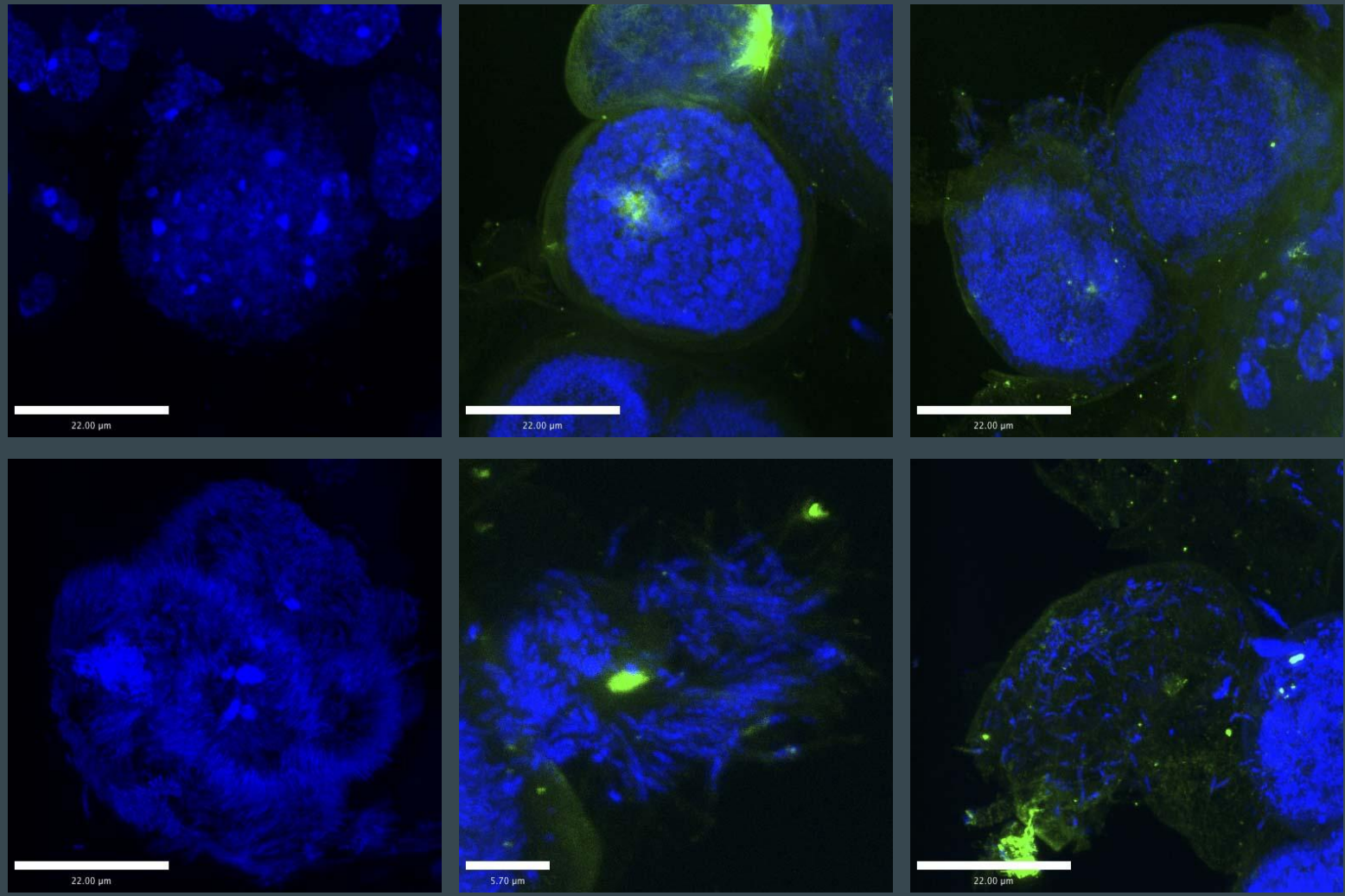
Hoechst stain oocysts (blue) with phase image, SP5, 63x 1x zoom

WT

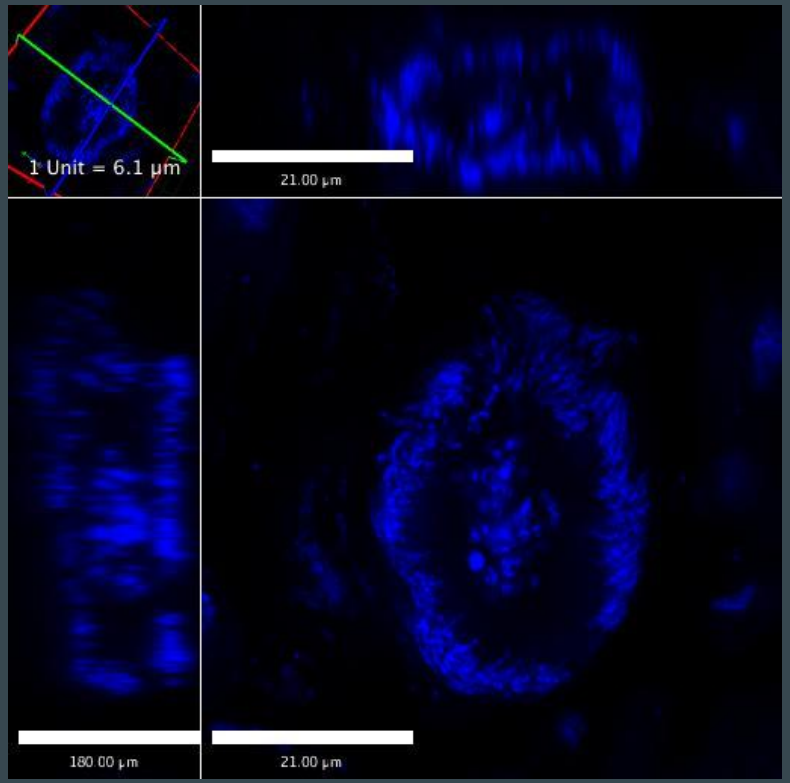
Morphological types of oocysts present.

Seen here, there are many stages of the oocysts development.

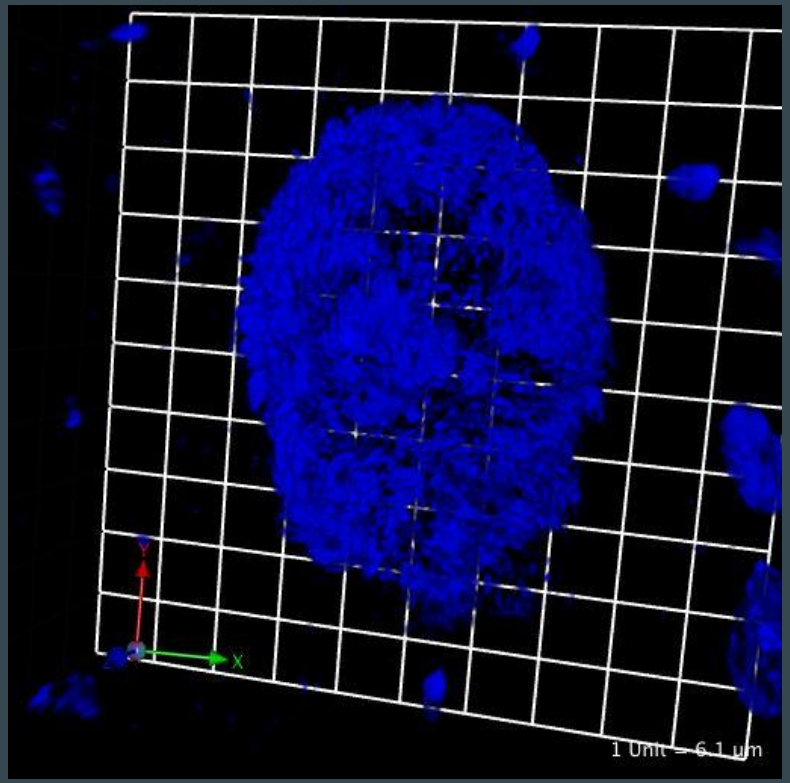
Images projection of z-series. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.



WT 3D reconstructions oocysts

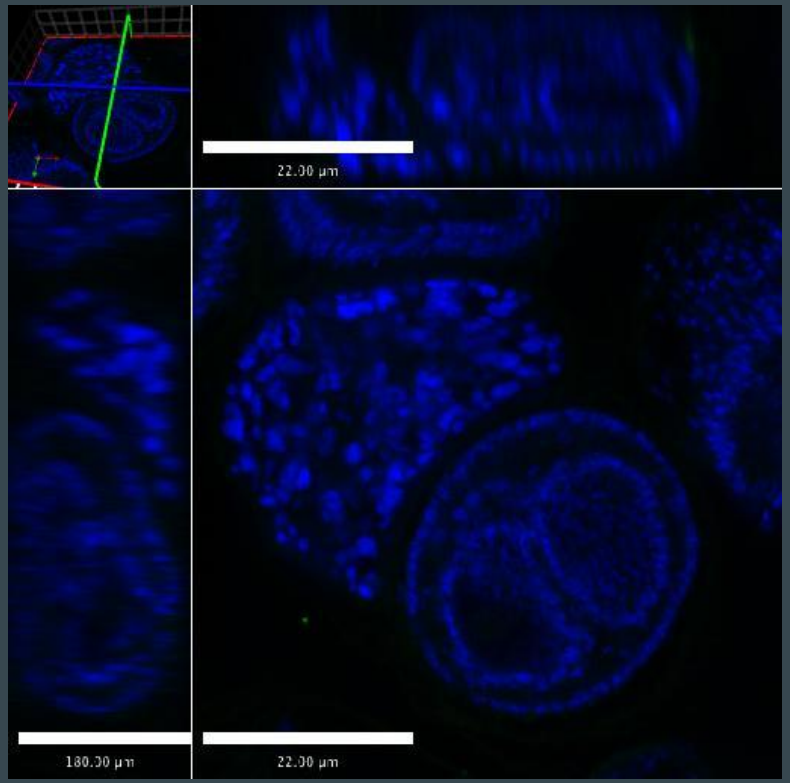


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

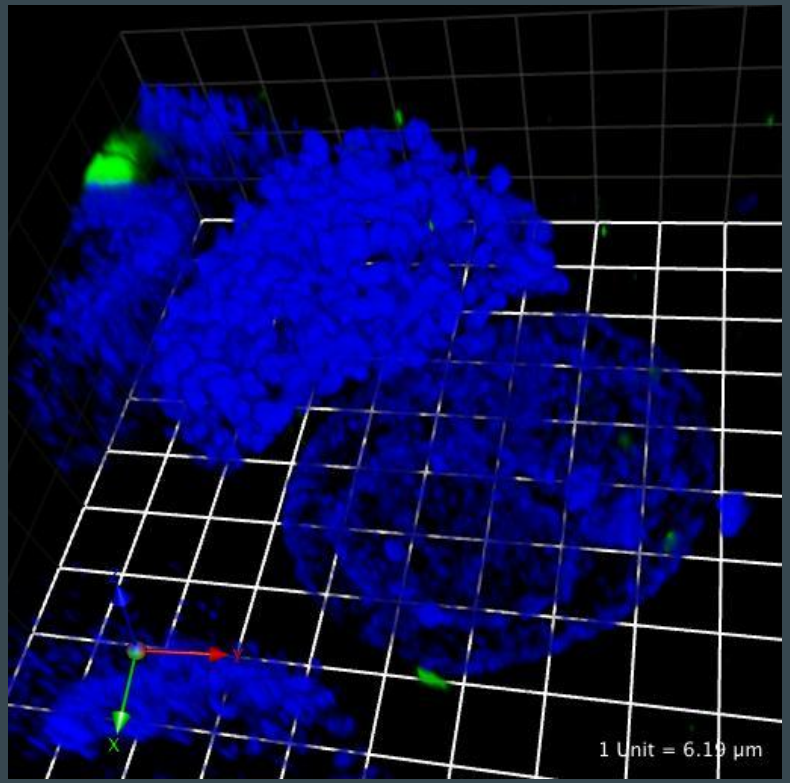


3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

WT 3D reconstructions oocysts

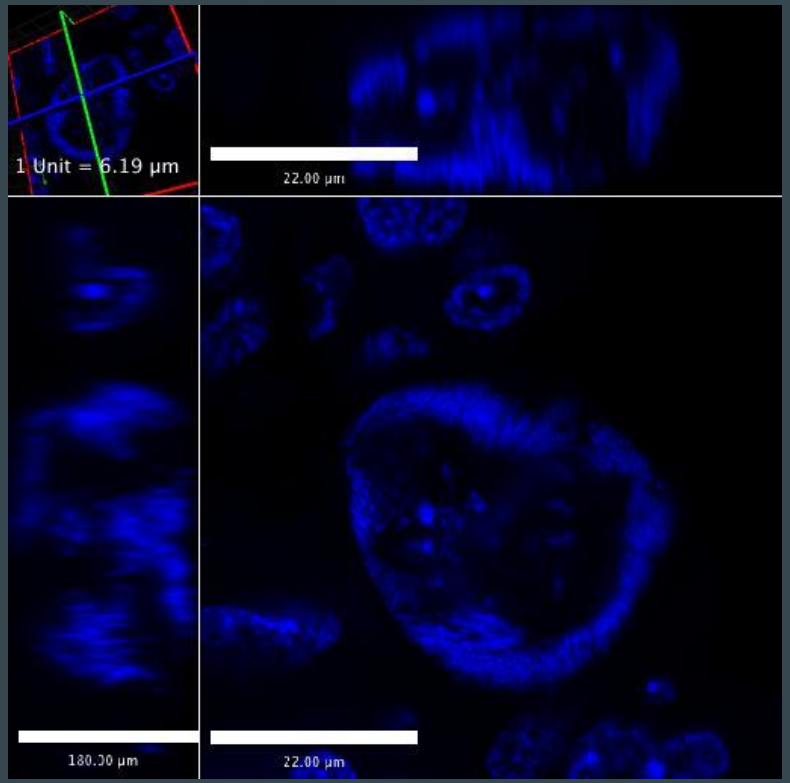


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

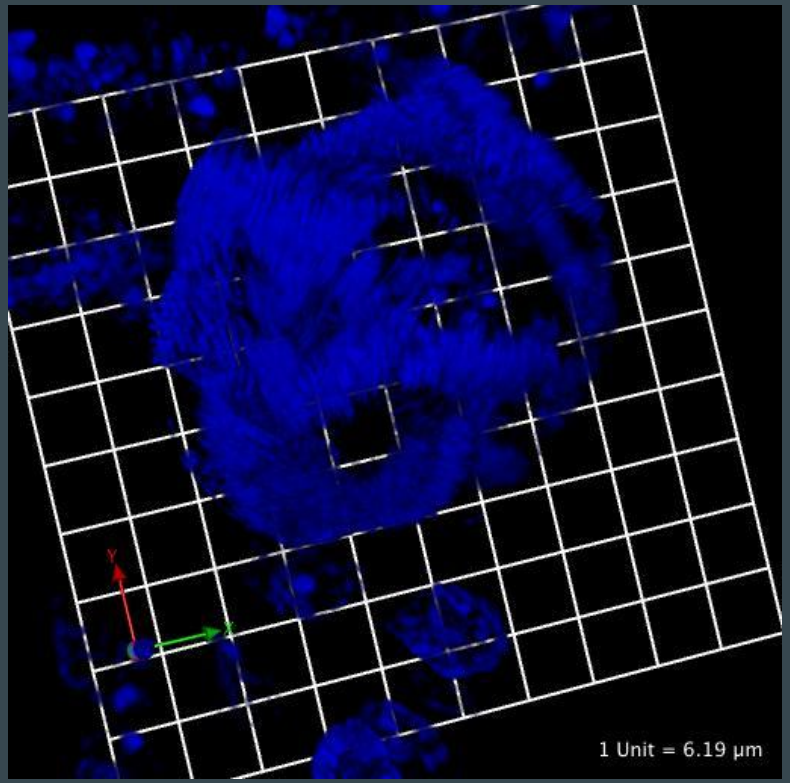


3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

WT 3D reconstructions oocysts

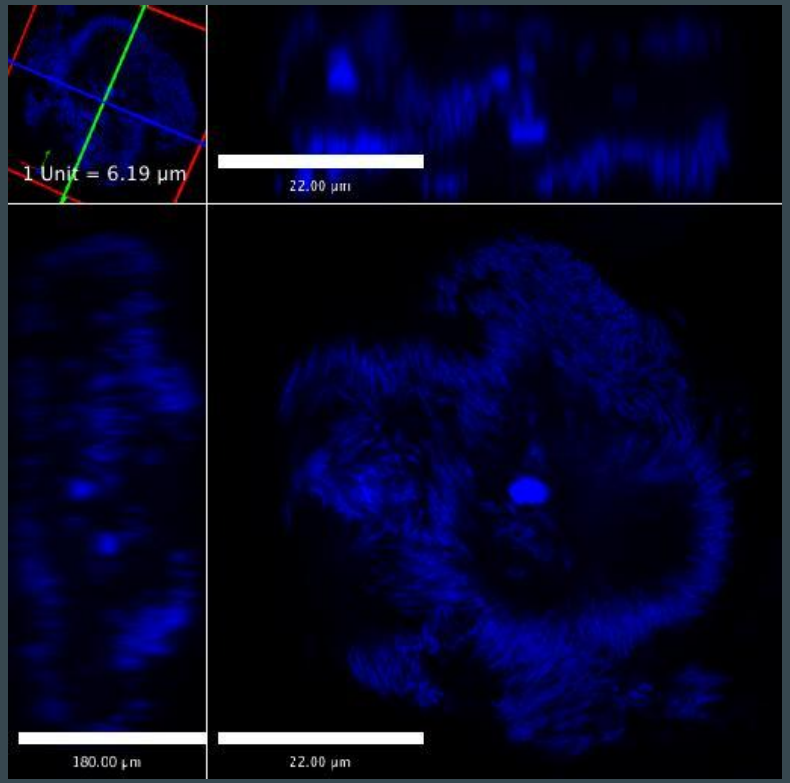


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

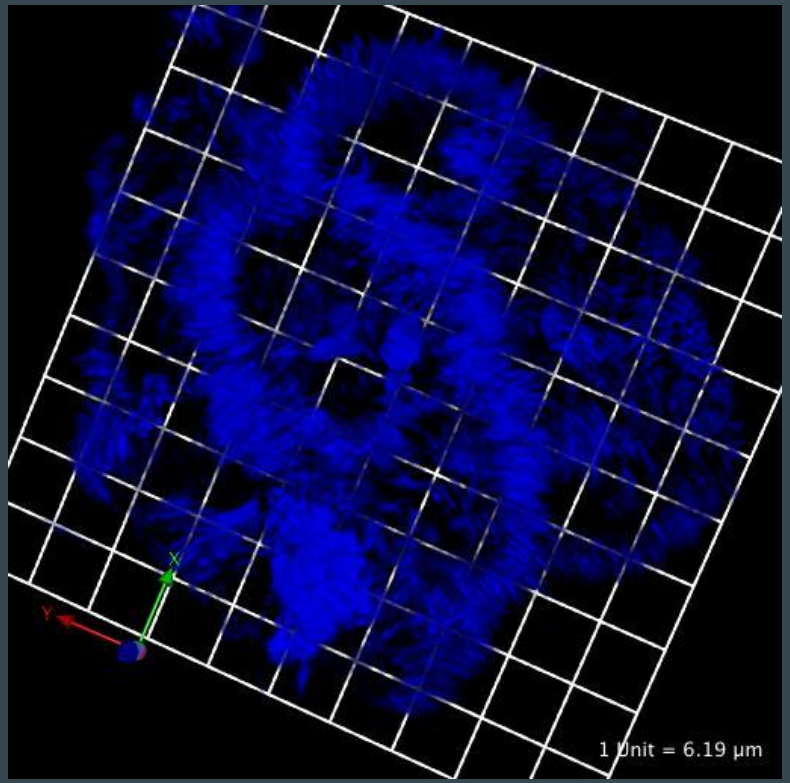


3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

WT 3D reconstructions oocysts

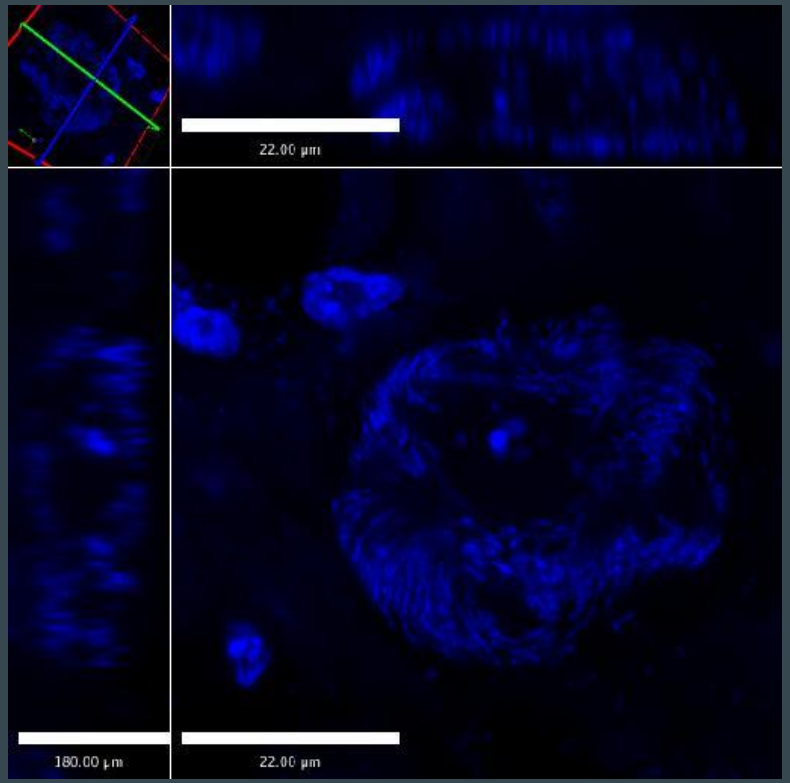


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

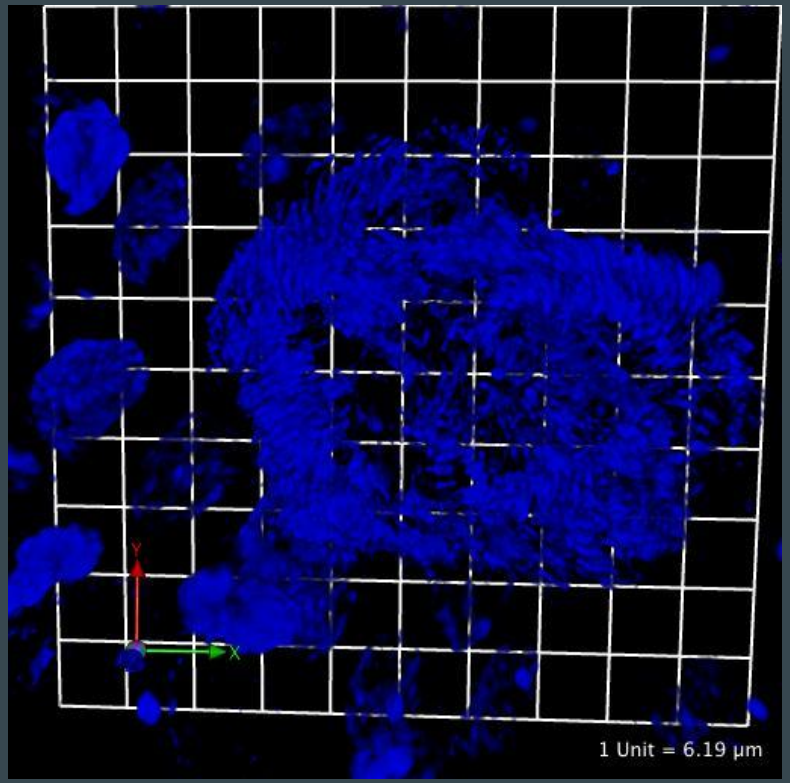


3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

WT 3D reconstructions oocysts



Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) SP5, 63x 4x zoom, Z-series 1 um slice.



3D reconstruction of oocysts. Hoechst stain (blue) SP5, 63x 4x zoom, Z-series 1 um slice.

Image Analysis

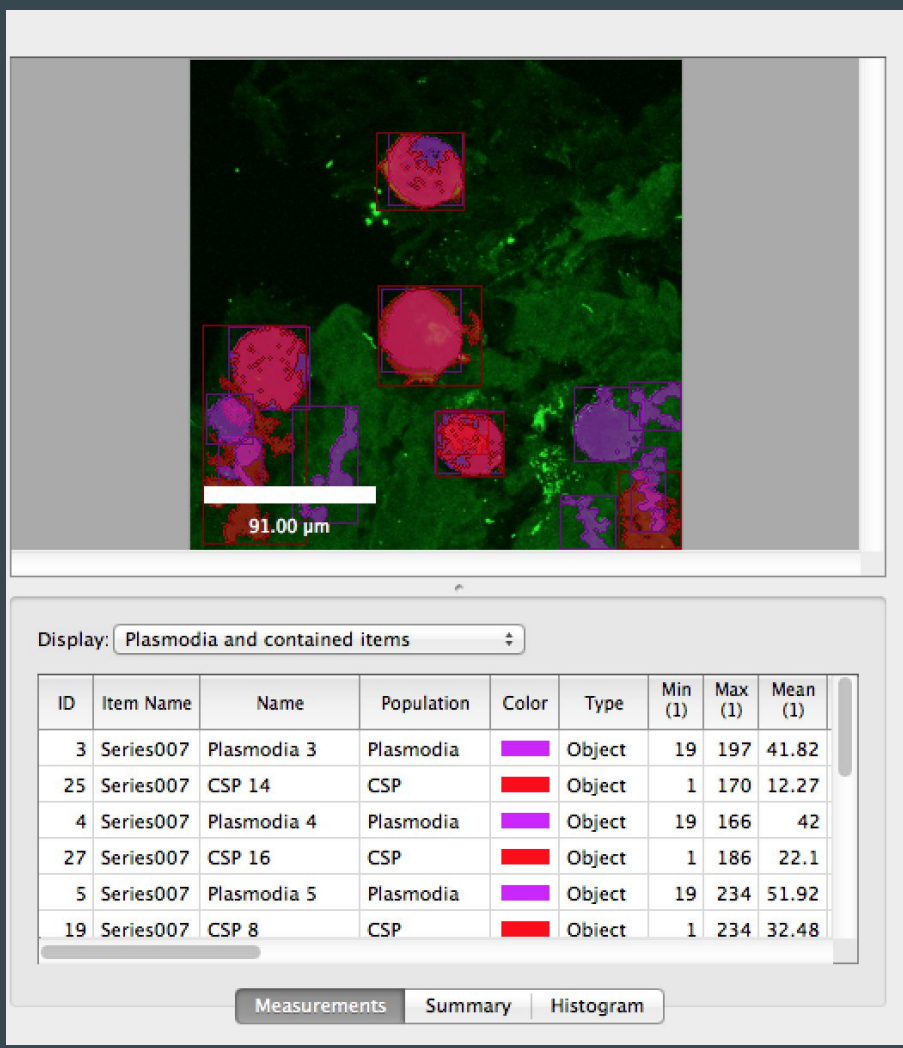
Was conducted using Volocity, a 3D image analysis program

Volocity is a software that specializes in 3D volumetric measurements. This software measured the volume of each oocyst in 3D. The protocol created to measure each oocyst can be reused and exported to instate the exact same parameters on each image.

Volocity CSP and Hoechst Analysis Protocol

1. Find objects Hoechst channel: SD lower 1.5, upper 100, minimum object size 0.2um^3
2. Exclude objects: smaller than 2,000 um^3
3. Find objects: CSP channel: SD lower 1.6, upper 100, minimum object size 10um^3
4. Separate touching objects: 170,000 um^3
5. Fill in holes in objects
6. Exclude objects: smaller than 400 um^3
7. Exclude object: larger than 20,000 um^3
8. Compartmentalize: CSP by Plasmodia

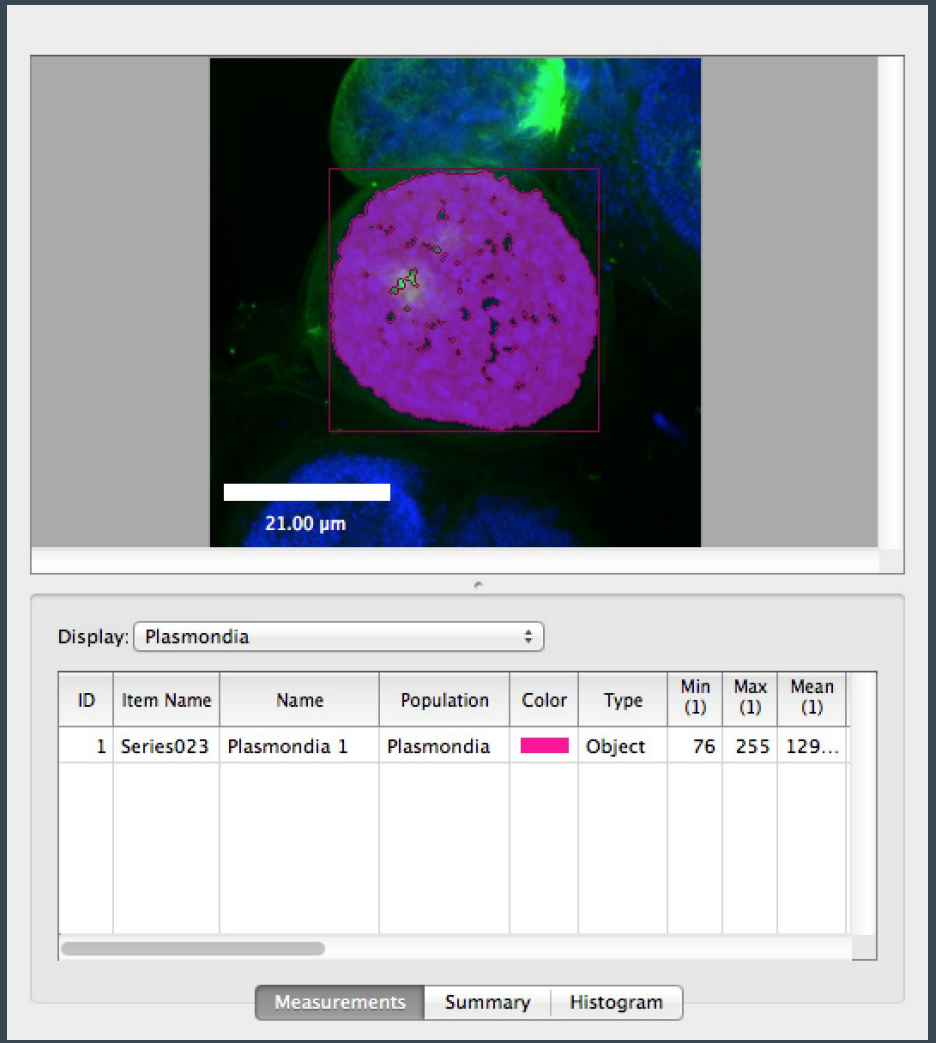
Right: Example image of what objects are being measured in one image. Plasmodia, hoechst stain (purple) CSP green stain (pink).



ID	Item Name	Name	Population	Color	Type	Min (1)	Max (1)	Mean (1)
3	Series007	Plasmodia 3	Plasmodia		Object	19	197	41.82
25	Series007	CSP 14	CSP		Object	1	170	12.27
4	Series007	Plasmodia 4	Plasmodia		Object	19	166	42
27	Series007	CSP 16	CSP		Object	1	186	22.1
5	Series007	Plasmodia 5	Plasmodia		Object	19	234	51.92
19	Series007	CSP 8	CSP		Object	1	234	32.48

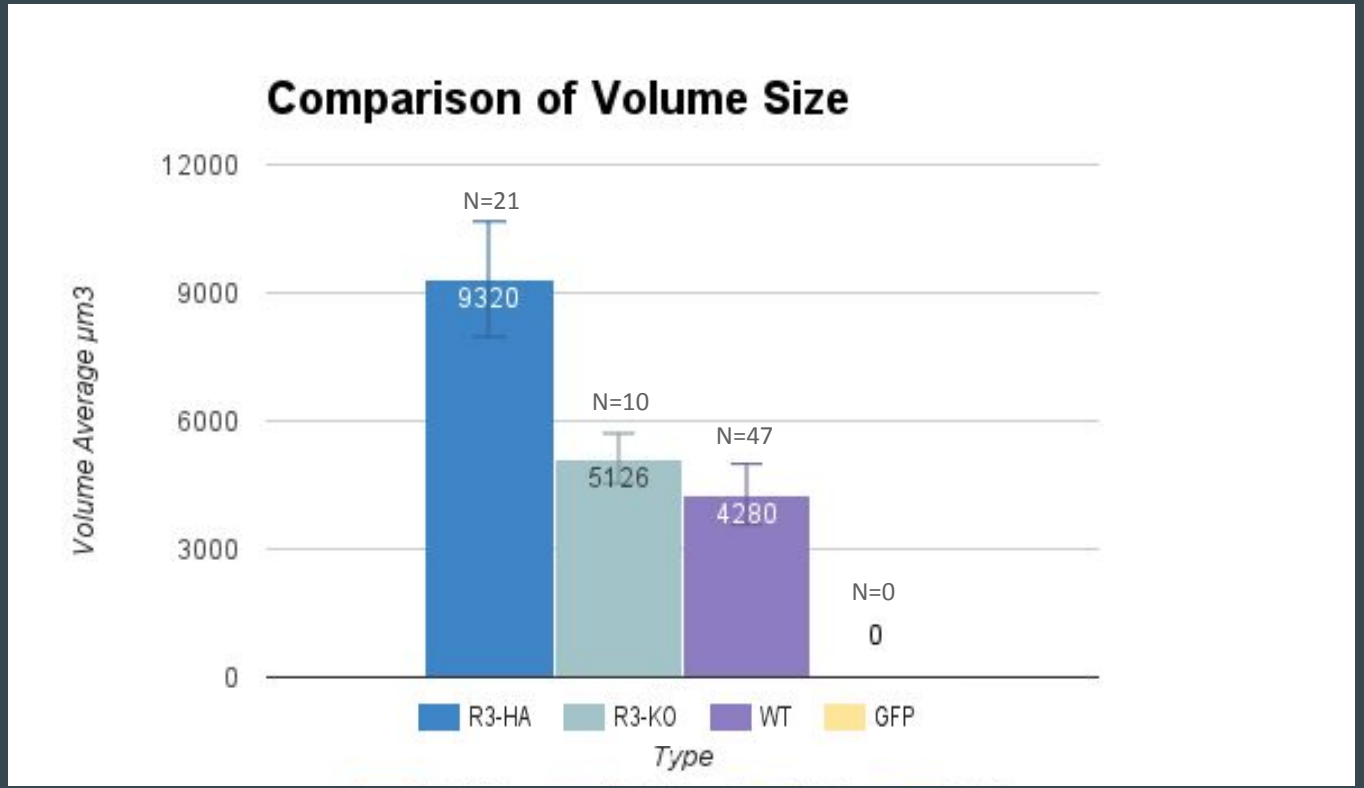
Volocity Hoechst Only Analysis Protocol

1. Find objects Hoechst channel: SD lower 1.4, upper 100, minimum object size 0.8um^3
2. Exclude objects: smaller than 2,000 um^3



Right: Example image of what objects are being measured in one image. Plasmodia, hoechst stain (purple)

Result of Compared Conditions Volume of Oocysts Size



Additional Images

Using the Leica SP5 to visualize individual parasite

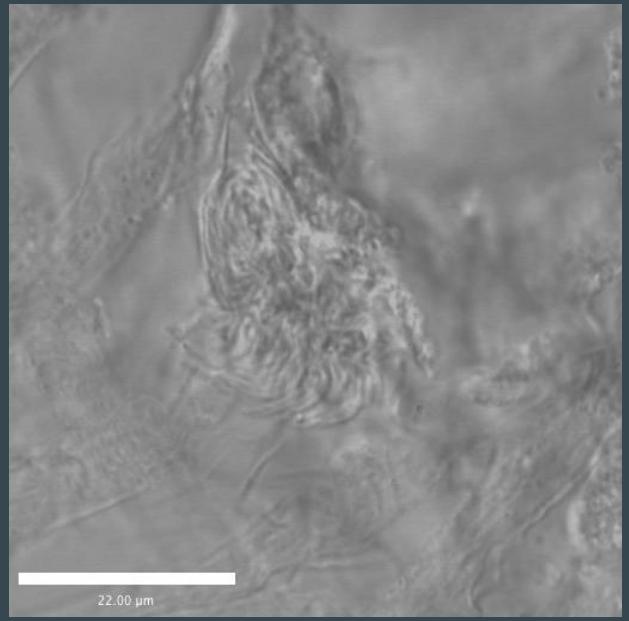
Although imaging the parasite was not a goal of the project, the individual parasites were quite interesting. It proved that the CSP staining worked, because CSP is located on the outside of the parasite.

All images were taken at 63x, with z steps of 0.6 um.

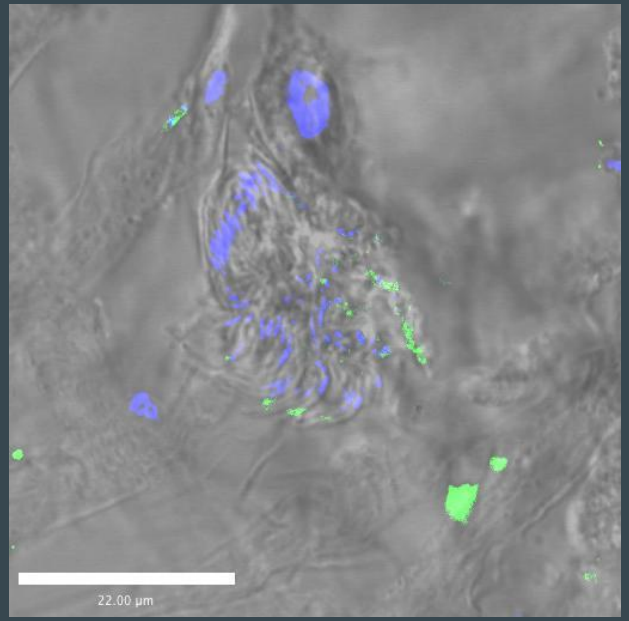
Images are shown as 3D max-projections and in 3D reconstructions in volocity.

Circumsporozoite Protein (CSP)

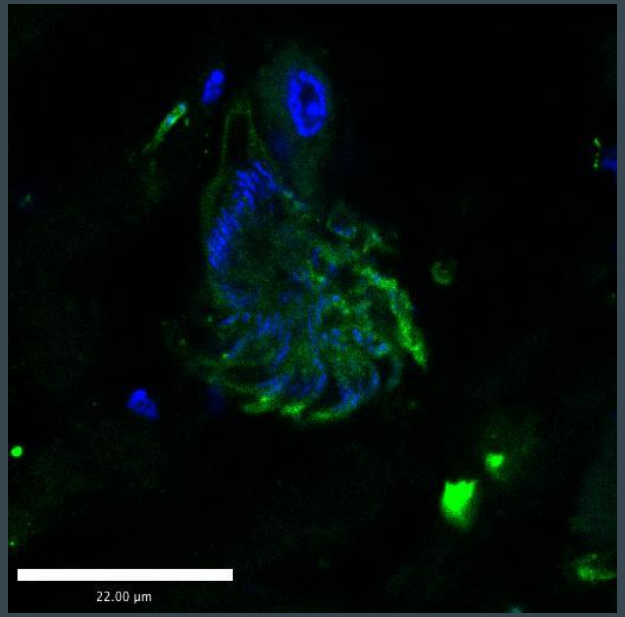
Examples of parasites inside the midgut



Phase of parasites bursting out of oocyst , SP5, 63x, 4x zoom.

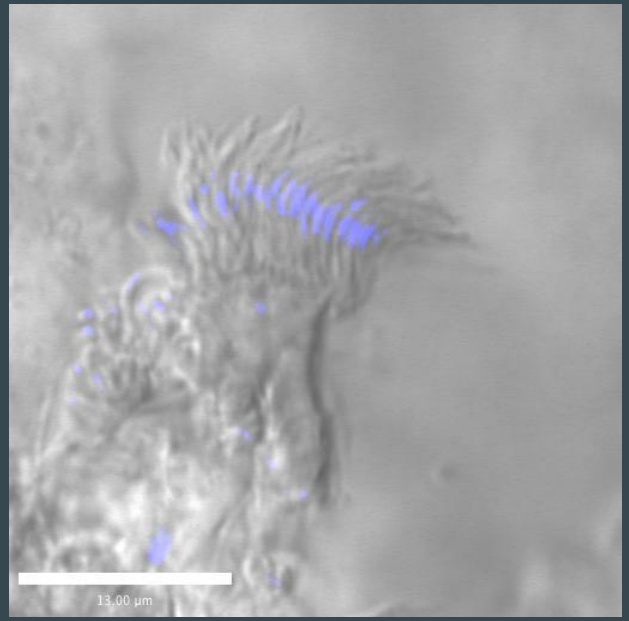


Hoechst stain oocysts (blue) CSP (Green) and phase , SP5, 63x, 4x zoom.

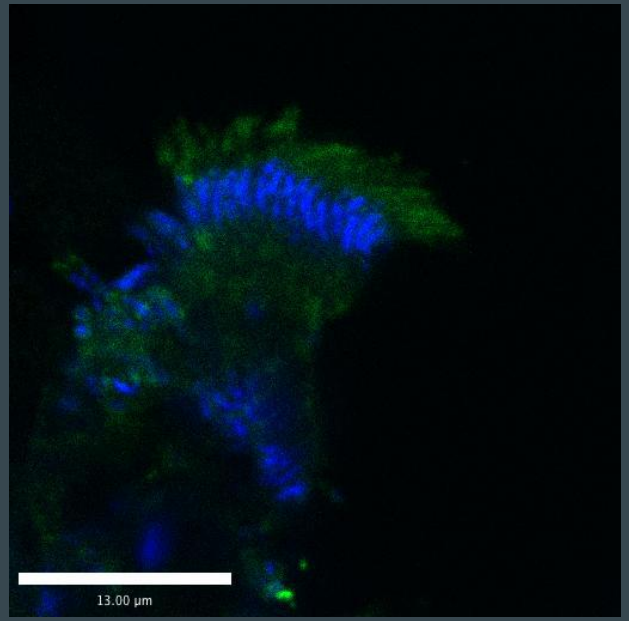


Hoechst stain oocysts (blue) CSP (Green) SP5, 63x, 4x zoom.

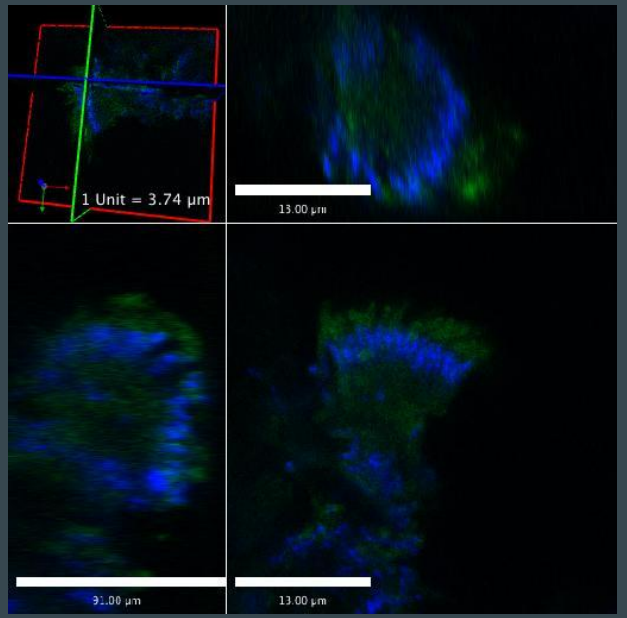
CSP Examples of parasites inside the midgut



Hoechst stain oocysts (blue) CSP (Green) and phase , SP5, 63x.



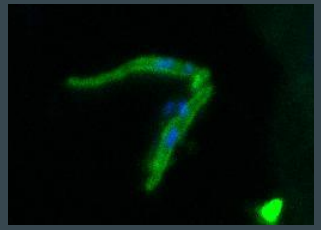
Hoechst stain oocysts (blue) CSP (Green) SP5, 63x.

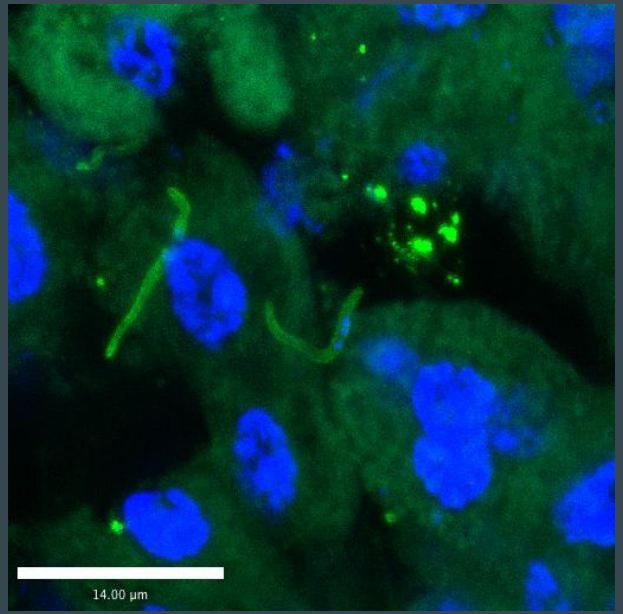


Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) CSP (Green), SP5, 63x, Z-series 1 um slice.

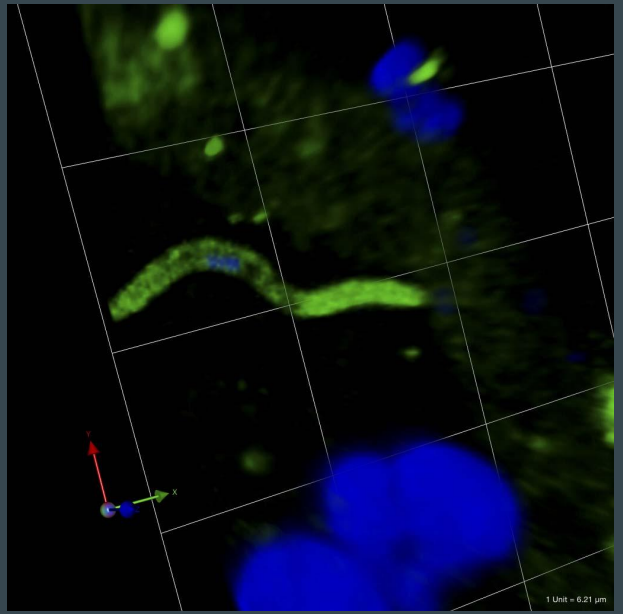
CSP

Four different examples of individual parasites inside the midgut

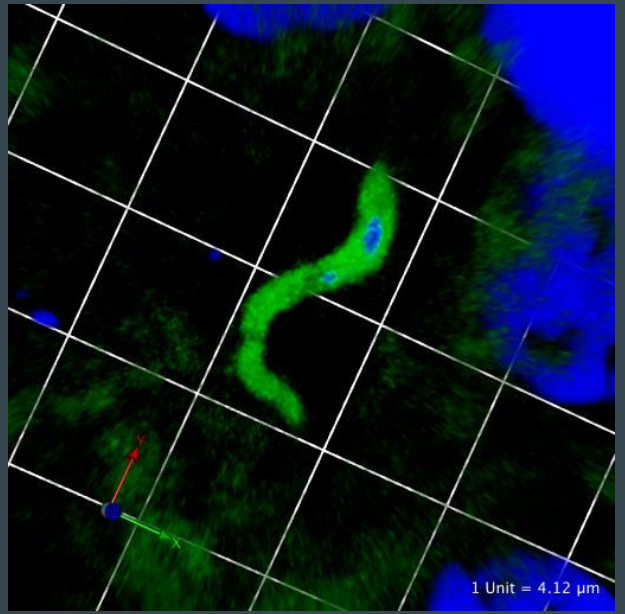




Images projection of z-series. Hoechst stained nuclei (blue) CSP (Green), SP5, 63x, Z-series 0.6 um slice.



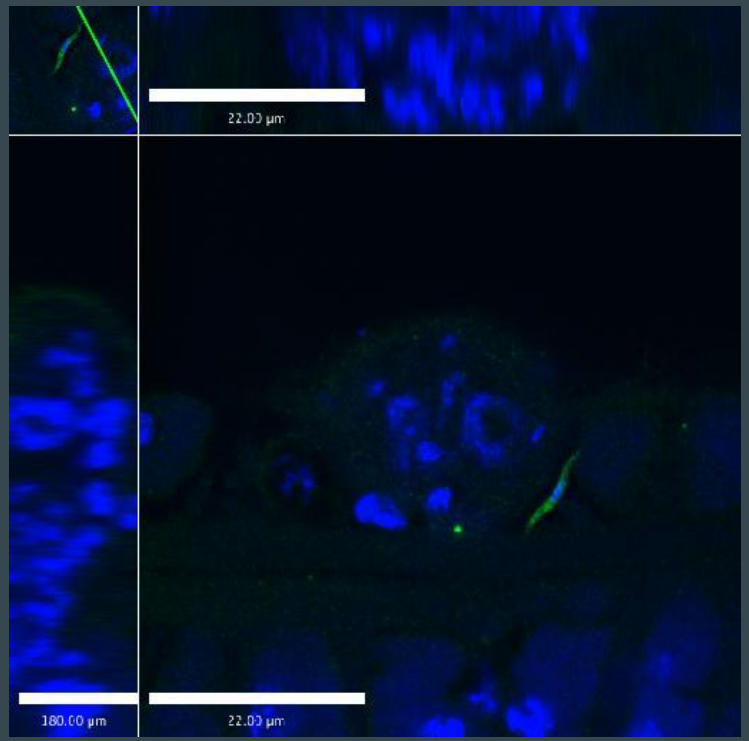
3D reconstruction of parasite. Hoechst stained nuclei (blue) and CSP (green) SP5, 63x, Z-series 0.6 um slice.



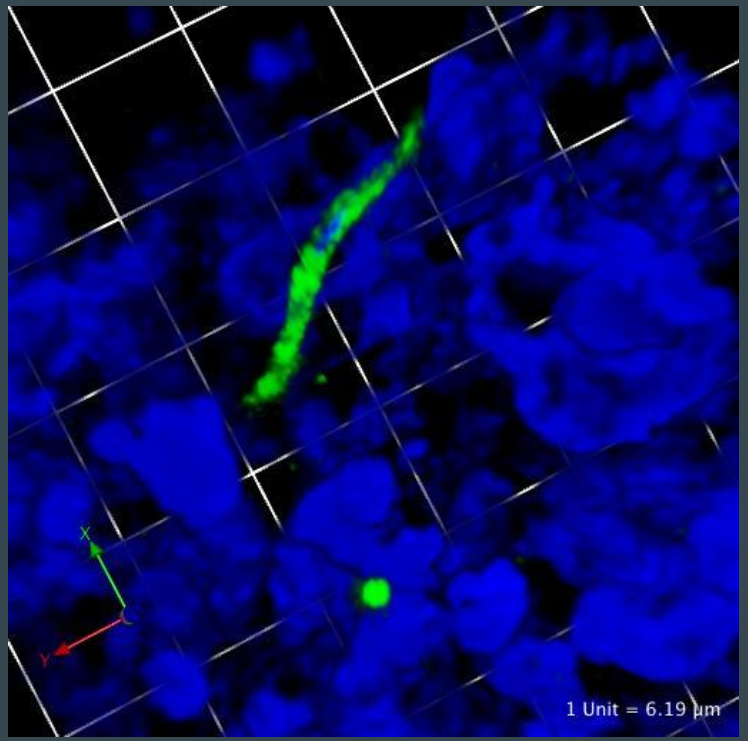
3D reconstruction of parasite. Hoechst stained nuclei (blue) and CSP (green) SP5, 63x, Z-series 0.6 um slice.

CSP

Examples of an individual parasite inside the midgut



Images projection of z-series, x and y axes. Hoechst stain oocysts (blue) CSP (Green), SP5, 63x, Z-series 1 um slice.



3D reconstruction of parasite. Hoechst stain (blue) and CSP (green) SP5, 63x, Z-series 1 um slice.

CSP

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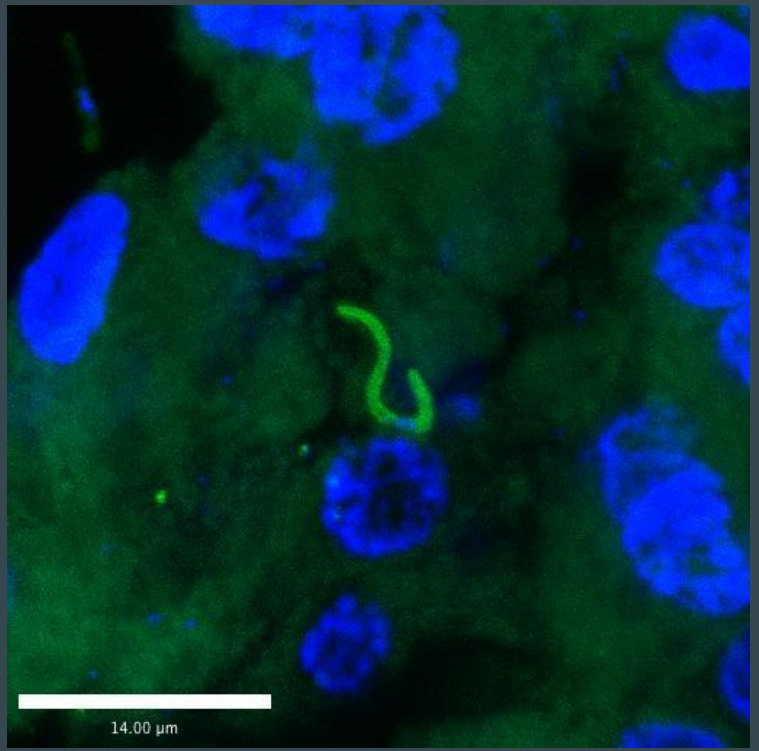
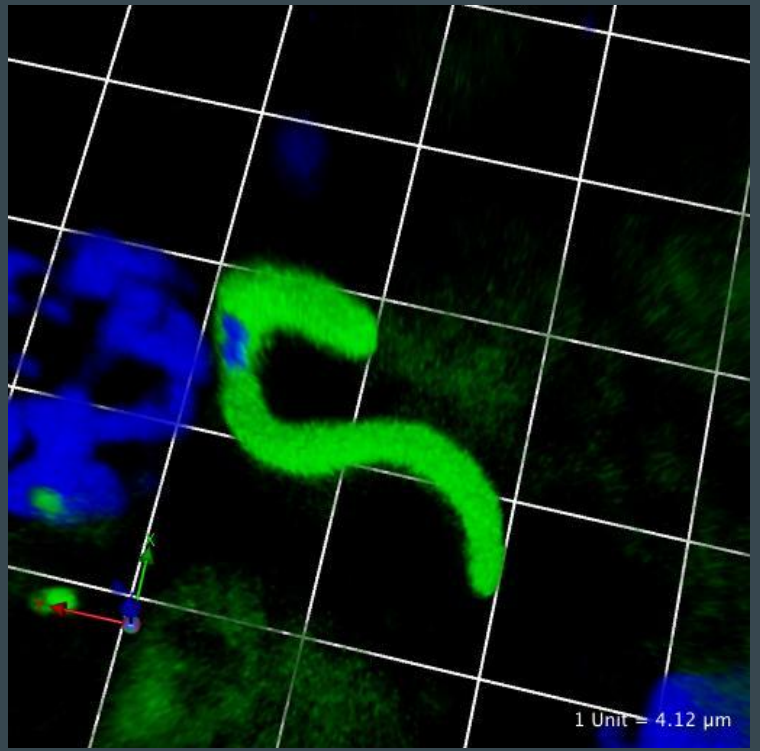


Images projection of z-series. Hoechst stained nuclei (blue) CSP (Green), SP5, 63x, Z-series 0.6 um slice.



3D reconstruction of parasite. Hoechst stained nuclei (blue) and CSP (green) SP5, 63x, Z-series 0.6 um slice.

CSP

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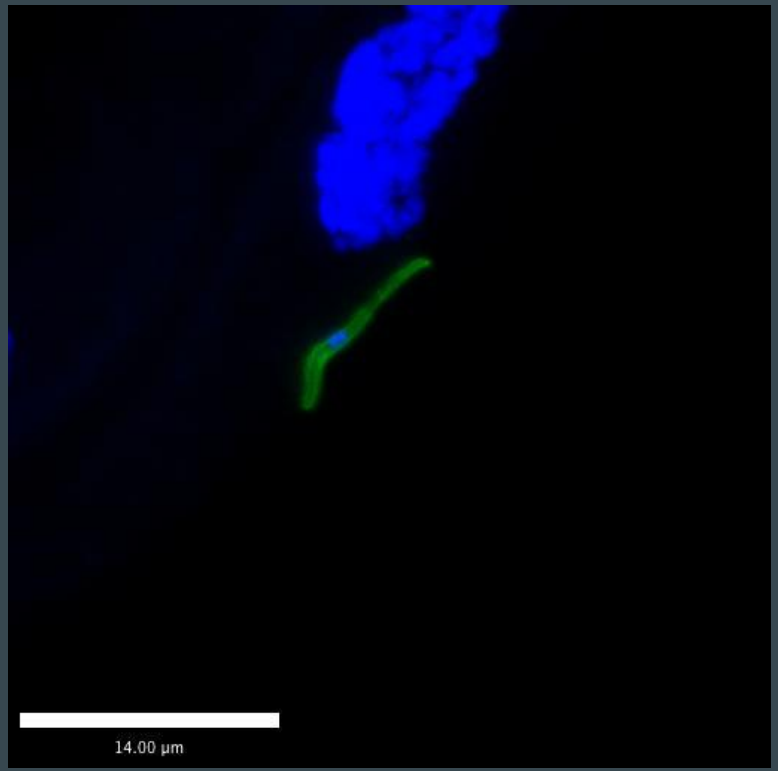
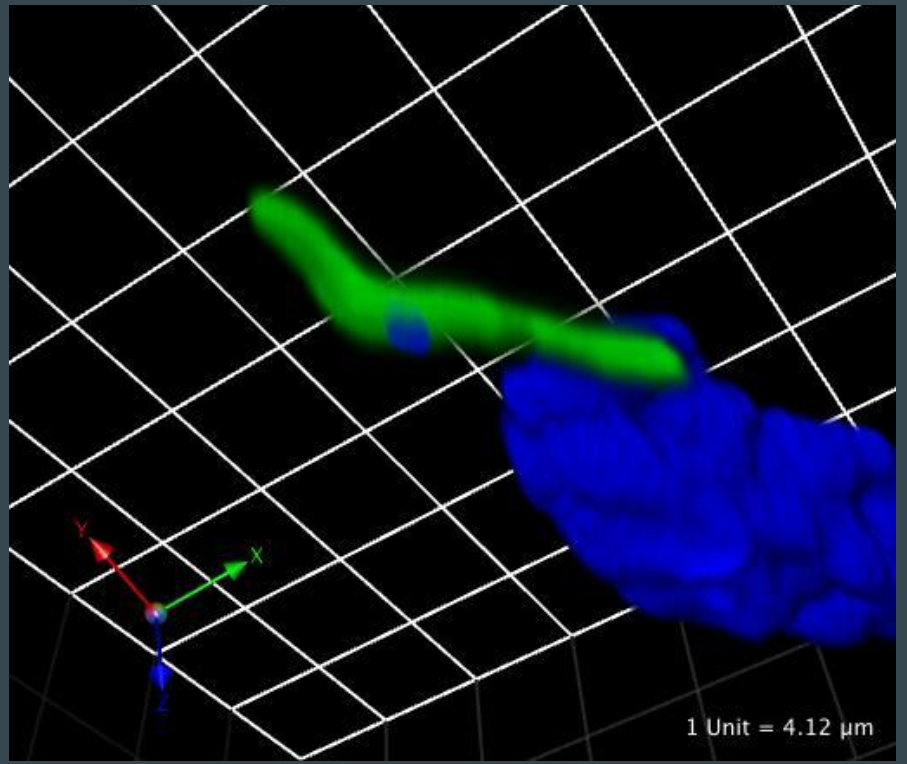


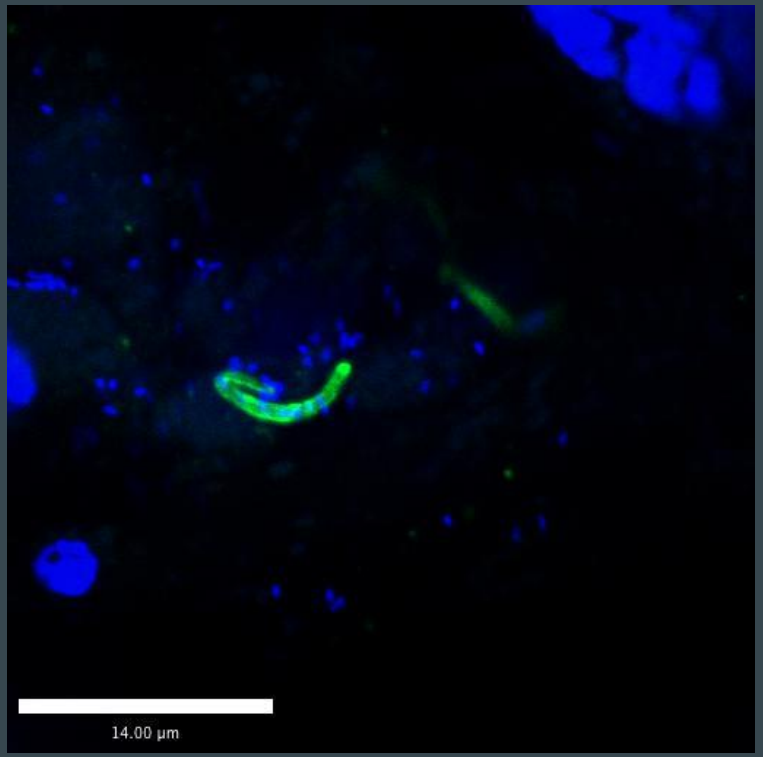
Images projection of z-series. Hoechst stained nuclei (blue) CSP (Green), SP5, 63x, Z-series 0.6 um slice.



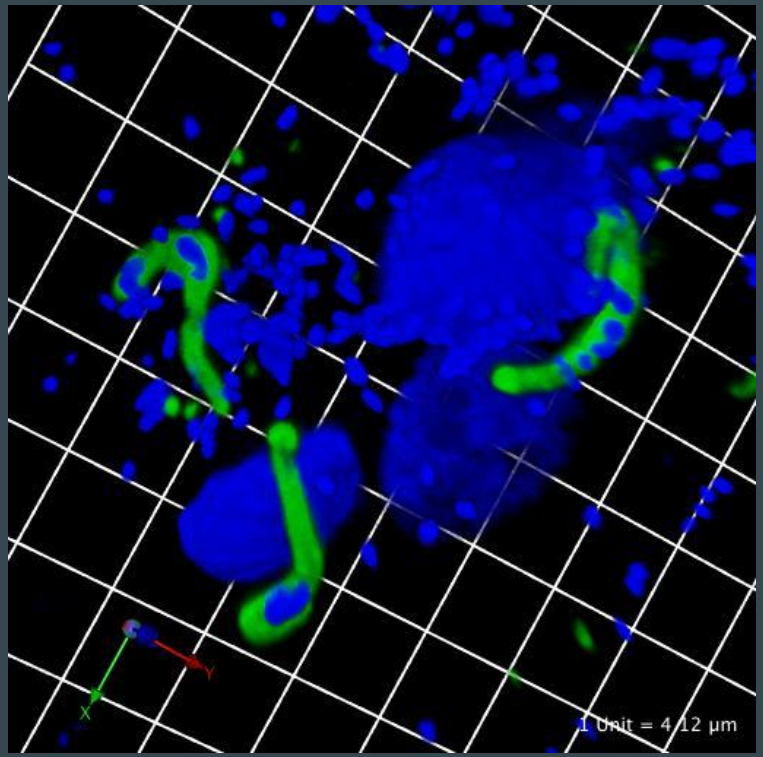
3D reconstruction of parasite. Hoechst stained nuclei (blue) and CSP (green) SP5, 63x, Z-series 0.6 um slice.

CSP

Examples of an individual parasite inside the midgut



Z-slice. Hoechst stained nuclei (blue) CSP (Green), SP5, 63x, Z-series 0.6 um slice.



3D reconstruction of parasite. Hoechst stained nuclei (blue) and CSP (green) SP5, 63x, Z-series 0.6 um slice.

Additional Images

Using the Nikon Super Resolution Microscope

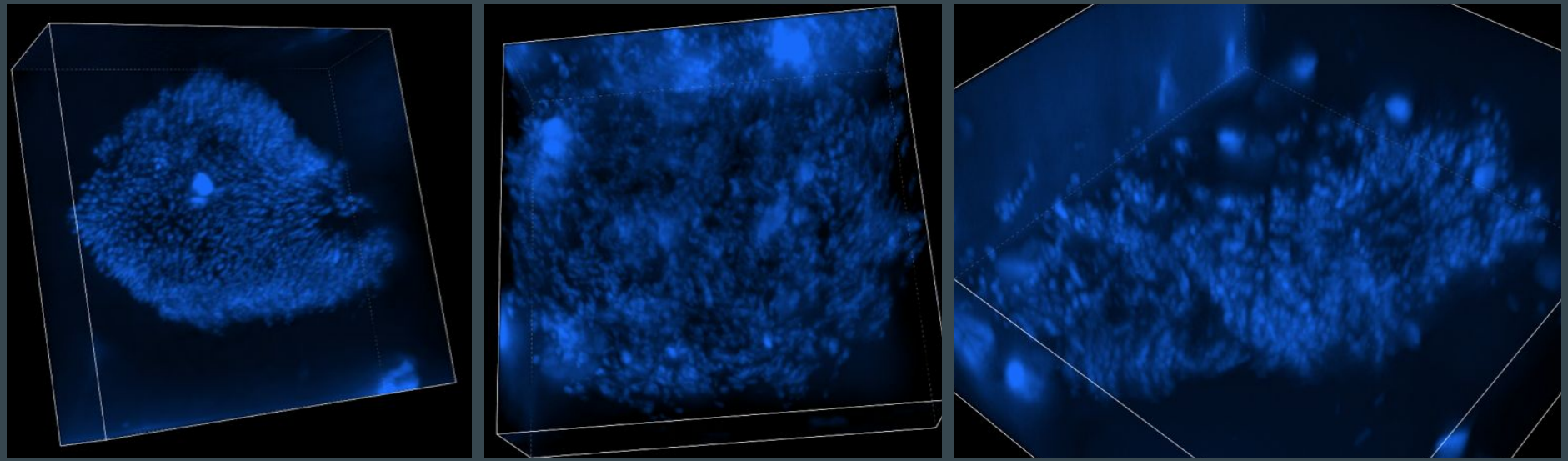
The technique of Structured Illumination Microscopy (SIM) was used for these images. SIM is a light microscopy technique that allows one to get closer to the resolution limit of light.

All images were taken at 100x, with a z steps of 0.03 um, imaging half of an oocyst.

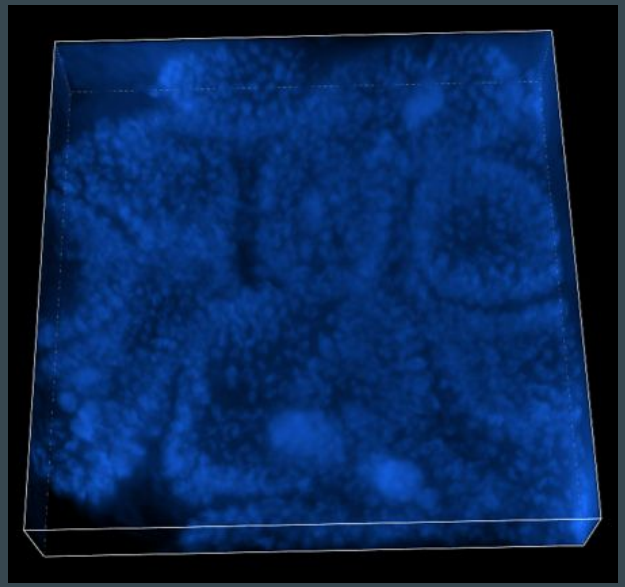
Images are shown as 3D projections.

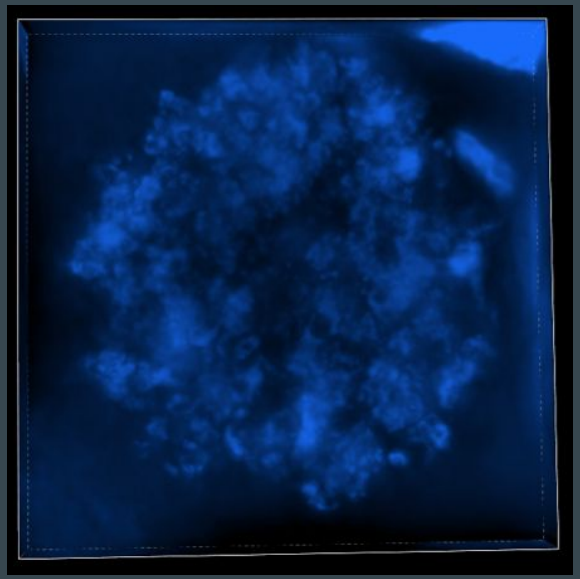
Particular interest was taken to a “tunnel” like structure observed, that Dr. Kami Kim had not known about previously.

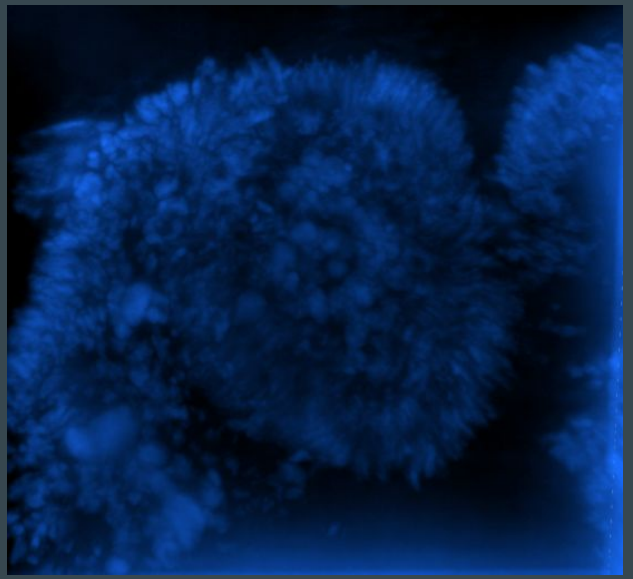
Examples of the different stages of oocysts observed in SIM



Examples of the different stages of oocysts observed in R3-HA in SIM

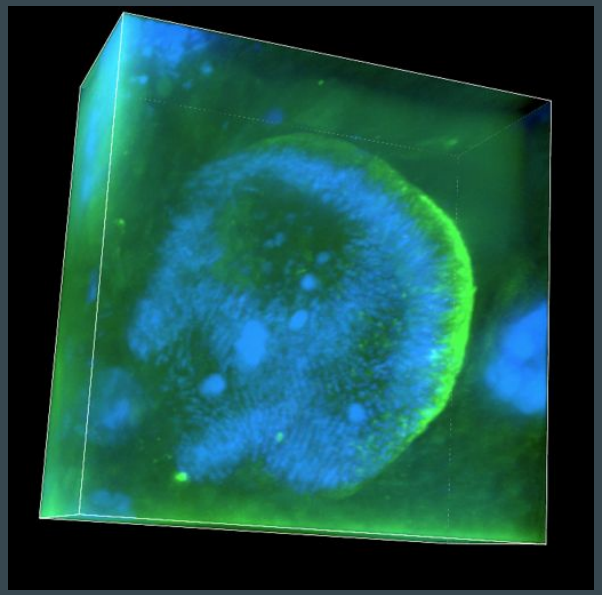


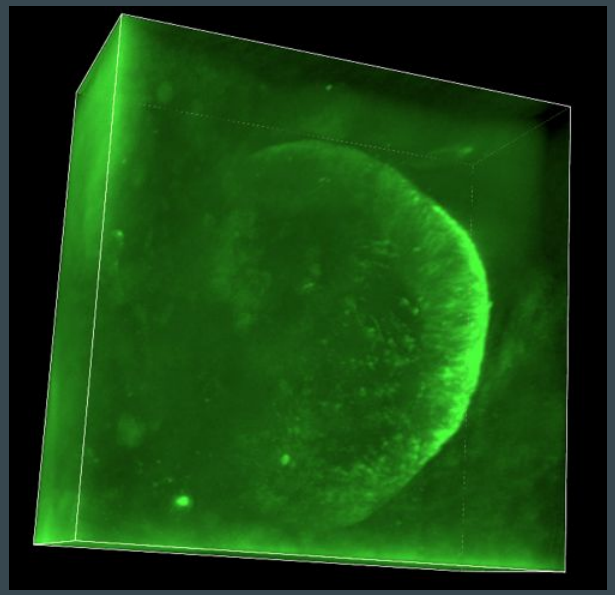


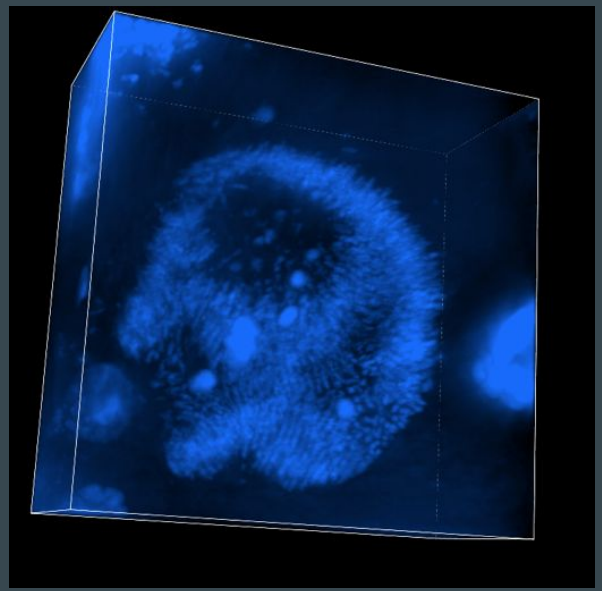


CSP and Hoechst

Example of a oocyst imaged in SIM

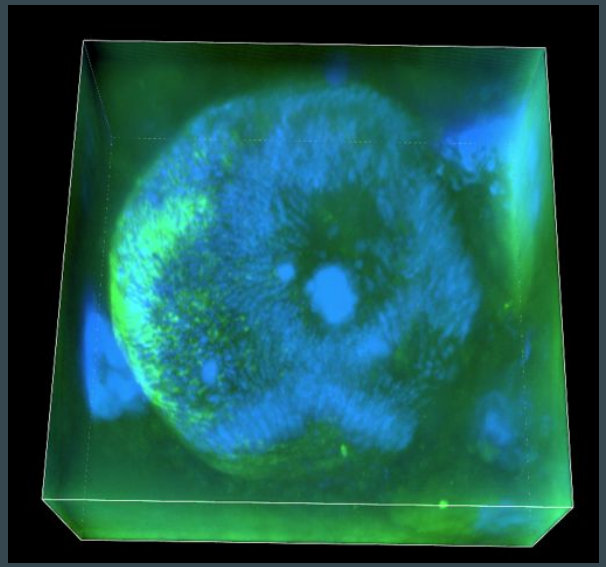


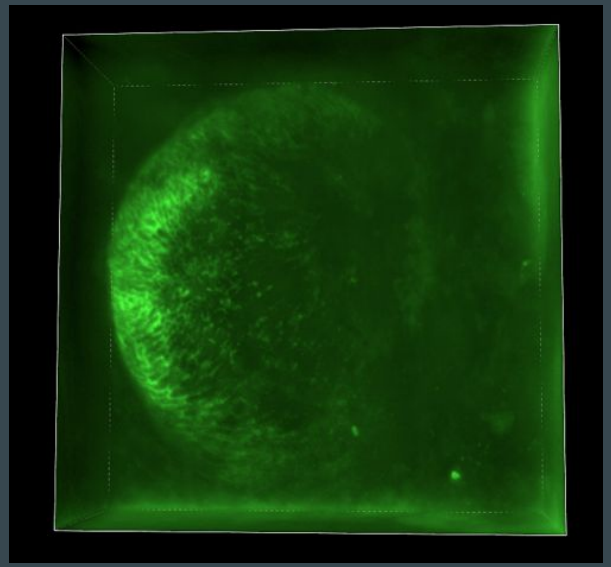


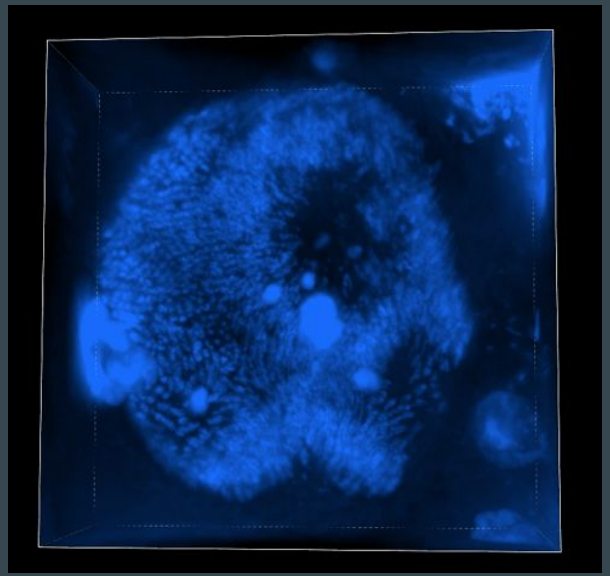


CSP and Hoechst

Example of a oocyst imaged in SIM

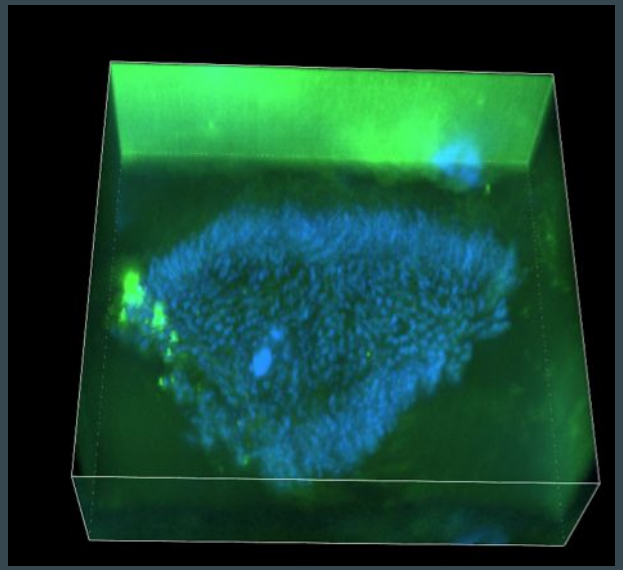


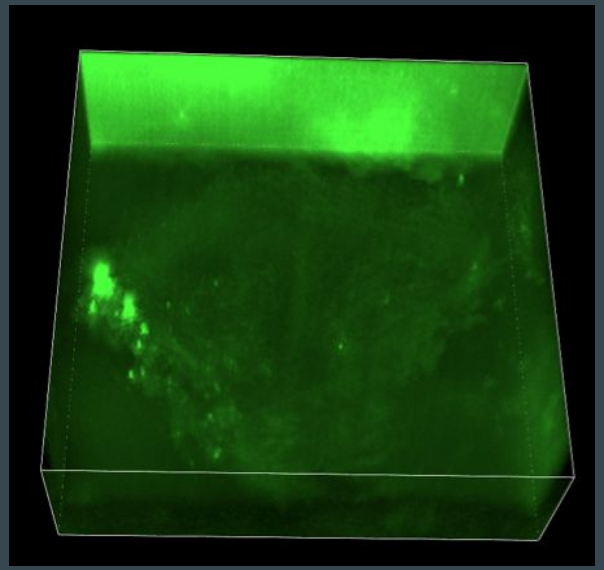


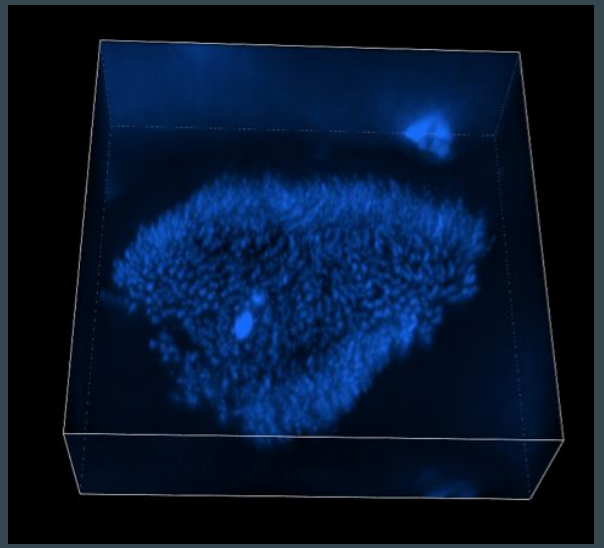


CSP and Hoechst

Example of a oocyst imaged in SIM

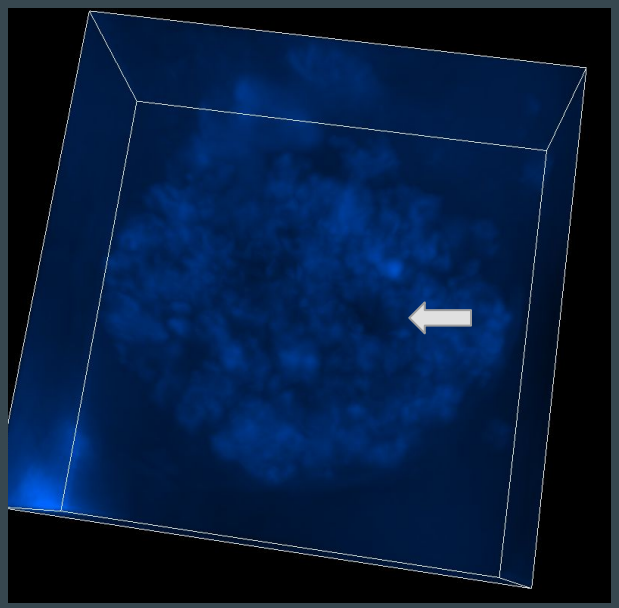


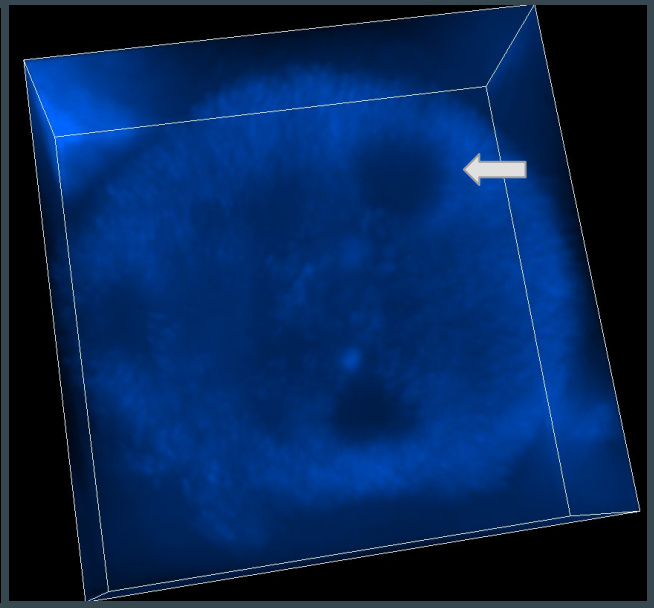


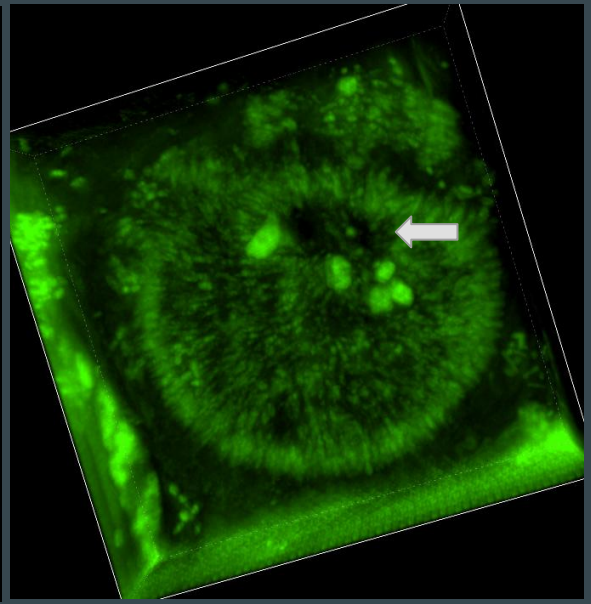


SIM Imaging

General examples of the tunnel structure observed in oocysts, indicated by the white arrow







SIM Imaging

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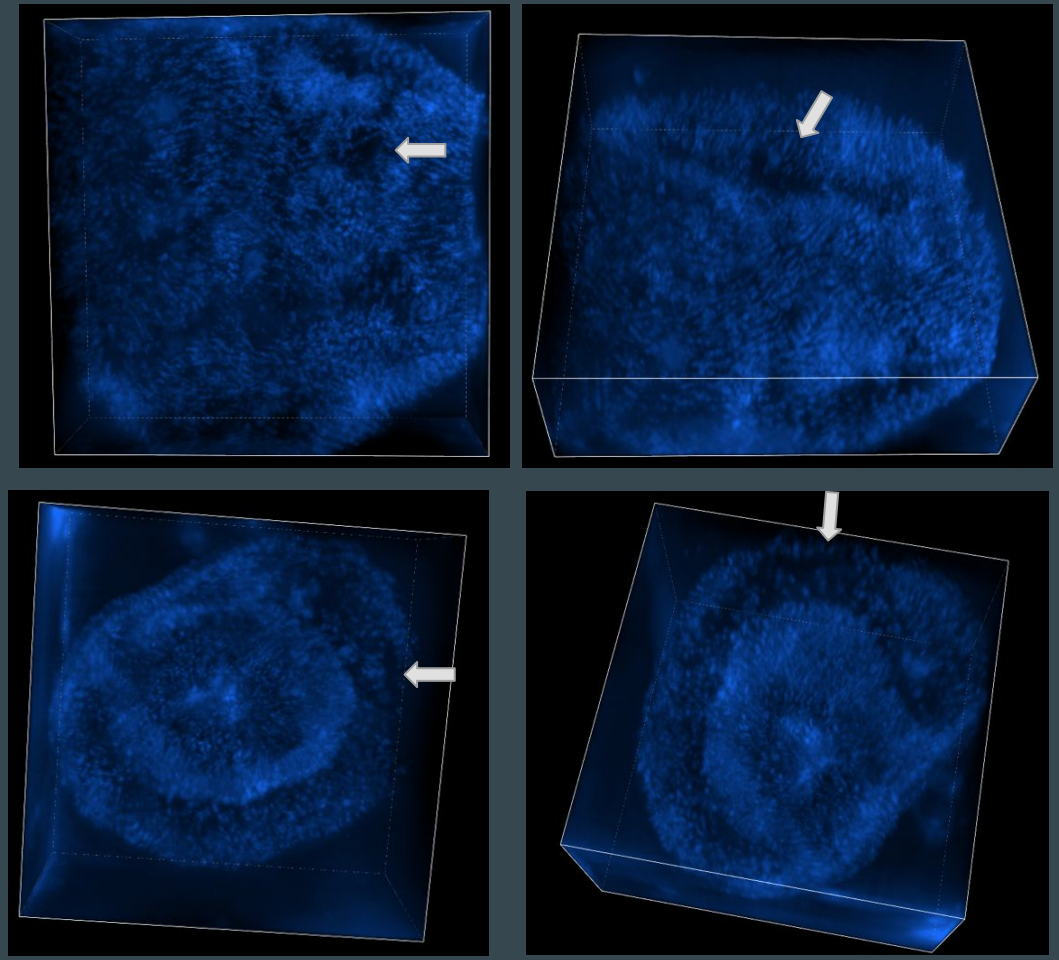
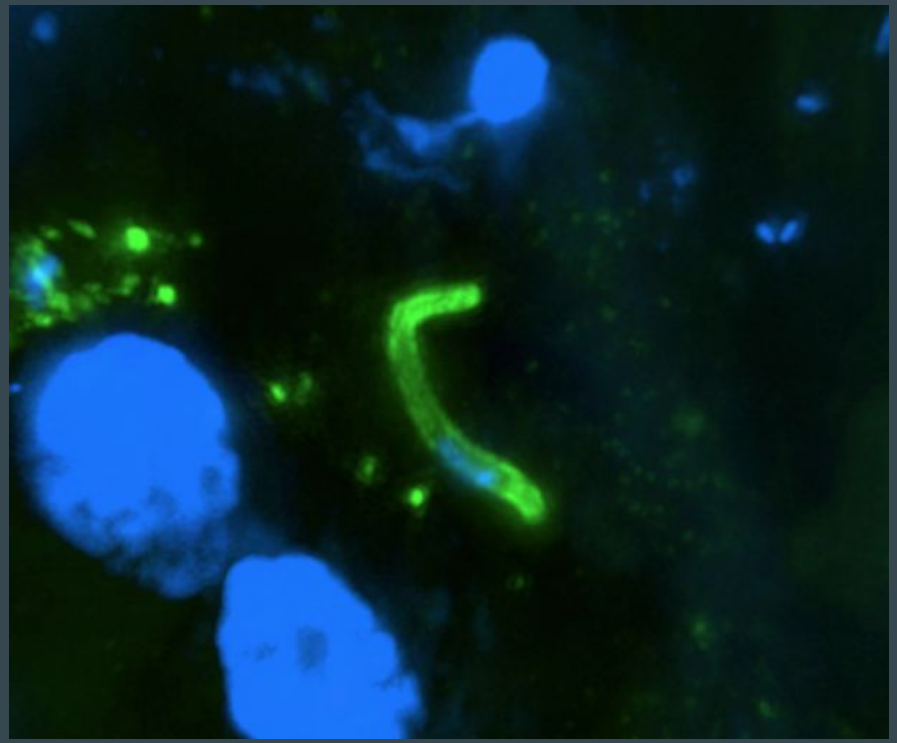
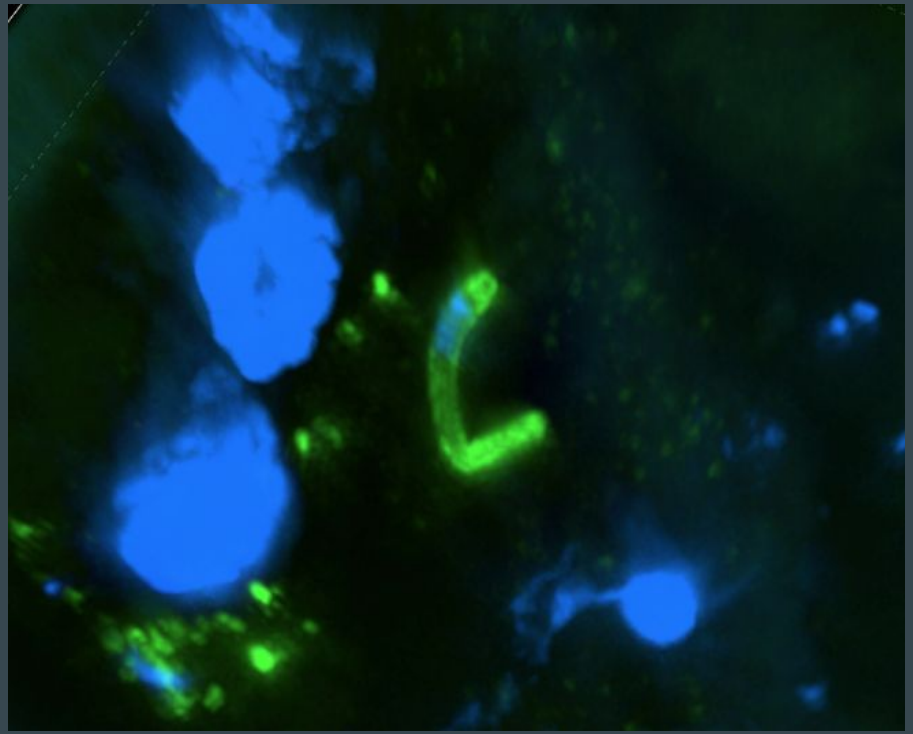


SIM Imaging

Examples of individual parasites using SIM technique





Future Directions

Staining: When staining for CSP use the antibody Alexa 561 instead of Alexa 488, because there is less inherent autofluorescence in the red channel in comparison to the green. Test a fresh anti-CSP C terminus antibody and see if it improves the signal.

Image analysis: Repeat the experiment and get more counts for the R3-KO and GFP, because the number of counted oocysts in comparison to the other condition were significantly less. The statistics could be improved with a higher oocysts count.

Imaging the Oocysts in the Midgut of the Mosquito

This project was completed by and submitted for Hillary Guzik's Masters of Science degree in Entomology from the University of Nebraska. It is submitted for review for the summer 2016 semester. All data and images generated during the duration of this project will further aid the future and current research of Dr. Kami Kim of Albert Einstein College of Medicine.