

# Système d'Informations Halieutiques

Action Paramètres biologiques

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## Stereology reading protocol when using quantitative histology for the determination of sexual maturity in fish ovaries



## Stereology reading protocol when using quantitative histology for the determination of sexual maturity in fish ovaries

The digital version of this document is available on the [ARCHIMER](#) Web site.

The digital version of complementary documents describing the histological structures found in the ovaries and during the oogenesis of different fish species are available on the [ARCHIMER](#) Web site.

<b>Version</b>	<b>Date</b>	<b>Comments</b>
1.0	July 2019	Conception of the document for the Plaice
2.0	May 2020	Conception of the document for the Megrin
2.1	June 2020	Conception of the document for the Four Spot Megrin
2.2	June 2020	Size rectifications
2.3	June 2020	Figure and Ido added with text
3.0	May 2022	Generalization of the reading protocol for all species
3.1	June 2022	Added a summary sheet
3.2	August 2022	Added Nucleo-cytoplasmic measures
3.3	March 2023	Correction of a mistake on p.13
3.4	May 2023	Added problems and solutions for QuPath scripts p. 17

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# 1. Context

This protocol was established for the evaluation of sexual maturity for exploited stock species, using quantitative histology (stereology). This document describes the reading method used on histological slides under the cross-platform QuPath.

This document follows the work of different workshops that aimed to improve the compilation of data on sexual maturity. The WKMATCH (Workshop for MATurity staging CHairs, 2012) and the WKASMSF (Workshop for Advancing Sexual Maturity Staging in stageFish, 2018) defined a universal evaluation grid for sexual maturity staging of different species, including teleosts. During the WKMATCH workshop, two main recommendations were made, underlining the need to improve and complete knowledge on sexual maturity, as well as to harmonize the practices used to determine these sexual phases.

Another workshop, the WKMATHIS (Workshop on sexual MATurity staging from HIStological tools) that took place in Caen in 2017, added new objectives : review the current knowledge on gonad histology, determine a sexual maturity scale at a cellular level, and histological studies should be made in order to improve the determination of sexual maturity on a macroscopic scale.

This protocol, drawn from these workshops and their conclusions, offers guidelines on how to read histological slides through the use of stereology. As of June 2022, this protocol is linked to the MATO (MATurité Objectif des poissons par l'histologie quantitative) project, a study restricted to female gonad and the different cellular structures found inside the ovaries for different species (*Pleuronectes platessa*, *Lepidorhombus whiffiagonis*, *Lepidorhombus boscii*, *Mullus surmuletus* and *Micromesistius poutassou*).

This protocol will present reading methods that favor an objective reading of histological slides, through stereology, and will allow less experienced readers to partake in this exercise. For a detailed description of the germinal cells, refer to the lexicon of the studied specie.

## 1.1. Terminologies and vocabulary

The specific histological terms, their abbreviations, the descriptions of the different types of germinal cells, as well as an accurate description of identifiable ovarian structures, may be found in the lexicons of the studied species (cf. Archimer).

For the MATO project, the terminologies used were that of Brown-Peterson *et al.* (2011), following the criteria set by the working groups of the International Council for the Exploration of the Sea (ICES) to define different maturity scales for European stock species.

For the description of female germinal cells that will become gametes, the terms **oogonia** (before meiosis) or **oocyte** (meiosis has begun) will be used. An oocyte is characterized by an **ooplasm** encased in an **oolemma** (plasmic membrane) (Tyler and Sumpter, 1996). A **zona pellucida** will also be present once the cell becomes dependent on follicle-stimulating hormone (FSH).

The term **follicle** will be used to designate an oocyte encased in somatic cells: follicular cells and theca cells (Tyler and Sumpter, 1996).

For the description of gamete development, the term **stage** will be used to designate the different gametogenesis stages (Brown-Peterson *et al.*, 2011) :

- Oogonia
- Primary growth oocytes
- Secondary growth oocytes
- Oocyte maturation
- Ovulation stage

The sexual maturity cycle is split into two **states**. The Sexually Immature (**SI**) state and the Sexually Mature (**SM**) state. The terminology **phase** will be used for the gonadal development. The reproduction cycle of fish is divided into seven **phases** (Brown-Peterson *et al.*, 2011, ICES, 2012 ; 2018).

- A - Immature
- B - Developing
- C - Spawning
- Da - Regressing
- Db - Regenerating
- E - Omitted spawning
- F - Abnormal

## 1.2. Processing of histological slides

For a more detailed explanation on the extraction and processing of fish ovaries, refer to « A macroscopic and stereological imaging dataset of *Pleuronectes platessa* ovaries » (Sauger *et al.*, 2020) as well as gonad extraction protocols (Sauger *et al.*, 2019 & Sauger *et al.*, 2022).

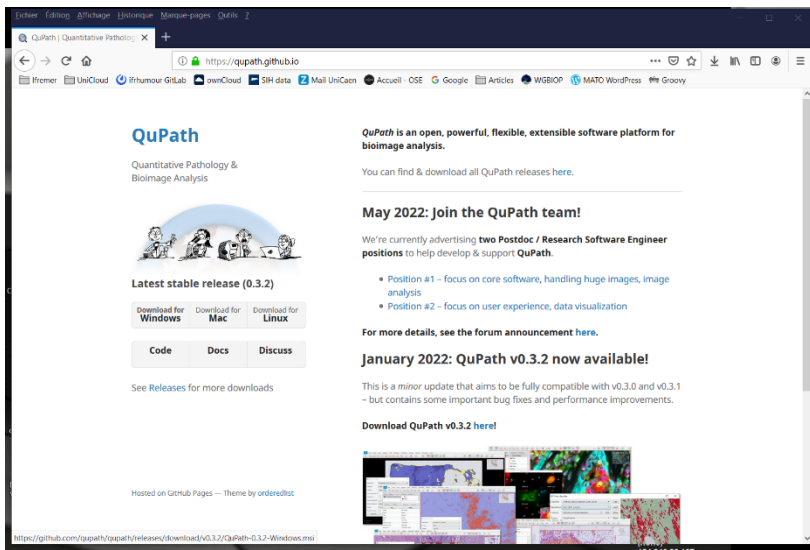
The ovaries of fresh female fish were processed in a Davidson solution, embedded into a paraffin block and 3µm cross sections were made with a microtome. The samples were then stained with Gabe's trichrome (Gabe, 1968), mounted between slide and slip, and digitized using a histology slide scanner. The pictures found in this protocol are screenshots of these scanned slides in the QuPath cross-platform interface used for the stereology counting process.

og (A)	Oogonium
po1 (Z)	Primary oocyte stage 1
po2 (E)	Primary oocyte stage 2
ldo (R)	Lipid droplet oocyte
cao (T)	Cortival alveoli oocyte
vtg1 (Y)	Vitellogenic oocyte stage 1 / Early vitellogenesis
vtg2 (U)	Vitellogenic oocyte stage 2
vtg3 (I)	Vitellogenic oocyte stage 3
oih (O)	Oocyte in hydration
ho (P)	Hydrated oocyte
POF (Q)	Post-Ovulatory Follicle
aoA (S)	Atretic oocyte alpha
aoB (D)	Atretic oocyte beta
L (F)	Lysis
gw (G)	Gonadal wall
ct (H)	Connective tissue
bv (J)	Blood vessel
Is (K)	Intercellular space
U (L)	Undetermined
E (M)	(unnatural) Emptiness

List of structures that may be found within the ovaries. The number of structures will be species dependent (QuPath interface)

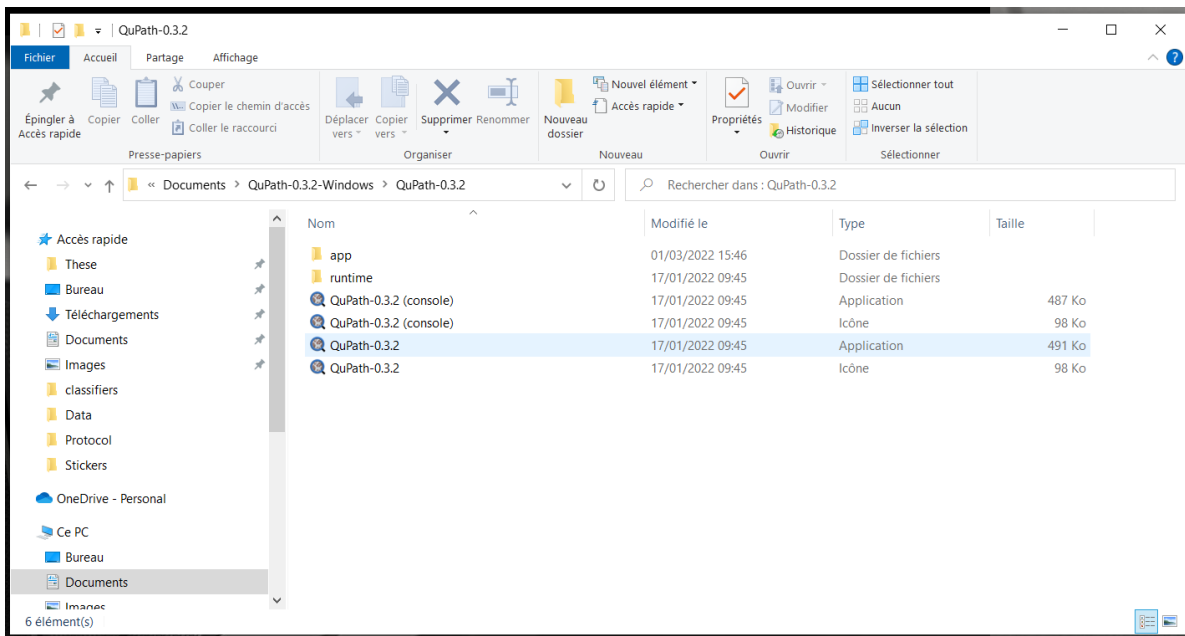
## 2. QuPath - Stereology Software

For the stereological readings, the software platform for bioimages analysis QuPath (Bankhead *et al.*, 2017, version 0.3.2) was used. Each histological slide was processed : the ovaries were automatically outlined (black line) and a grid of crosses (in blue) covered the sampling zone. For each of the 500 to 600 sampling points (blue crosses) a single structure was assigned (box to select inside the *Stereology: Cross Grid* window). The automatic outline of the ovary was made possible by running a script under QuPath, allowing the detection of the ovarian tissues and the implementation of the counting grid. This script, coded by Nicolas Elie (CMABIO3), allows for anyone that has a slide scanner to replicate the stereology method used in this study.



QuPath is an open source software and may be downloaded from their website : [\(https://qupath.github.io/\)](https://qupath.github.io/)

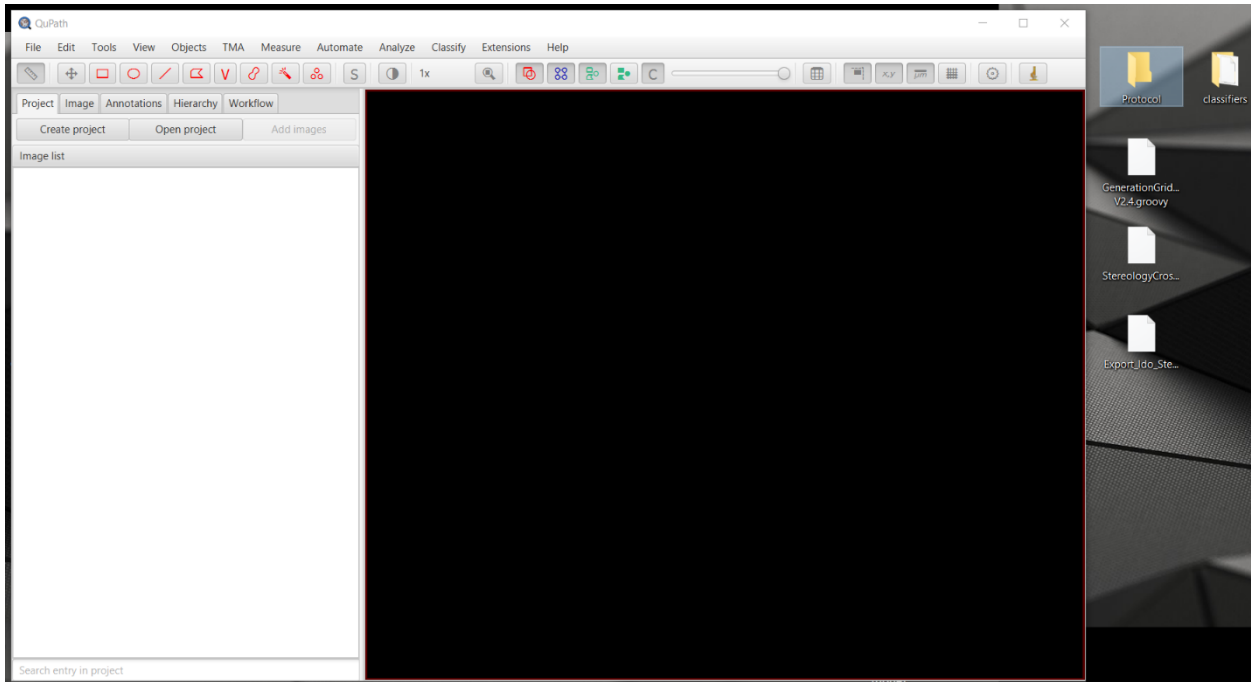
### 1. Launch QuPath



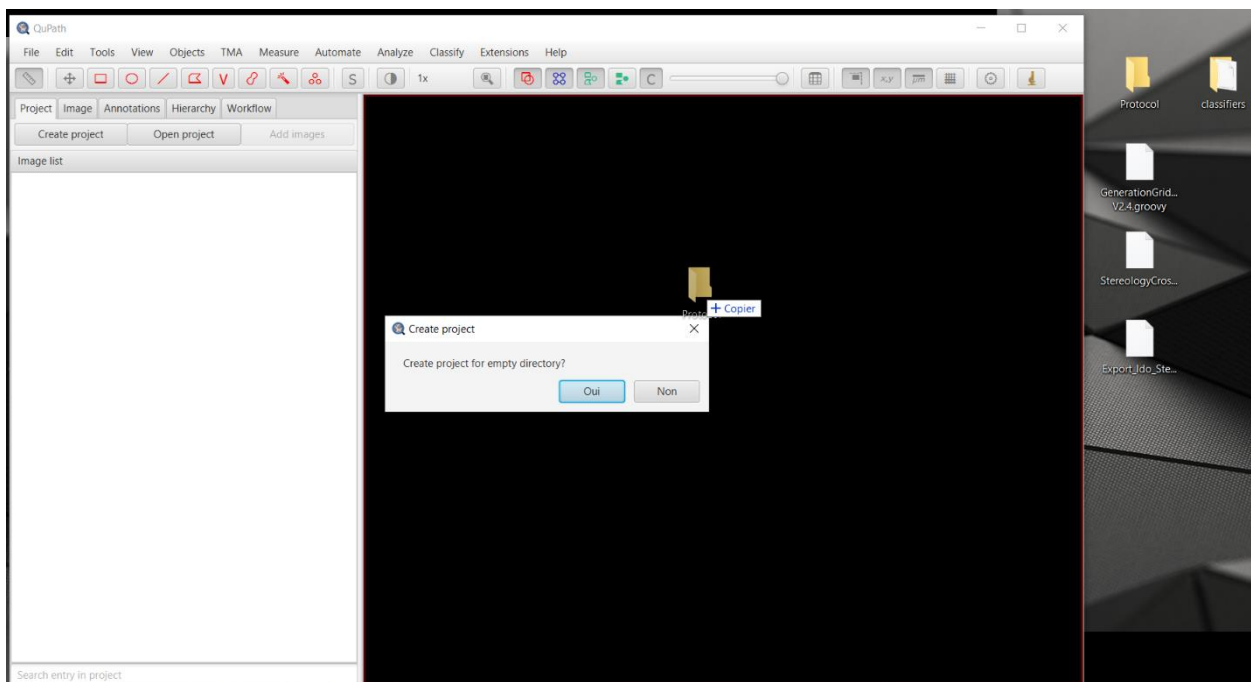


## 2. QuPath interface with mandatory files :

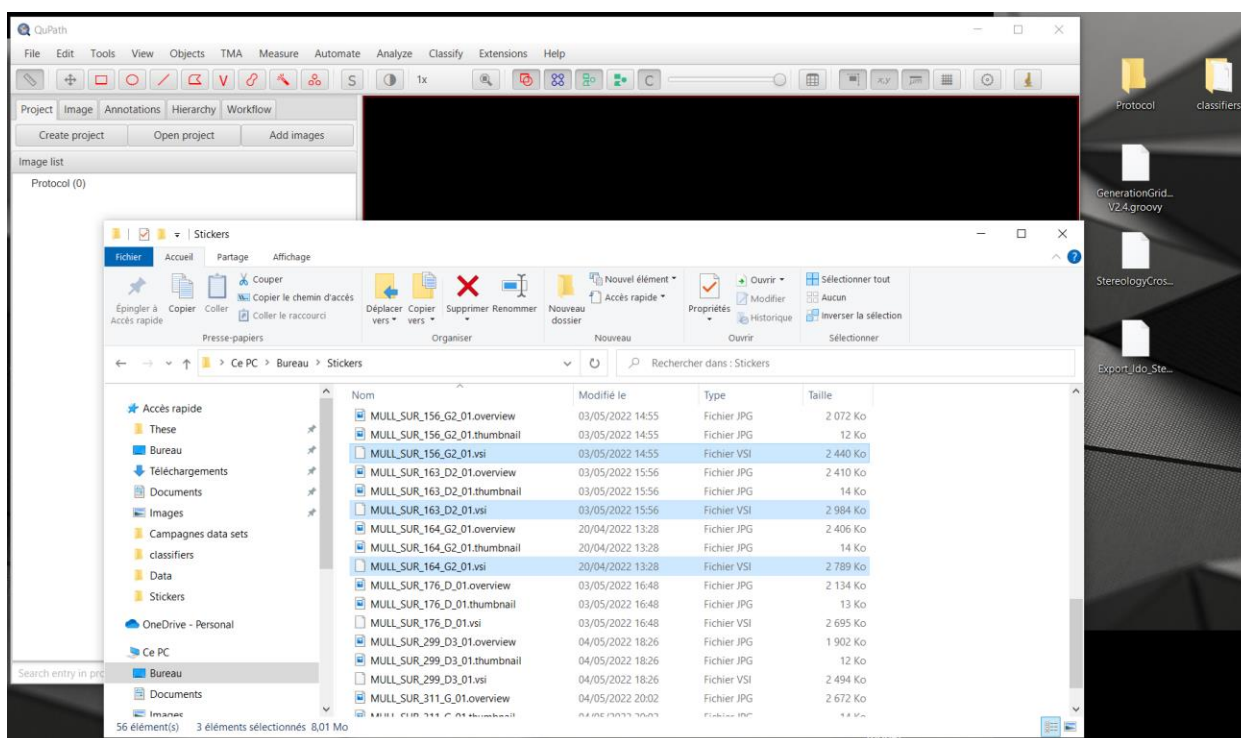
- Protocol : an empty folder for the future project
- GenerationGridCross.groovy : script that will outline the ovarian section
- StereologyCross.groovy : script to add the sampling grid
- Export\_StereoToXML.groovy : script to export the reading results into an xml file



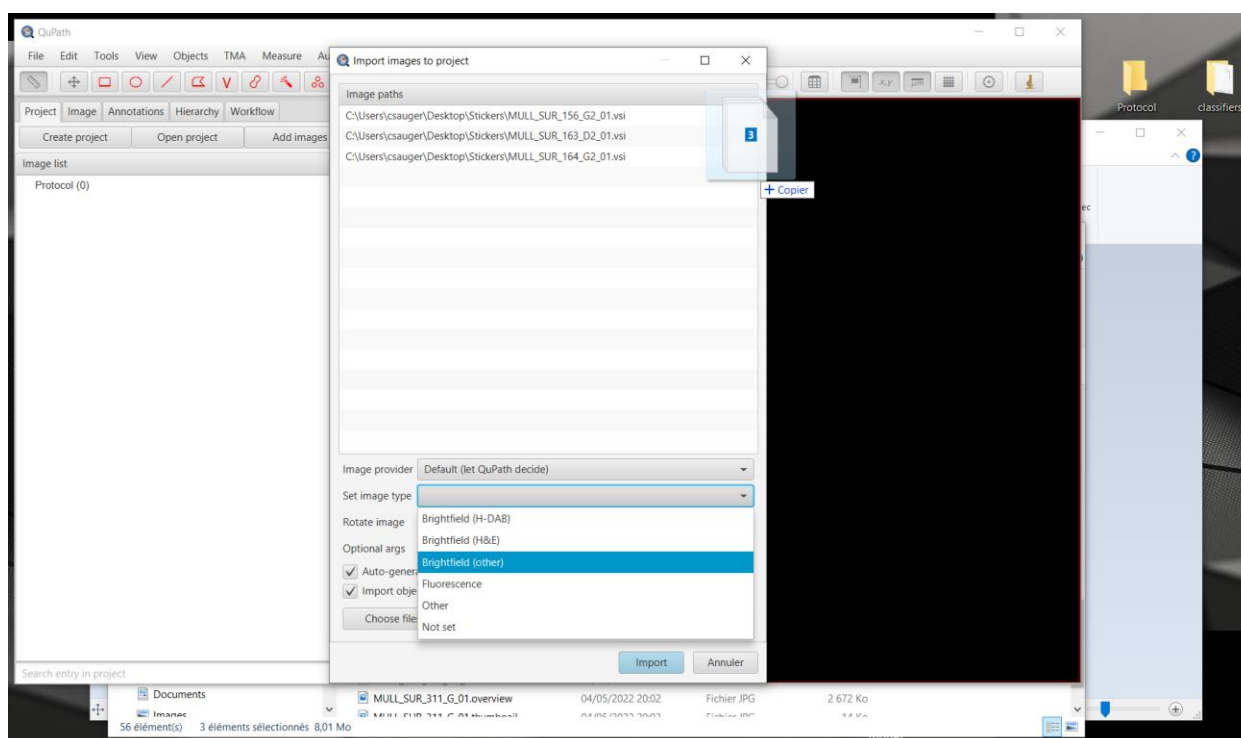
3. Click and drag the empty folder (where the future project will be stored (Protocol)) over the QuPath interface
4. Select 'Yes/Oui'



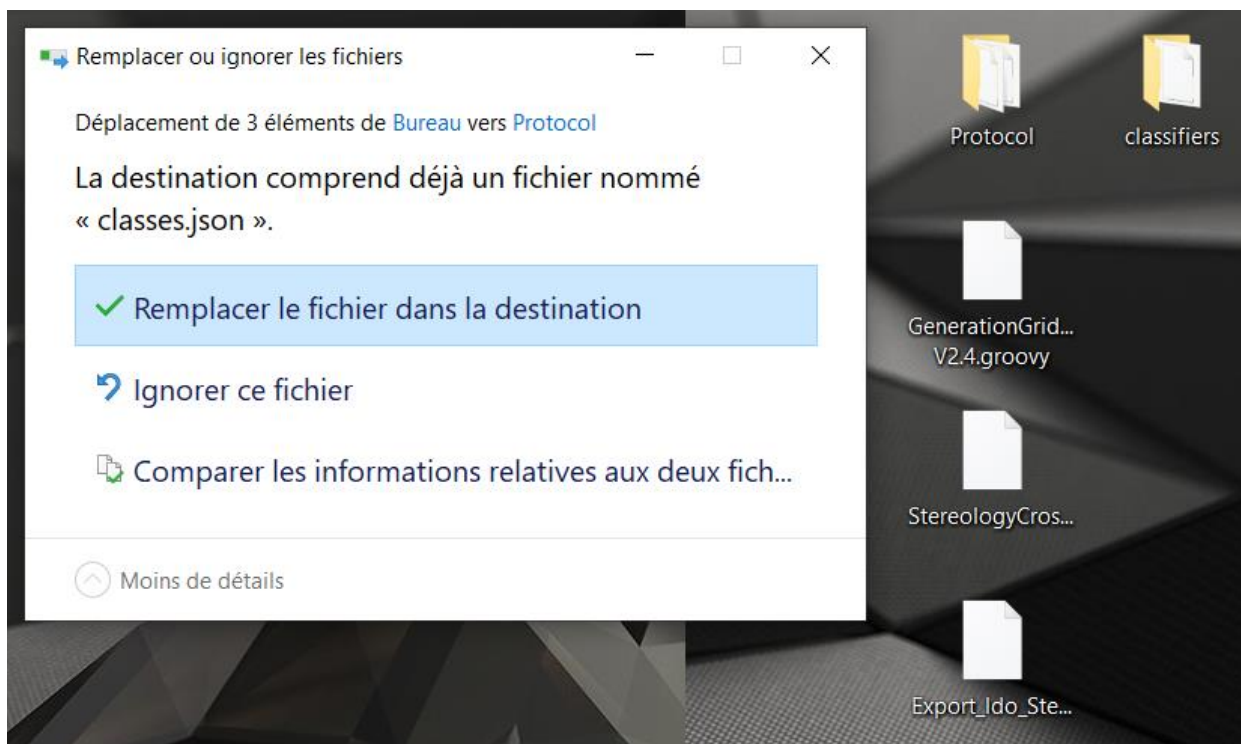
- Open the folder where the scanned slides are and select the .vsi files of the slides you want to analyze



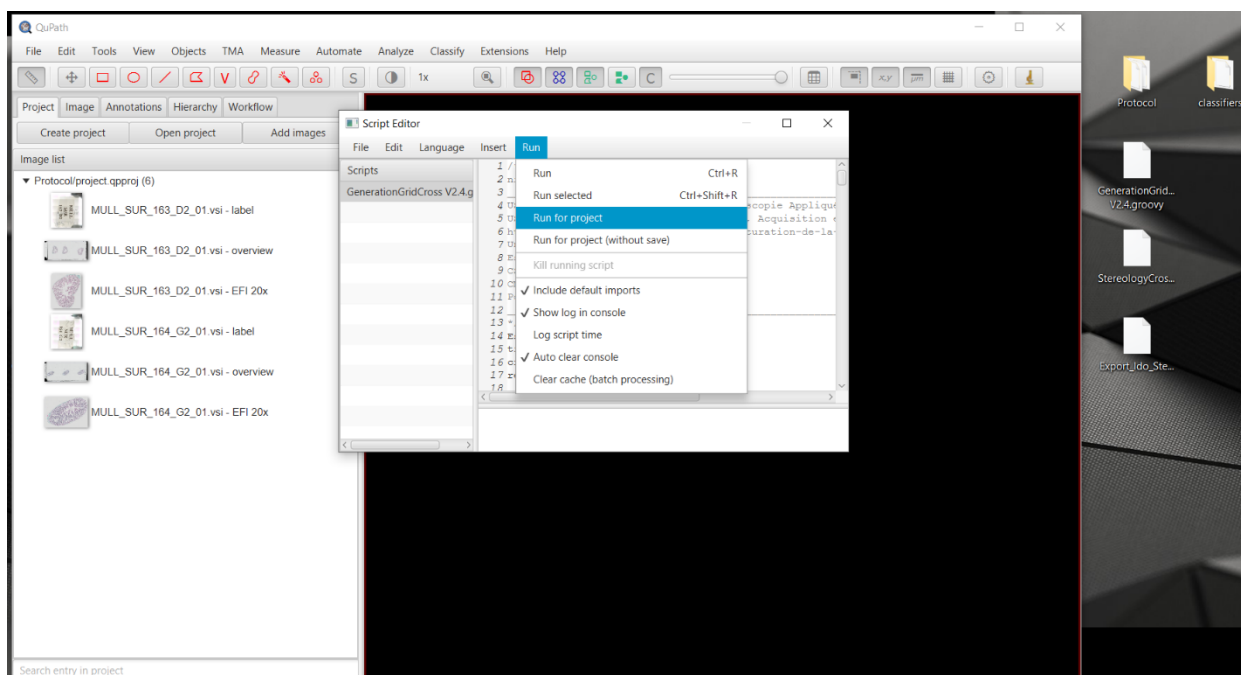
- Click and drag those files over the QuPath interface
- The 'Import images to project' window will open with the names of the .vsi files selected
- In the 'Select image type' choose 'Brightfield (other)'
- Click on 'Import'



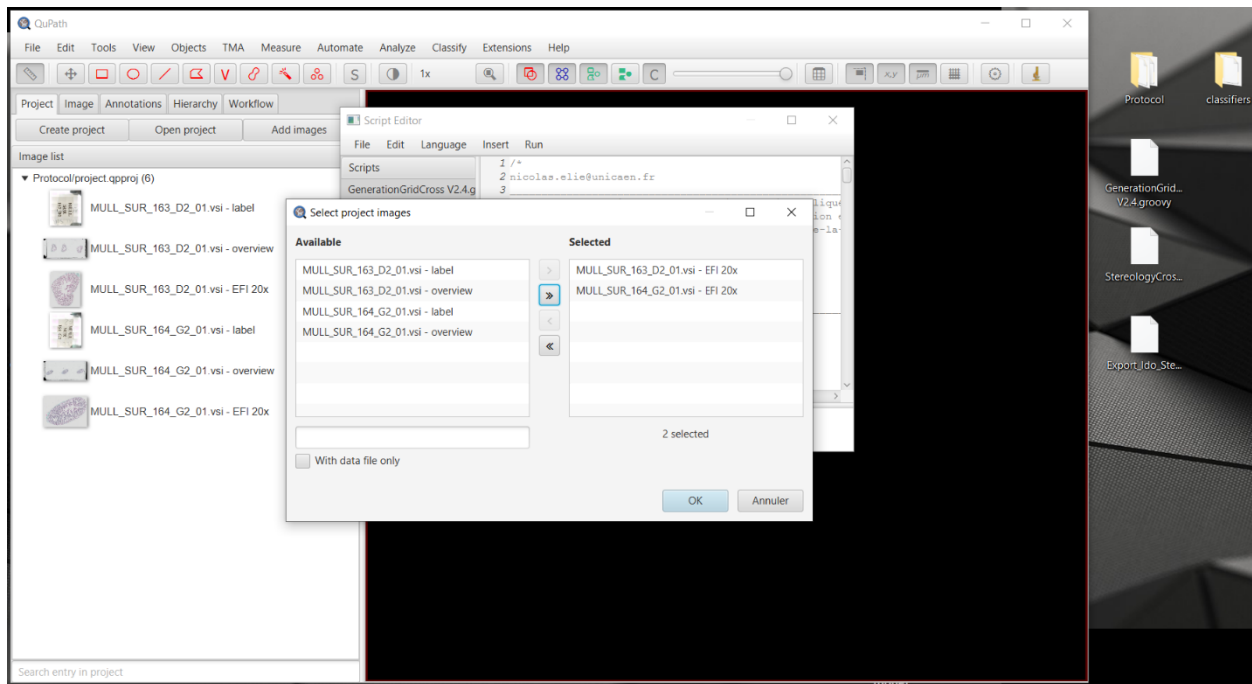
**10.** Back to the mandatory files, replace the 'classifiers' file inside the main project folder (Protocol) with the 'classifiers' files provided with the other mandatory files



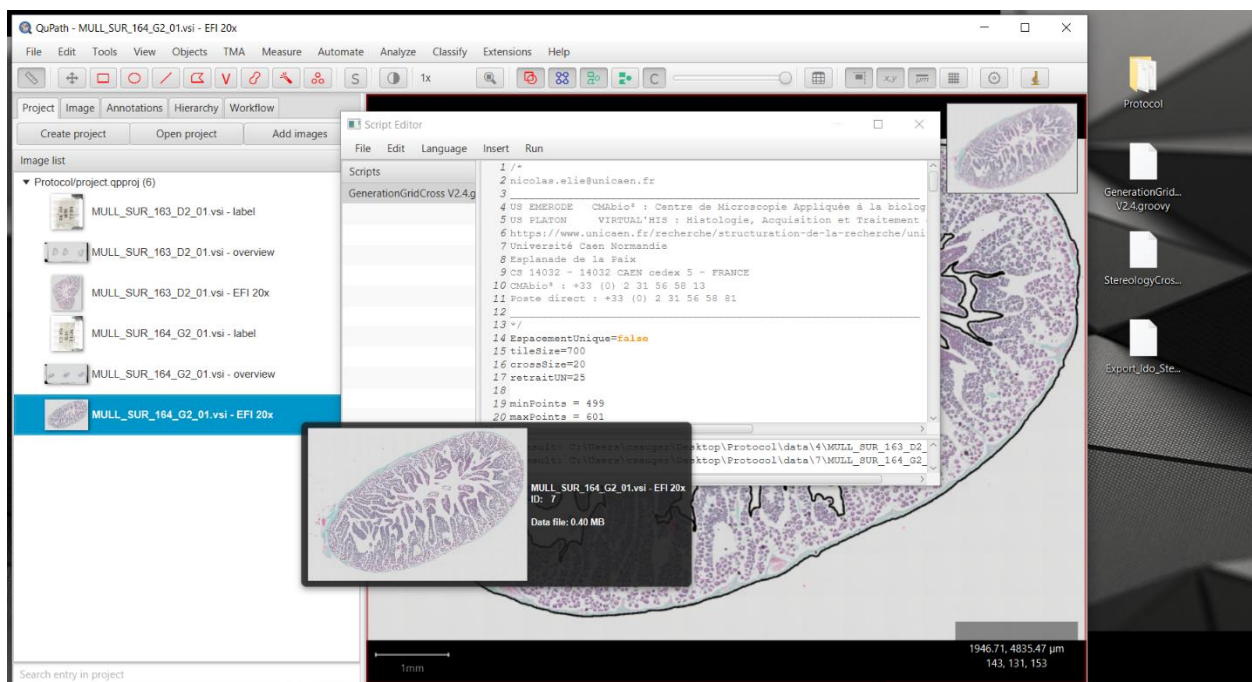
**11.** Click and drag the GenerationGridCross.groovy script over the QuPath interface  
**12.** The 'Script Editor' window will open  
**13.** Select the 'Run' tab and click on 'Run for project'



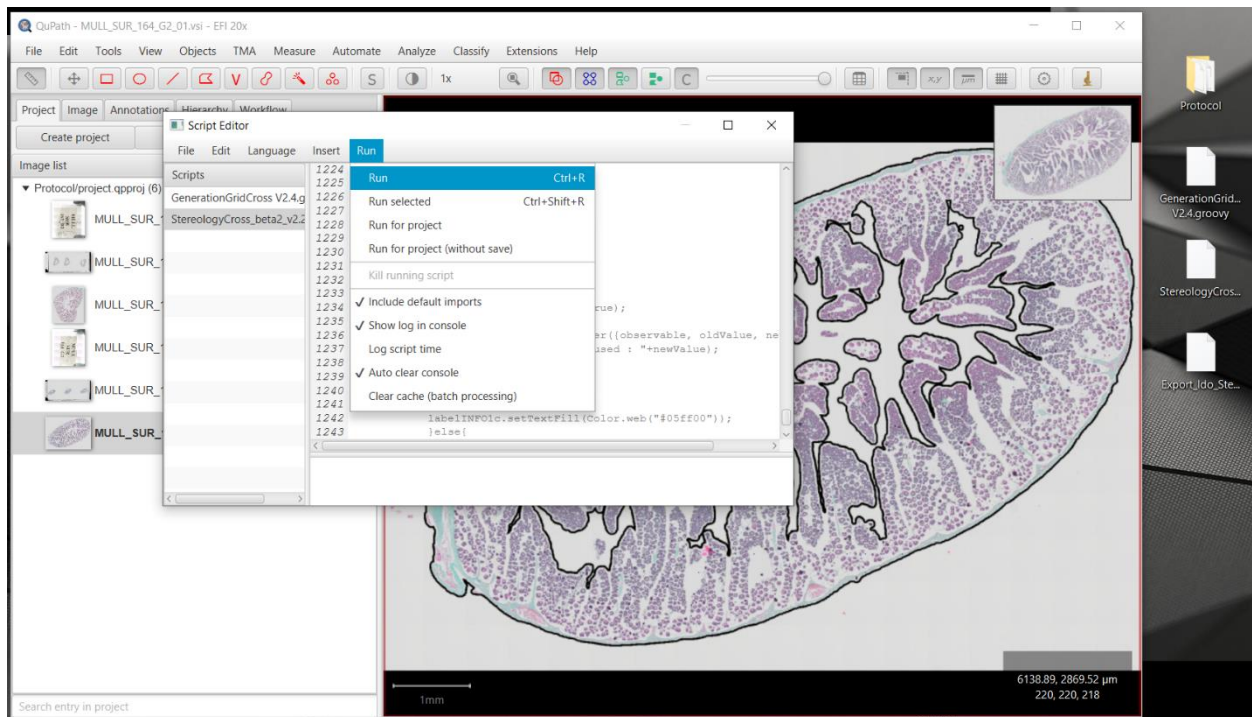
14. The 'Select project images' window will open
15. Select the EFI files
16. Click on 'OK'



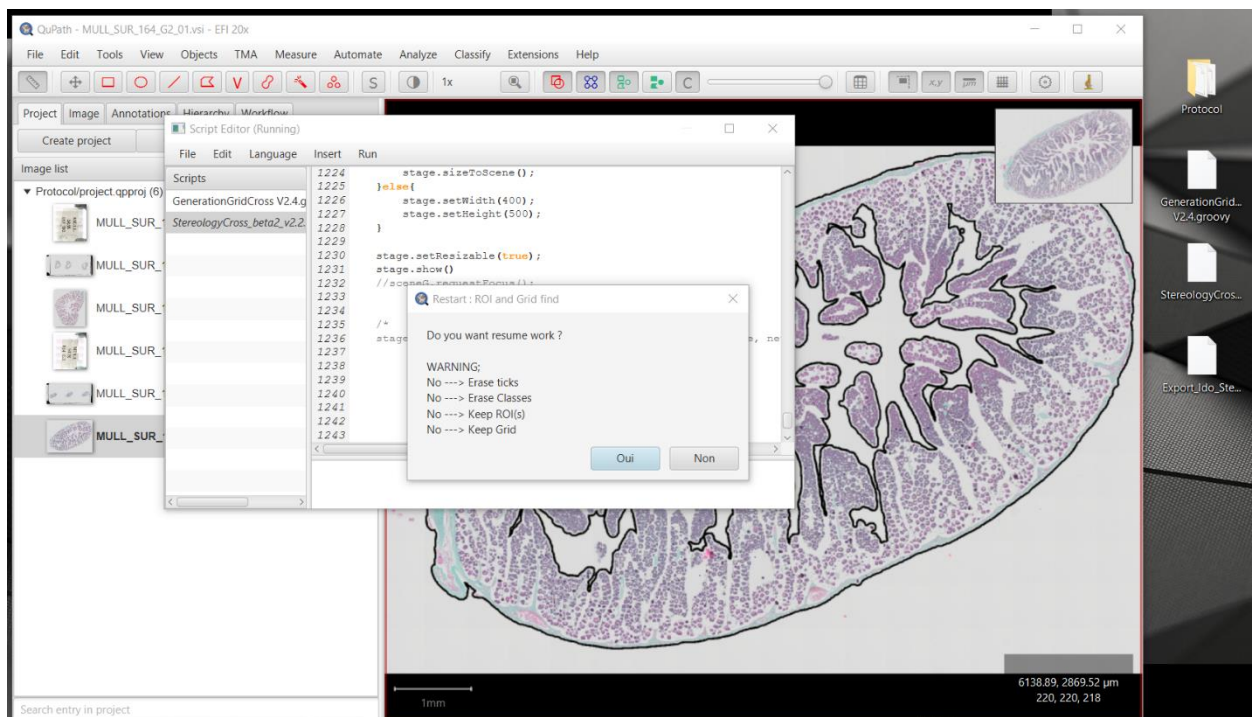
17. On the left window (Image list window) of the QuPath interface, double click on the scan you want to read.
18. The scan of the cross section should appear outlined in black. Blue crosses should have been laid out.



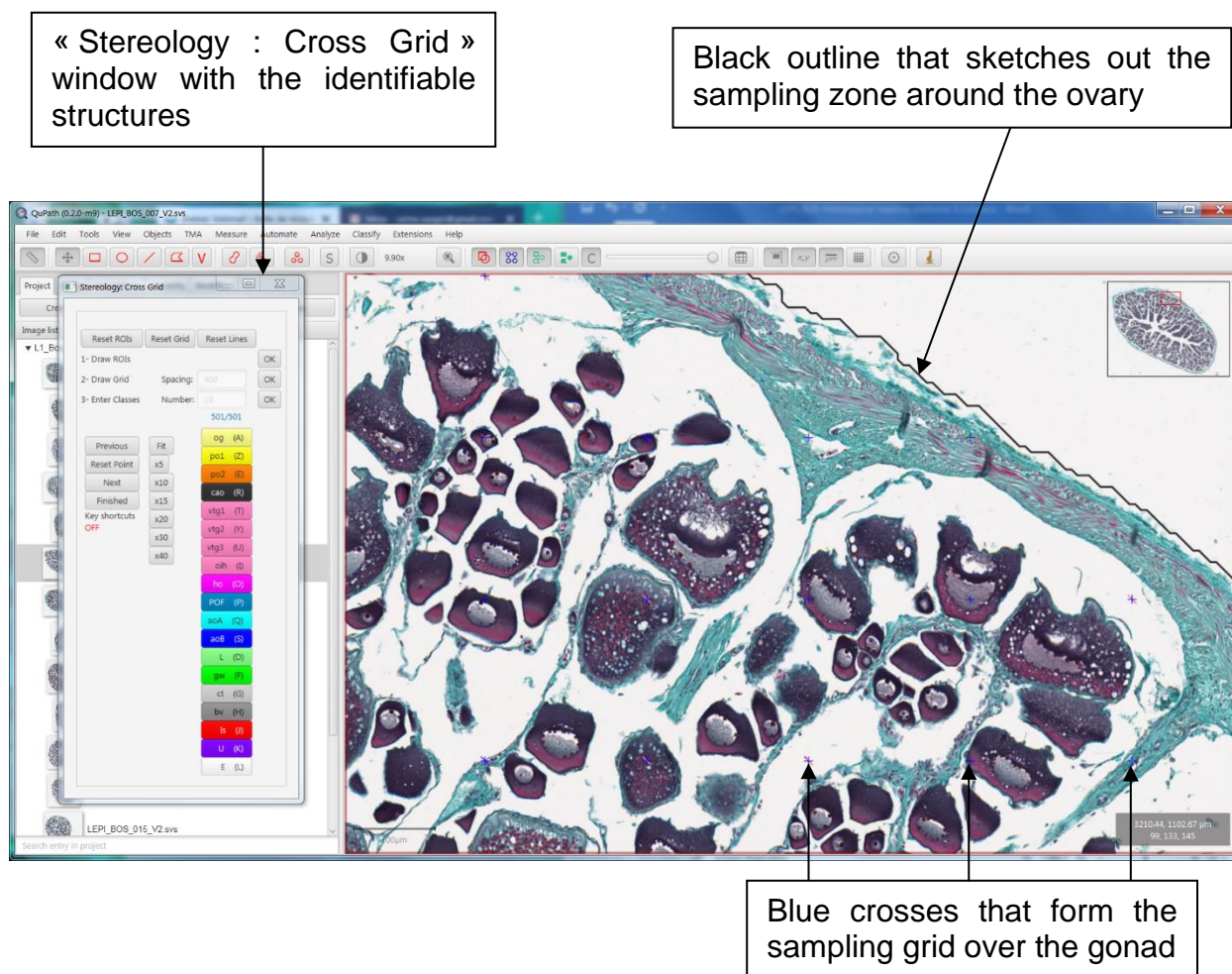
- 19. Click and drag the StereologyCross.groovy script over the QuPath interface
- 20. The new script should appear selected in the 'Script Editor' window
- 21. Select the 'Run' tab and click on 'Run'



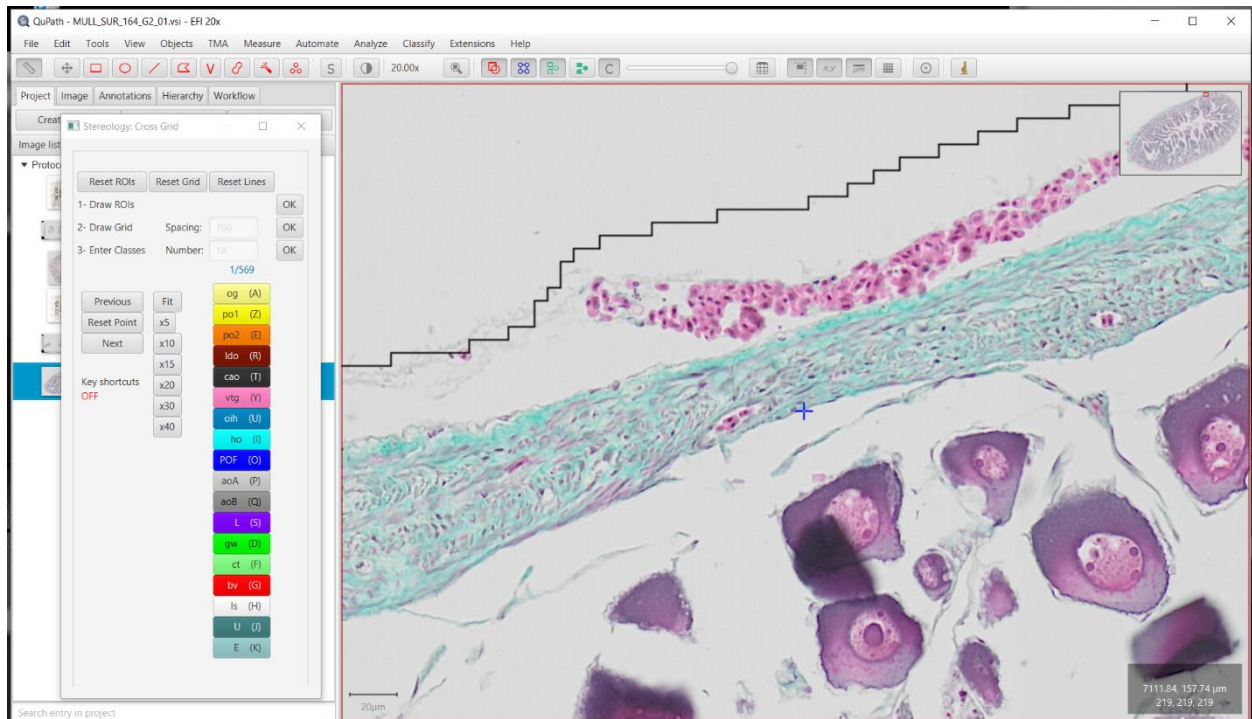
- 22. The 'Restart : ROI and Grid find' window will open
- 23. Click on 'Yes/Oui'



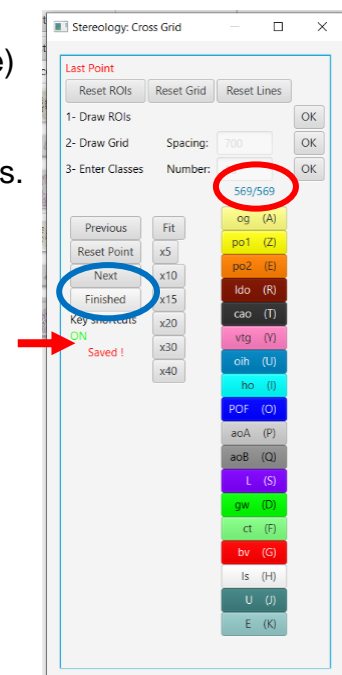
24. The 'Stereology Cross Grid' window will open and the picture interface will zoom in on the first reading cross
25. Select one cellular structure identified by the blue cross (Keyboard shortcut available)
26. Once a structure selected, the next cross will automatically become active



- 27. To go back to the previous cross, select the 'Previous' button in the 'Stereology Cross Grid' window, or use the keyboard left arrow
- 28. To go to the next cross, select the 'Next' button in the the 'Stereology Cross Grid' window, or use the keyboard right arrow
- 29. When a structure is selected, a colored dash will appear over the blue cross. To remove an answer, select the 'Reset Point' button in the 'Stereology Cross Grid' window

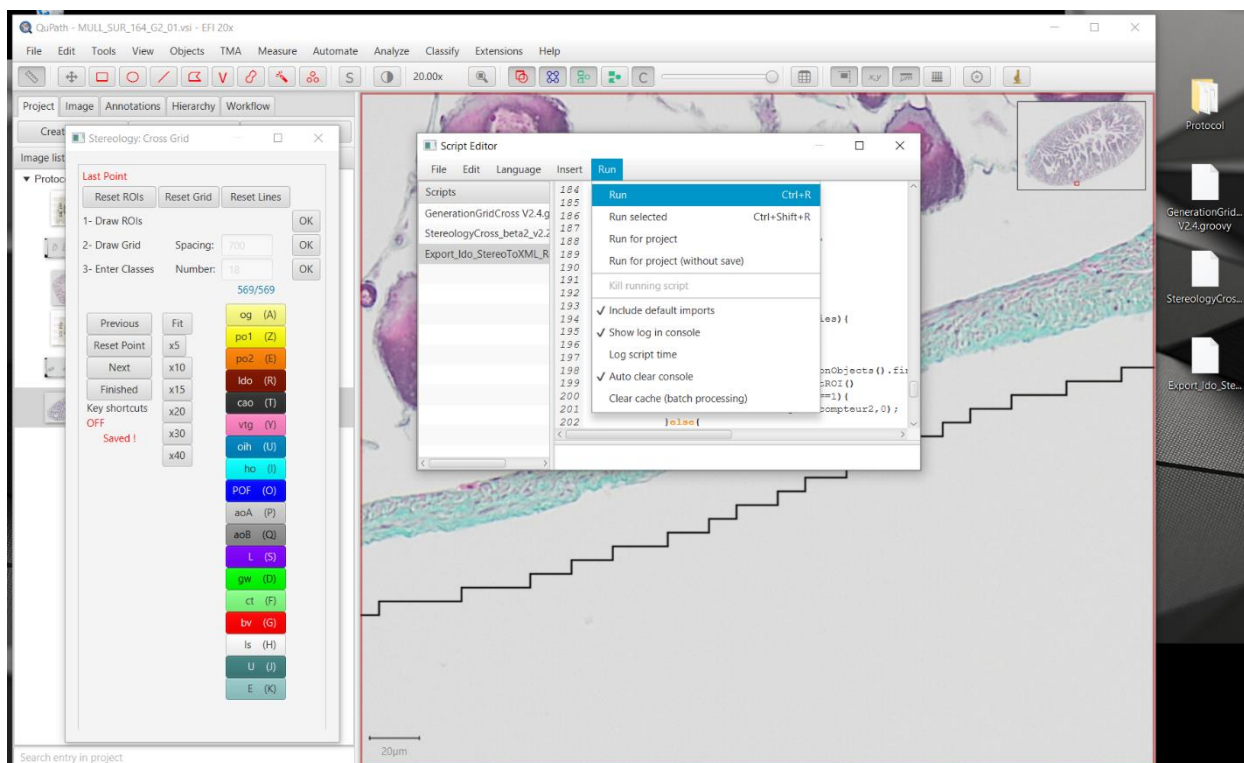


- 30. The number of crosses with an identified structure will appear in the 'Stereology Cross Grid' window (red circle)
- 31. Once all the crosses have been assigned a structure, a 'Finished' button will appear (blue circle)
- 32. Click on the 'Finished' button to save your reading results.
- 33. A 'Saved!' message should appear under (red arrow)

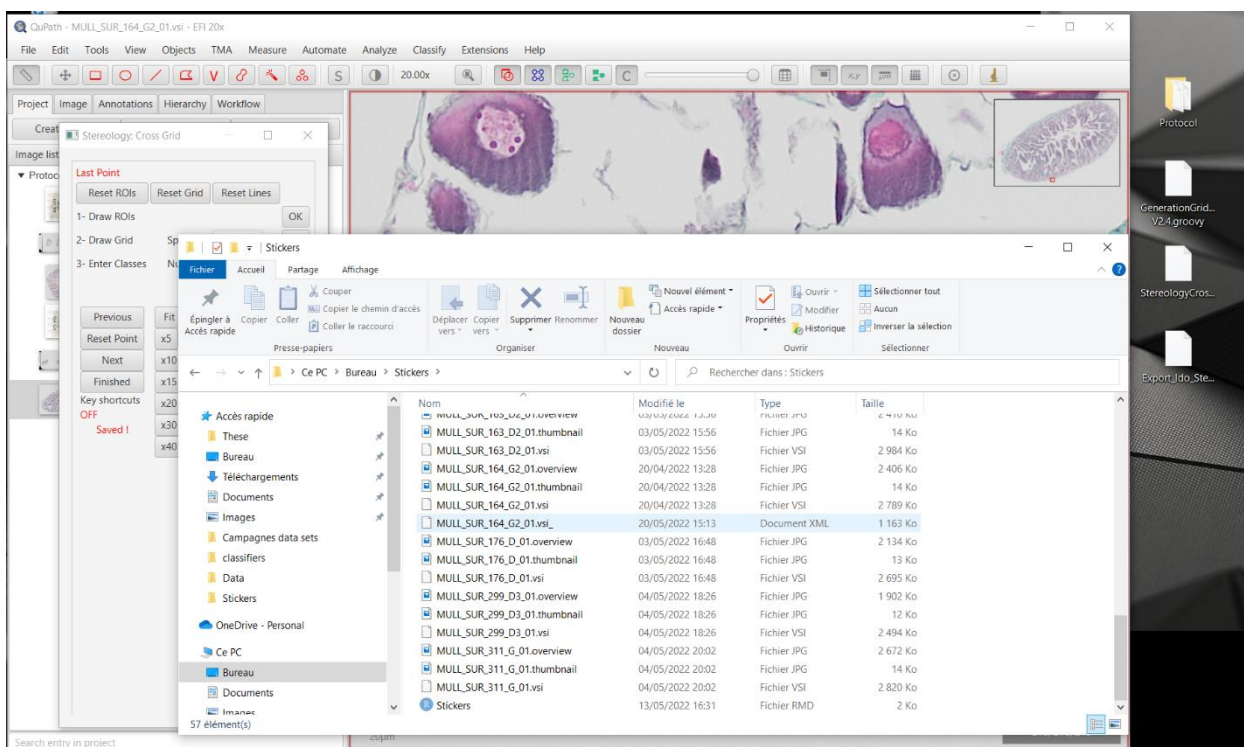


**34. Extract the reading answers and their coordinates :**

- Click and drag the 'Export\_StereoToXML.groovy' file over the QuPath interface
- The new script should appear selected in the 'Script Editor' window
- Select the 'Run' tab and click on 'Run'



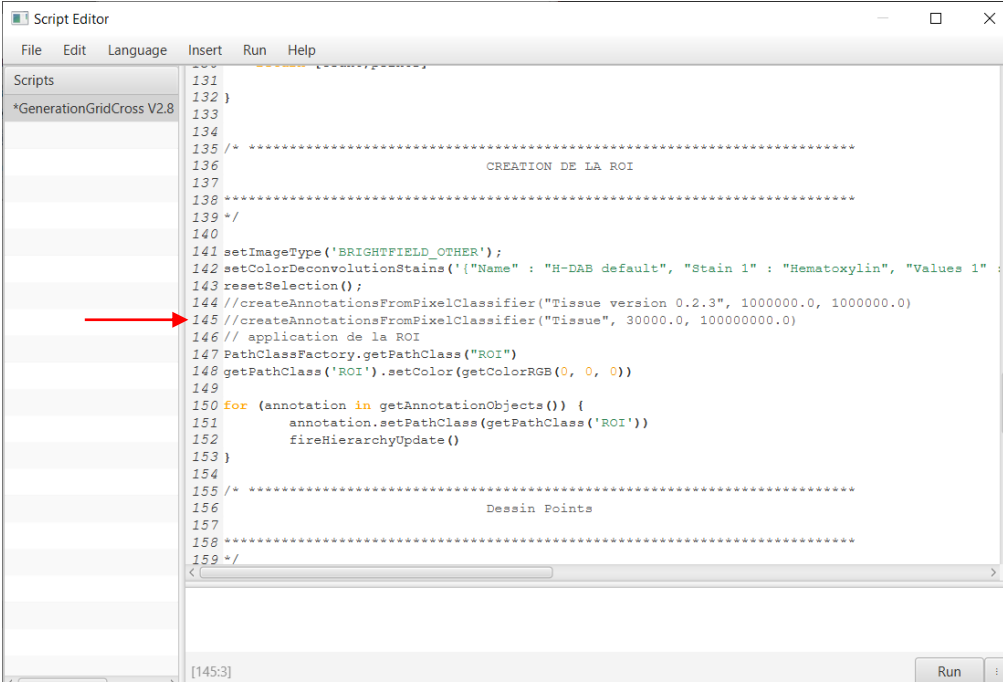
**35. An .xml document with the reading answers should have appeared inside the folder with the scans slides**





## Possible problems:

- **Step 18 message 'Erreur' appears in the Scrip Editor console.** The generation of the grid of crosses by the GenerationGridCross.groovy script can't be done. The outlined area is too small. To counter this, in the GenerationGridCross.groovy script, lower the values of TileSize (l.15) and the retraitUN (l.17).
- **A small isolated element is outlined but you don't want to have it taken into account in your counting grid.** The GenerationGridCross.groovy script sees it as an element big enough to include it in the counting area. To remove all elements of that size and lower, create a polygon annotation to get the volume area of the element, then change the first value of createAnnotationsFromPixelClassifier (l.145) into a slightly higher value than the area volume you just annotated.
- **A small isolated element isn't outlined but you want to have it taken into account in your counting grid.** The GenerationGridCross.groovy script sees it as an element that is too small to include it in the counting area. To include all elements of that size and higher, create a polygon annotation to get the volume area of the element, then change the first value of createAnnotationsFromPixelClassifier (l.145) into a slightly lower value than the area volume you just annotated.
- **White area inside of the section isn't taken into account and you want to quantify it.** The GenerationGridCross.groovy script sees it as an area too big and devoid of elements and doesn't count it as the part of the outlined section. To take into account the large white areas in the center of a closed off section, increase the second value of createAnnotationsFromPixelClassifier (l.145).
- **The GenerationGridCross.groovy script can't outline the desired area or outlines only parts of the histological section.** The histological section you are trying to outline is most likely not closed off, with scattered elements surrounded by white areas. You will have to manually outline the area by creating a closed annotation (□ ○ ◀ ▶). Then, in the GenerationGridCross.groovy script, comment the line generating the outlined area (createAnnotationsFromPixelClassifier l.145) by adding '//' at the beginning of the line. This will add the counting grid (blue crosses) in the manually outlined area.



```

131
132 }
133
134
135 /* .....
136
137
138 .....
139 */
140
141 setImageType('BRIGHTFIELD_OTHER');
142 setColorDeconvolutionStains(['Name' : "H-DAB default", "Stain 1" : "Hematoxylin", "Values 1" :
143 resetSelection();
144 //createAnnotationsFromPixelClassifier("Tissue version 0.2.3", 1000000.0, 1000000.0)
145 //createAnnotationsFromPixelClassifier("Tissue", 30000.0, 100000000.0)
146 // application de la ROI
147 PathClassFactory.getPathClass("ROI")
148 getPathClass("ROI").setColor(getColorRGB(0, 0, 0))
149
150 for (annotation in getAnnotationObjects()) {
151     annotation.setPathClass(getPathClass("ROI"))
152     fireHierarchyUpdate()
153 }
154
155 /* .....
156
157
158 .....
159 */

```

### 3. Reading rules summary

#### Follicle identification

- **See lexicons for follicle descriptions**
- Doubt between 2 follicle types? **Check cell size**. Still in doubt? **U**
- No nucleus for **po1**, **po2**, **ldo** and **cao**? Classify under **U**
- No nucleus for **vtg**, **oih** and **ho**? **Identify the follicle**

#### Sample cross location

- 75%+ of the color? **Identify that structure**
- 50%-50% color? Prioritize the **colored structure**
- 50%-50 between 2 different follicles? **U**
- Cross on a theca? **Identify the follicle**
- Cross in a “white space” between a disconnected theca and its follicle? **Identify the follicle**
- Cross inside an empty but complete theca (the ‘inside’ of the cell has been ripped away)? **U**
- Cross near an incomplete theca? **Is can be used**. Still in doubt? **U**
- Less than 50% of the inside of a **vtg** or **ioh** follicle? Classify under **U**
- 2 structures overlap? **Identify the under most structure**. Still in doubt? **U**

#### Gonadal wall (gw)

- Cross between two layers of **GW** cells? Classify under **GW**
- Cross in the **GW** on muscle tissue? **GW**
- Cross in the **GW** on a blood cell or inside a blood vessel? Classify under **bv**
- Cross section is inside out with the **GW** in the middle? **GW for the entire zone**
- **GW** in a discolored zone? Classify under **GW**

#### Connective tissue (ct)

- Cross between a theca and **ct**? **Identify the follicle**
- Cross on a **ct** surrounding a **bv**? Classify under **ct**

#### Blood vessels (bv)

- 50%-50% on a **bv** and **ct**? Classify under **bv**
- Cross in a **bv** in the theca of a follicle? **Identify the follicle**
- Cross in the white of a blood vessel? Classify under **bv**
- Discolored blood cells? Classify under **bv**. Still in doubt? **U**

#### Intercellular space (is)

- “White space” between structures (outside of complete thecas or between **ct** pieces)? **Is**
- When the **GW** is broken and follicles are ‘out in the open’? Classify under **Is**
- When the **GW** is broken and follicles are ‘on the other side of the GW’? **Is**
- **GW** disconnected from the lamellas? **Is between GW and inner cellular structures**

#### Unnatural emptiness (E)

- **GW** is unbroken? **E between the last layer of GW cells and the sample outline**
- **GW** is broken with no follicles ‘outside’? Classify under **E**

#### Undetermined (U)

- Blurry structure? Classify under **U**
- Structure not found in the Lexicon? Classify under **U**
- Structures/Follicles in a discolored area? Classify under **U**
- Discolored area in the ovarian light? Classify under **U**
- **When in doubt, classify under U**

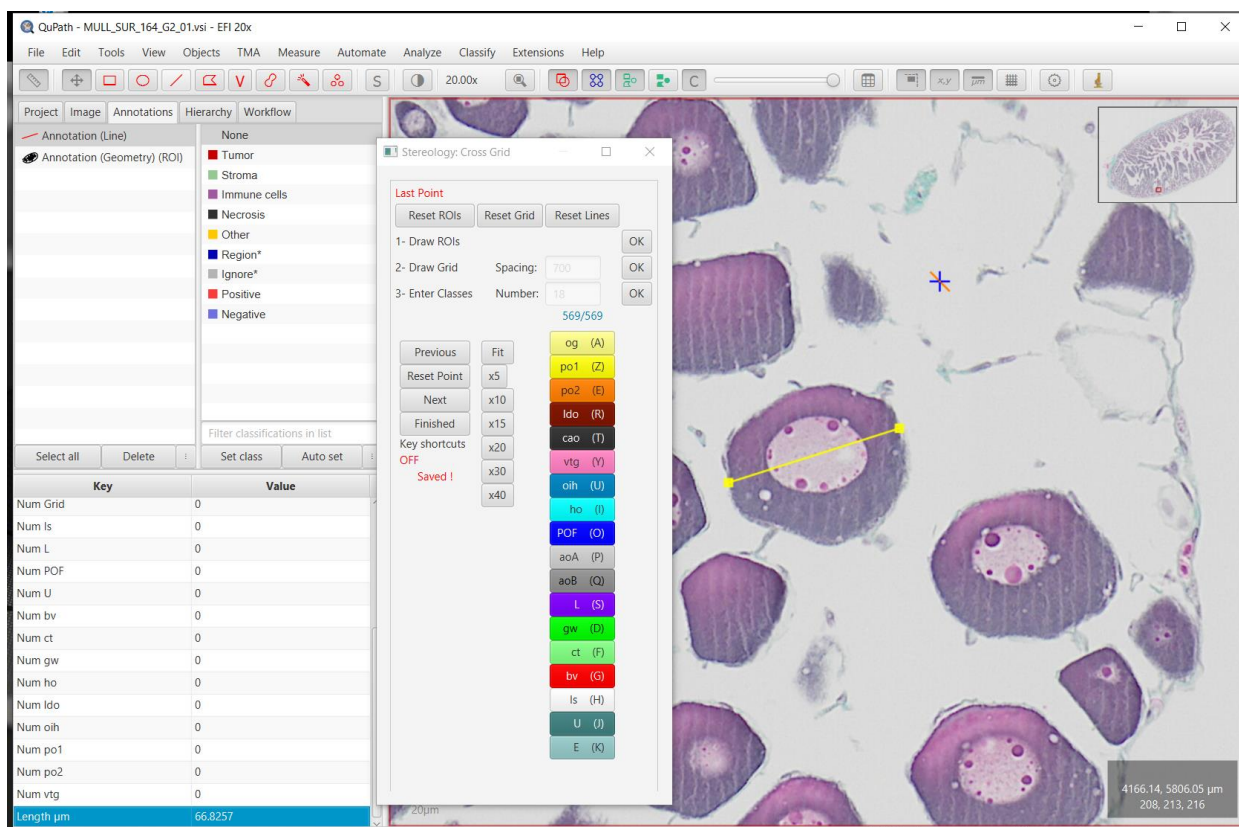


## 4. Structure identification

The list of the different cells and structures potentially present inside the ovaries are described within lexicons established for each individual species and may be found on the [Archimer](#) website. These lexicons allow the identification of the different cell types found throughout fish ovaries and are illustrated with pictures of « perfect looking cells ». Albeit, in a histological slide the presence of artefacts, deformed structures and poorly cut cells (no visible nucleus) are recurring problems.

### 4.1. Measuring cells

Other than the lexicons, in order to properly ascertain the different cell types, this software can be used to measure the structure that is to be identified. If the reader hesitates between two different cell types (like between **po1** and **po2**), it is possible to measure the oocyte in order to settle his choice.



### 36. Measuring a structure in QuPath :

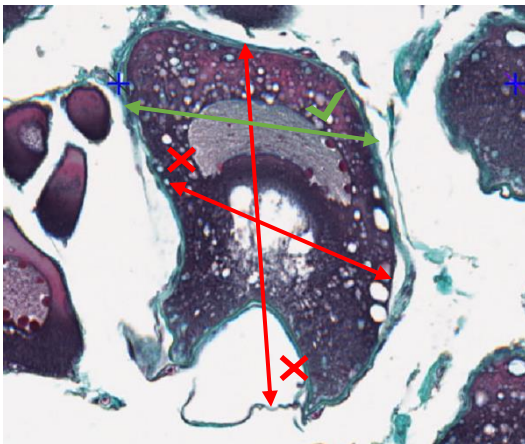
- Select the 'Line' icon (5<sup>th</sup> icon from the left in the top tool bar)
- Draw a line over the structure
- Select the 'Annotations' tab
- Scroll down to find the 'Length µm' row



The measurement of a follicle goes

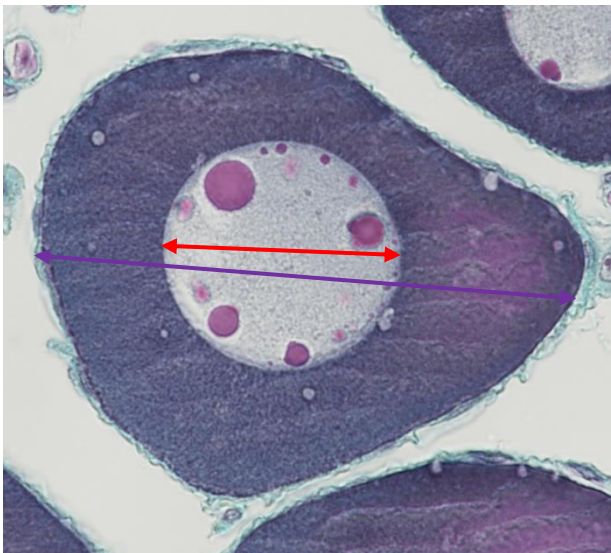
- **straight across the cell**
- from the **exterior membrane of the theca** to the other exterior membrane of **the same theca**
- while **passing over the nucleus**

If the follicle is distorted (ripped or stretched out), **do not measure in the direction of the distortion**



Measuring the cell can help with categorizing the cell type.

To measure a cell, go from the exterior cell layer of the theca to the opposite exterior cell layer of the theca, passing by the nucleus (center of the follicle). Avoid distorted, stretched or severed parts of the follicle

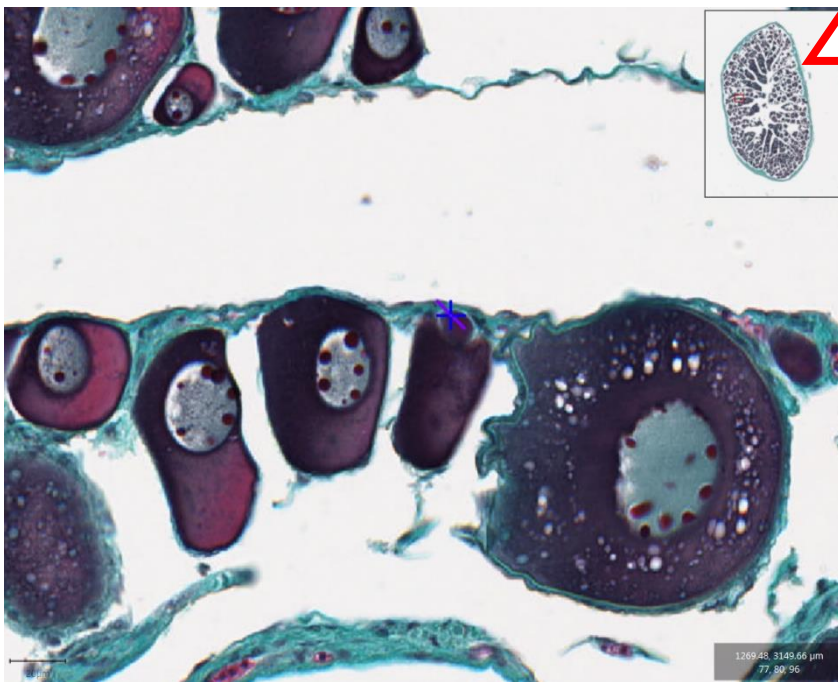


To measure the nucleo-cytoplasmic ratio, compare the diameter of the nucleus (red) with the diameter of the cytoplasm (purple).

Nucleus diameter is superior to 50% of the cytoplasm's diameter > **og** or **po1**

## 4.2. Follicle identification

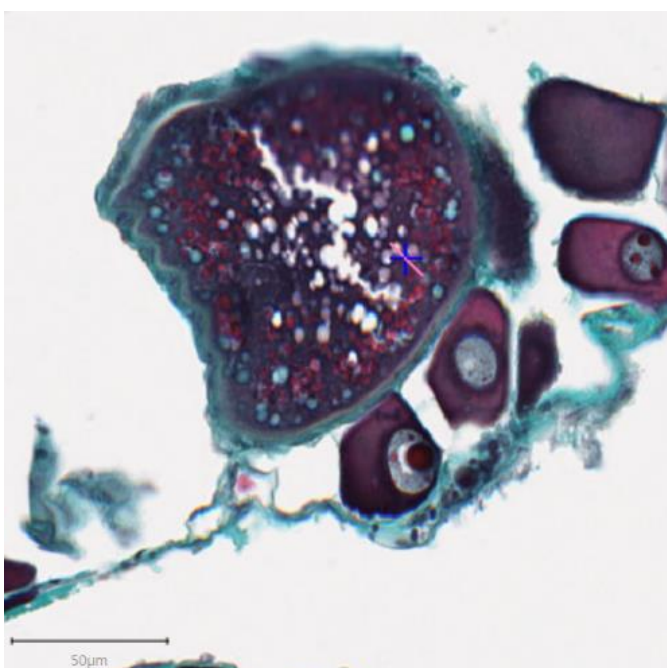
For a detailed description of the follicles and other cellular structures that may be found within the ovaries of a fish species, refer to the lexicon of the studied species. Lexicons for species studied under the **MATO** project may be found on the [Archimer](#) website.



Cross on a follicle with no visible nucleus. Put this cross in the **U** category.

Put into the **U** category the following follicles that do not have a nucleus : **po1**, **po2**, **ldo** or **cao**

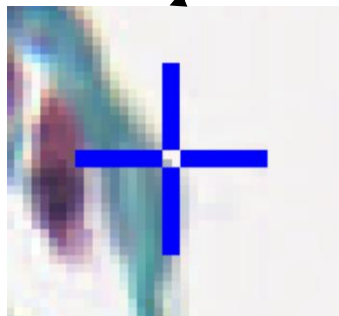
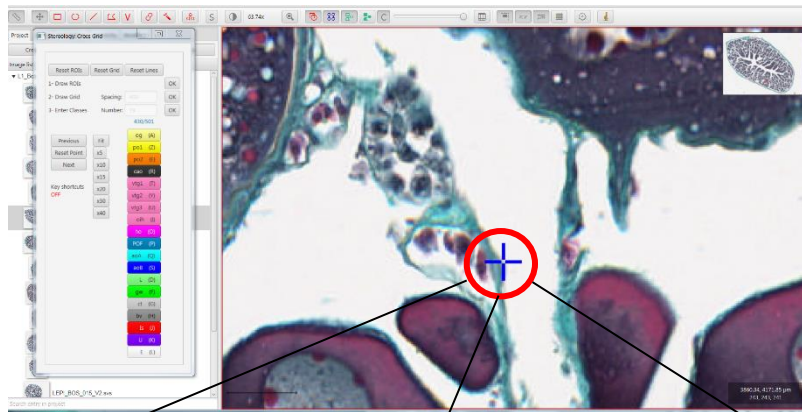
Measuring a follicle can help determine the cell type. If there is a doubt, mark the structure in the **U** category



The cross is on a follicle with no nucleus, but the green **zona pelucida** and presence of **vitellus droplets** confirms that this is a **vtg1**. Place this cross in the **vtg1** category

### 4.3. Sampling cross locations

The structures are identified by a blue cross ( + ), with a four pixel zone in its middle. These crosses are equidistant from one another, and the starting point of the grid is randomized at its creation. Each gonad has a sampling grid of 500 to 600 crosses. The center of the cross, on a 4 pixel-precise scale, is used to point out the structure that is to be identified.



Zoom to the center of the cross.  
 Colored pixels :  
 75% white, 25% colored

The white structure dominates. Put the cross in the **Is** category



Zoom to the center of the cross.  
 Colored pixels :  
 50% white, 50% colored

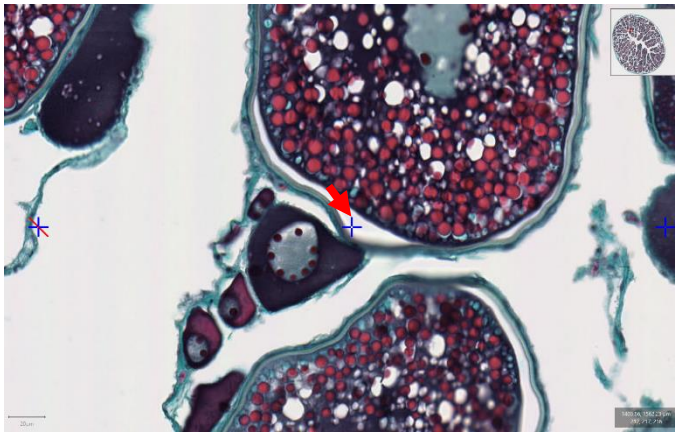
Always favor the colored structure, not the **Is**. Put the cross in the **ct** category




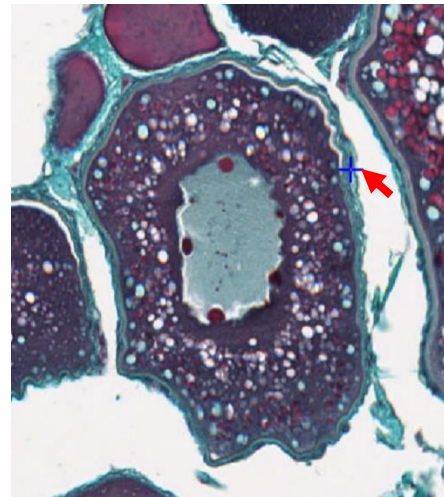
Zoom to the center of the cross.  
 Colored pixels :  
 25% white, 75% colored


The colored structure dominates. Put the cross in the **ct** category

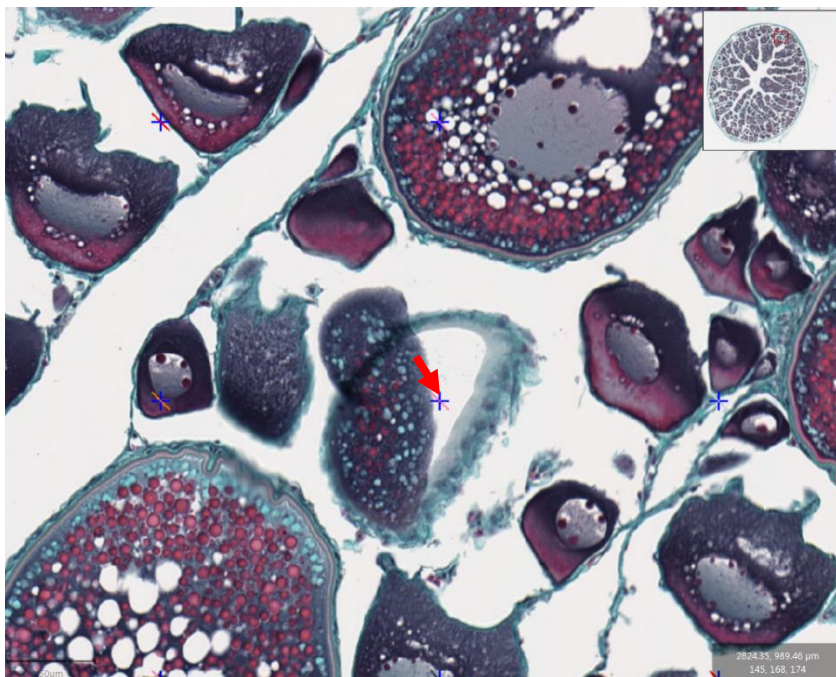
For this study, we will identify the **follicles**. This means that we will take into consideration the **oocyte** and its somatic tissue. If the center of a cross falls on the **theca** of an **oocyte**, we consider that the structure that is to be identified is the **oocyte encased inside this theca**.




Cross (  ) in a “white space” between the **zp** and the **theca** of a **vtg1** follicle. Put this cross in the **vtg1** category

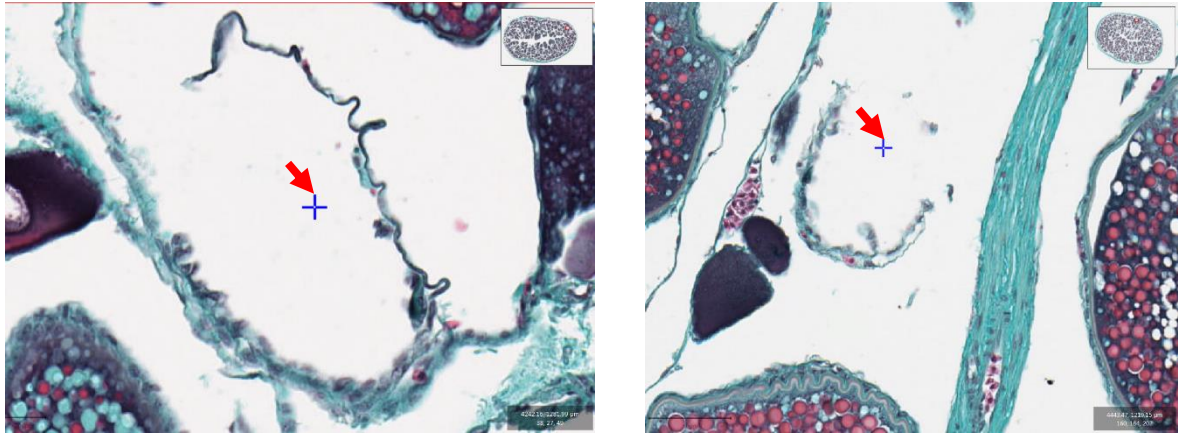


Cross (  ) on the **theca** of a **vtg1** follicle. Put this cross in the **vtg1** category

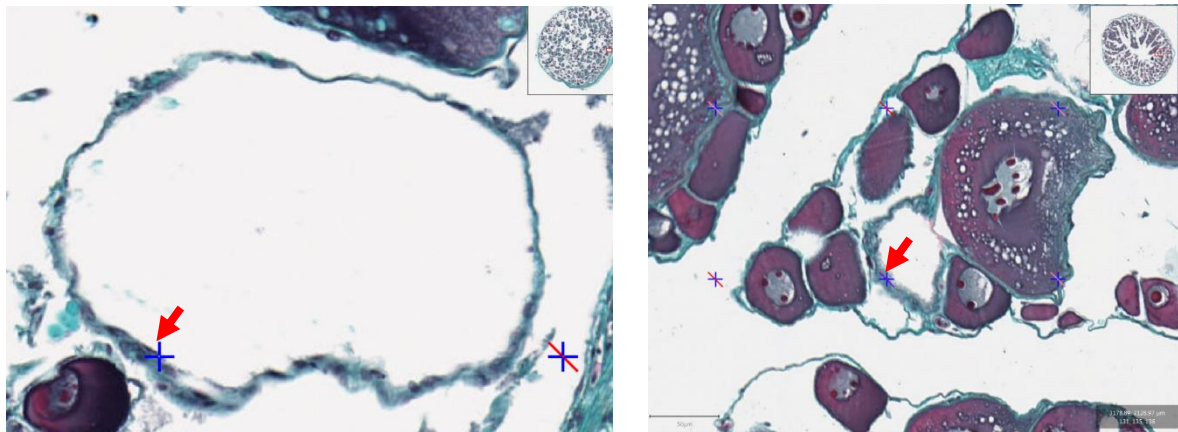


This cross (  ) falls on “white space” left by the shift of the cellular contents of the follicle. In this case, the cellular contents are still attached to the follicular cells (**ct + theca**), allowing the reader to identify the follicle correctly (presence of **vitellus droplets**). Put this cross under the **vtg1** category

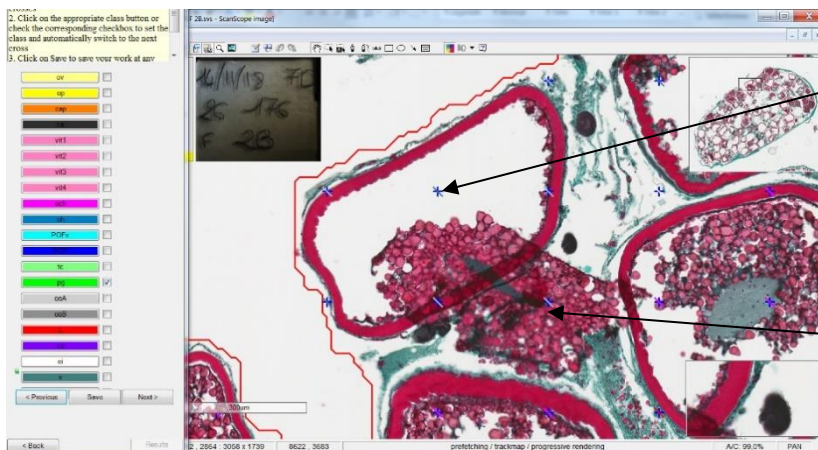
Moreover, if the cross falls between the **theca** and the **zona pellucida** (with the **theca** detached from the **zona pellucida**), we still identify the cross as being on the **follicle**.



Cross (↘) near an incomplete and broken piece of **theca**. Put this cross in the **Is** category. In doubt, put **U**



Cross (↘) on (or in) a complete **theca**. The oocyte was ripped away during the mounting, making it impossible to determine the cell type. Put this cross in the **U** category

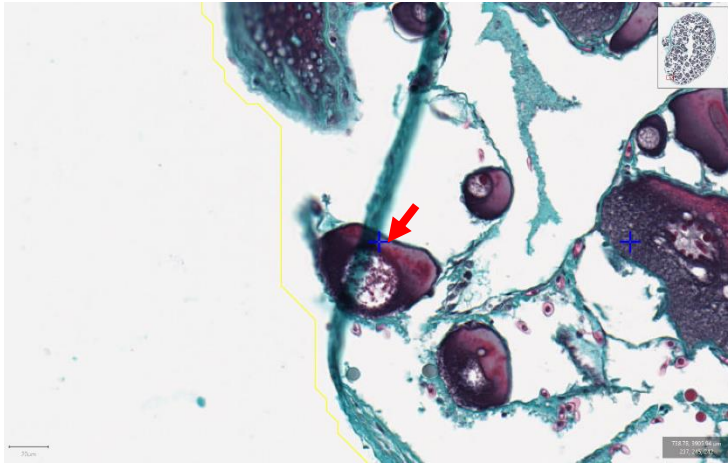


Cross inside a follicle with the **vd** that have started to shift out of the theca. Put this cross in the **vit4** category

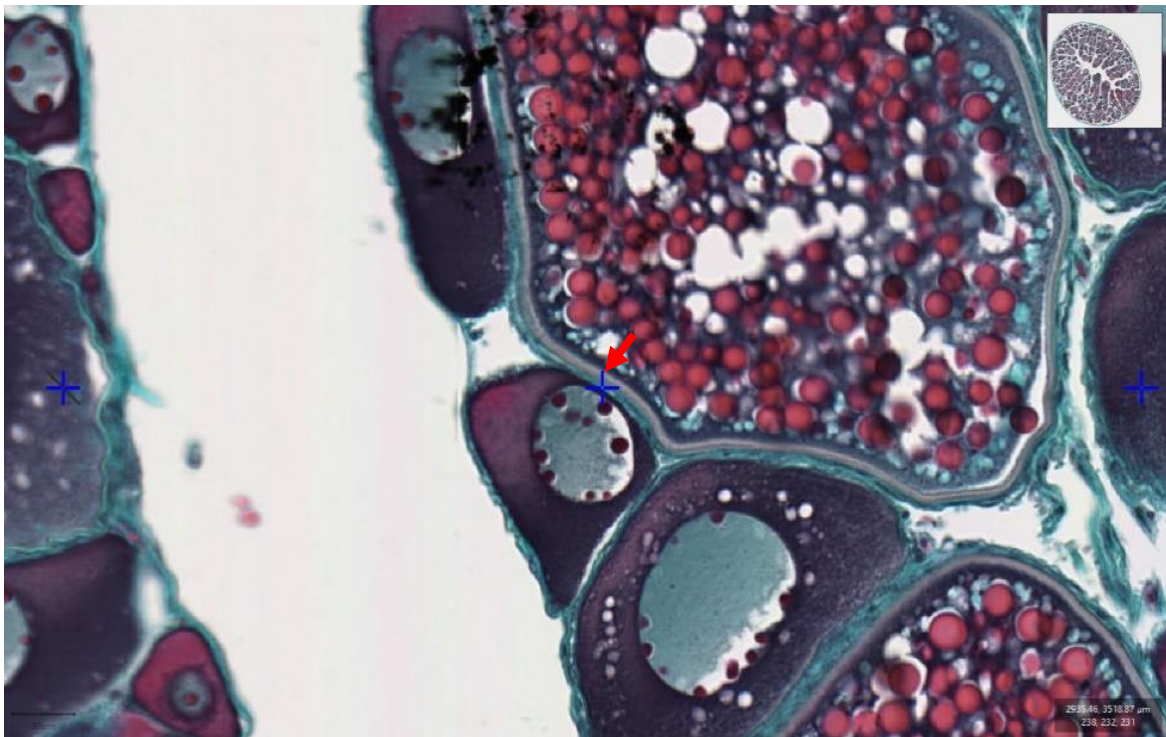
Cross outside of the follicle's theca, but inside the shifted **vd**. Put this follicle in the underneath most category : **Is**



If a cross falls on a structure that is **covered entirely or partially by another structure**, (a cell may have been dislocated or the gonadal wall may have been folded over oocytes), **the structure to identify will be the one most underneath**.

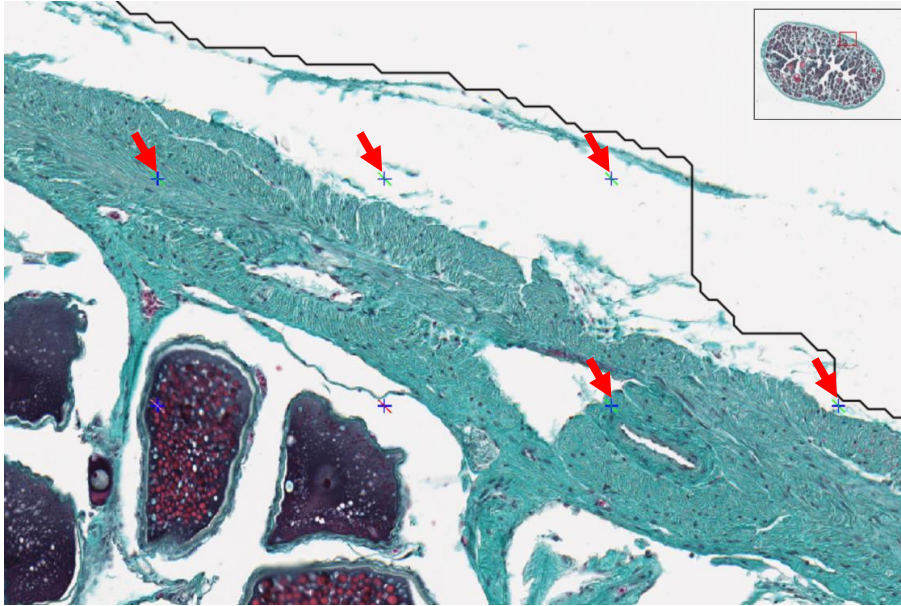


The cross (↙) falls on the gonadal wall (gw) that overlaps a **po2** follicle. In this case, identify the underneath most structure. Put this cross under **po2**



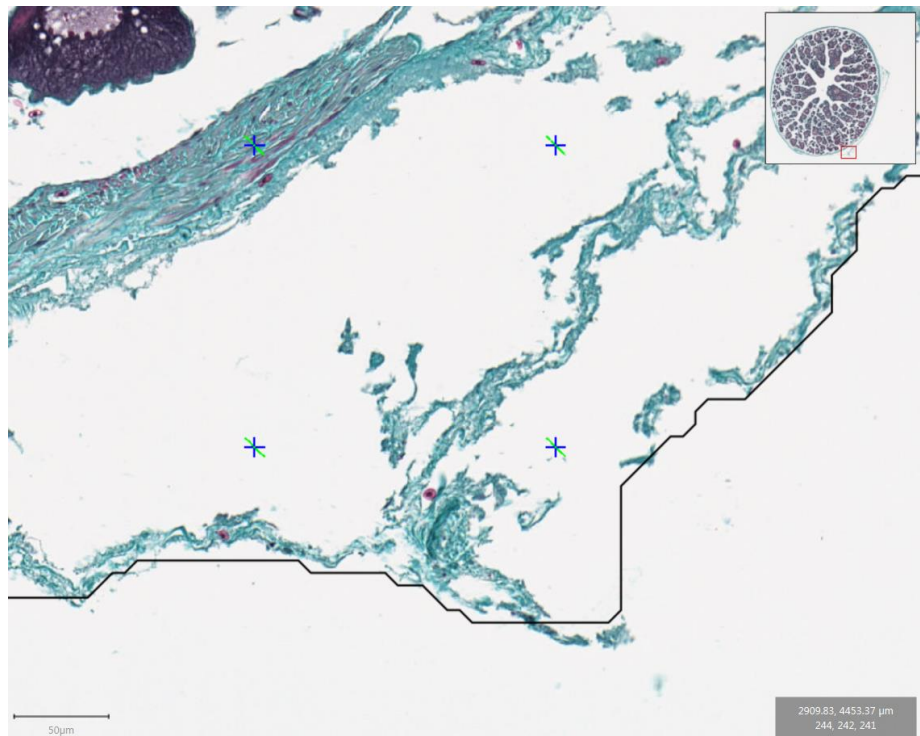
If a cross falls between two different follicle types (↙), place it under **U**

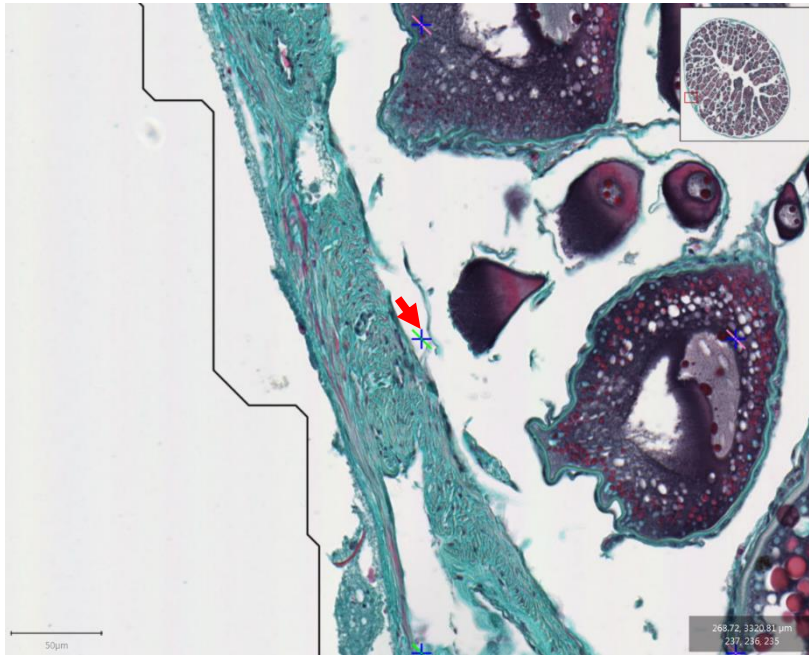
#### 4.4. Gonadal wall (gw)



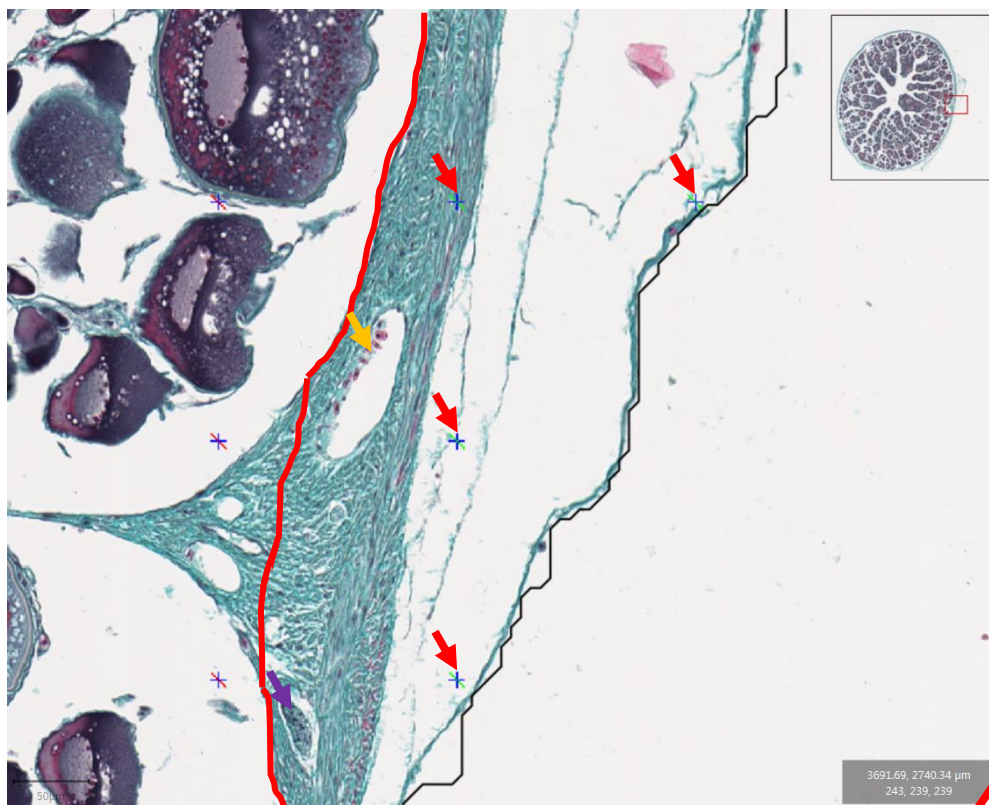
Crosses (↘) on the **gw**. The top right two crosses are under a layer of cell from the **gw**, so even if they are in a « white zone » they will be categorized as **gw**

All the crosses here are between the external and internal cell layer of the **gw**. Put all of these crosses into the **gw** category

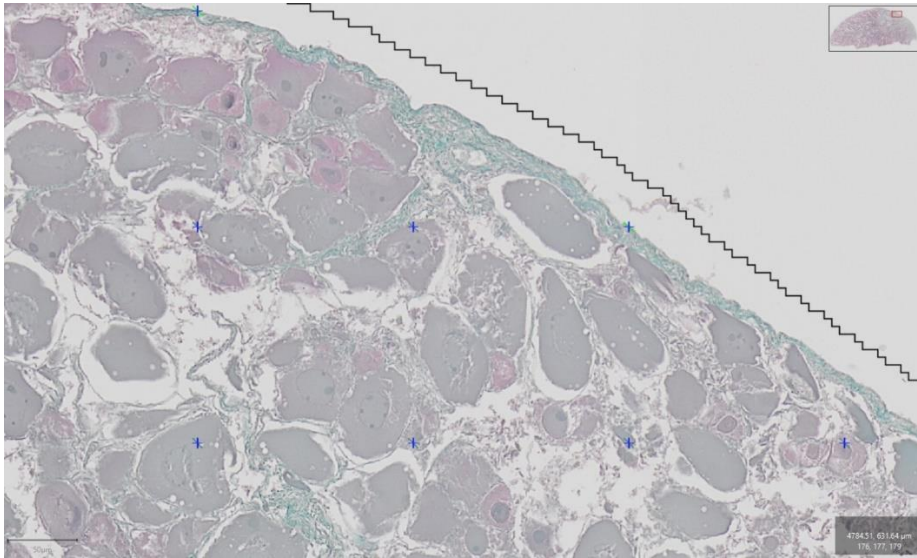




Cross (↘) under the inner most cell layer of the gw.

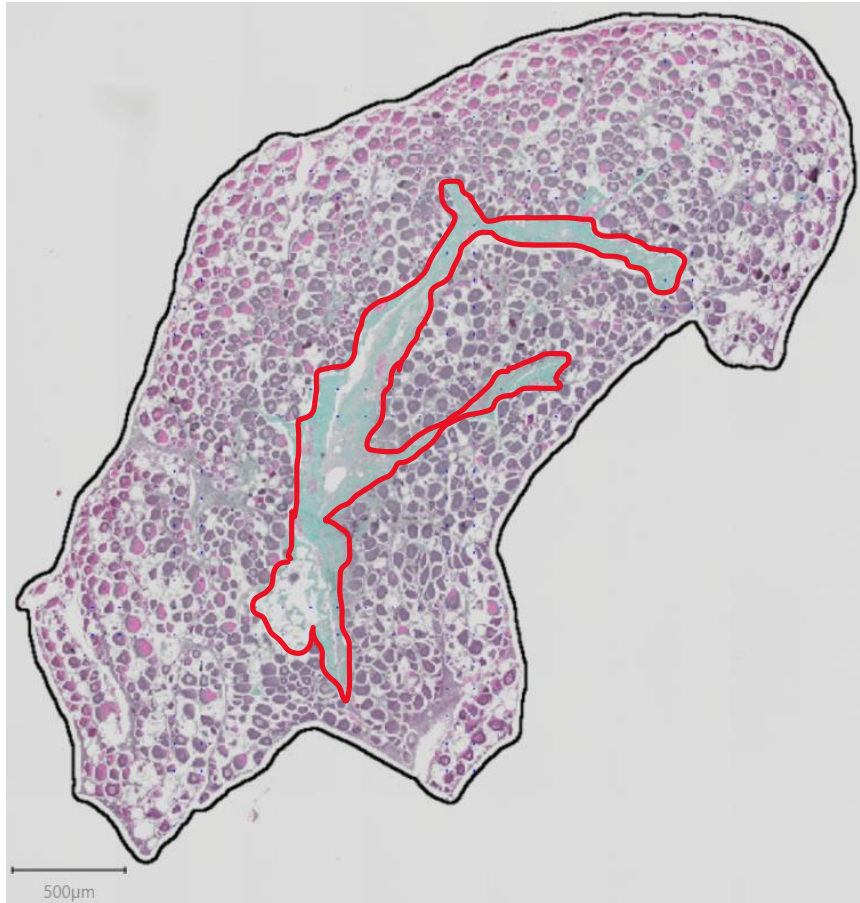


The ovarian lamellae are connected to the gonadal wall (gw) by connective tissue (ct). The gw ends where the ct enters the lamella. In this example, the gw is delimited by the red line. Crosses (↘) on gw tissue. If a cross falls on a muscle tissue (↙) inside the gw, put the cross in the gw category. If a cross falls on a blood vessel (bv / ↘) inside the gw, put this cross in bv category



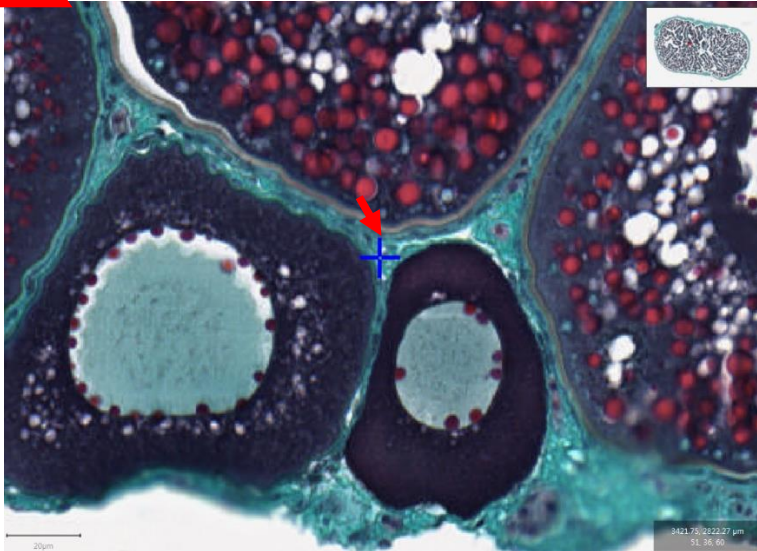
The area is discolored, but the **gw** will be properly visible and colored in green. Even in a discolored area, identify the **gw** if a cross falls on it.

The sample was flipped inside out, and the gonadal wall is now 'on the inside'. All crosses that fall in the area (outlined in red) should be classified under **gw**





### 4.5. Connective tissue (ct)



The cross ( ) is on **ct**, between the **thecas** of the follicles. Put this cross in the **ct** category.

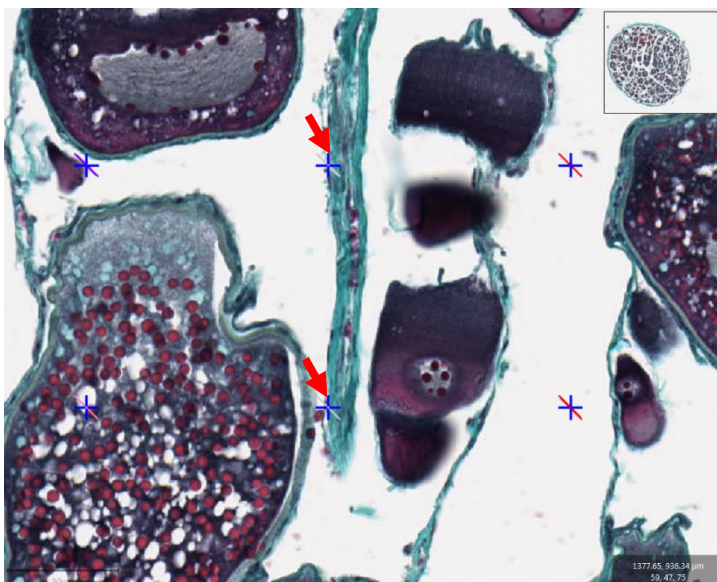
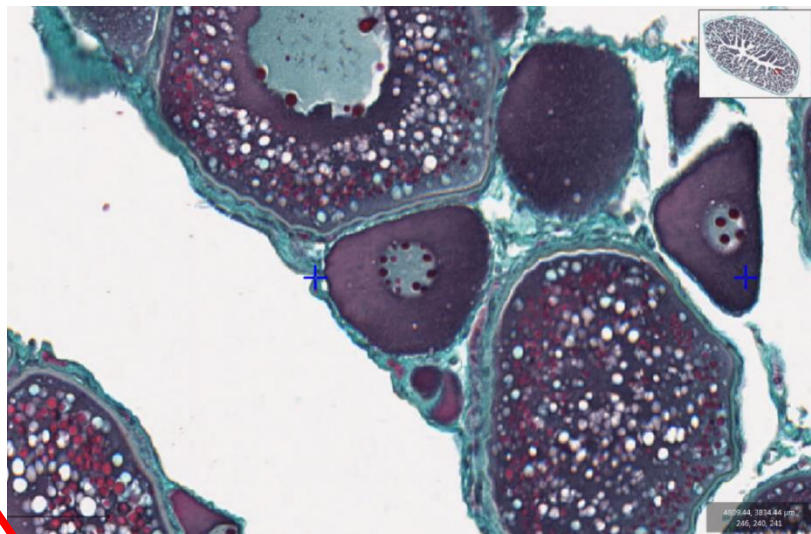
If it can not be determined whether the cross is on **ct** or on the **theca**, favor the follicle.

If it can not be determined where the cross falls when it is between two cells at different stages, put the cross under the **U** category

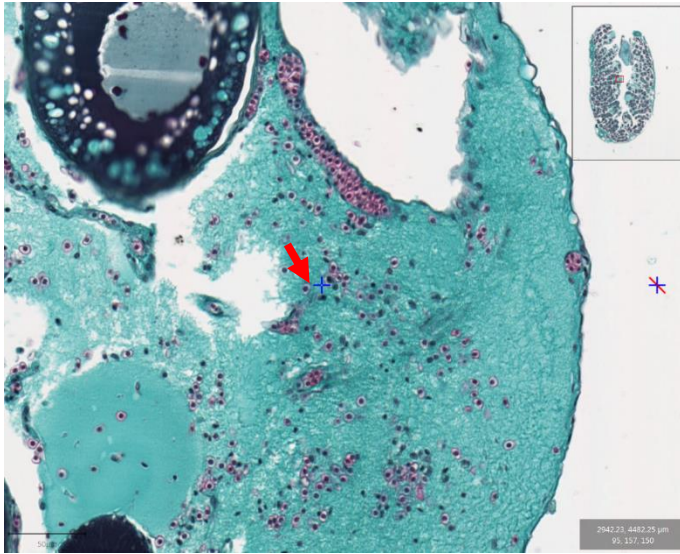
Do not confuse **ct** with the **theca**!

Here the cross is on the germinal epithelium that surrounds the cells into the ovarian lamella.

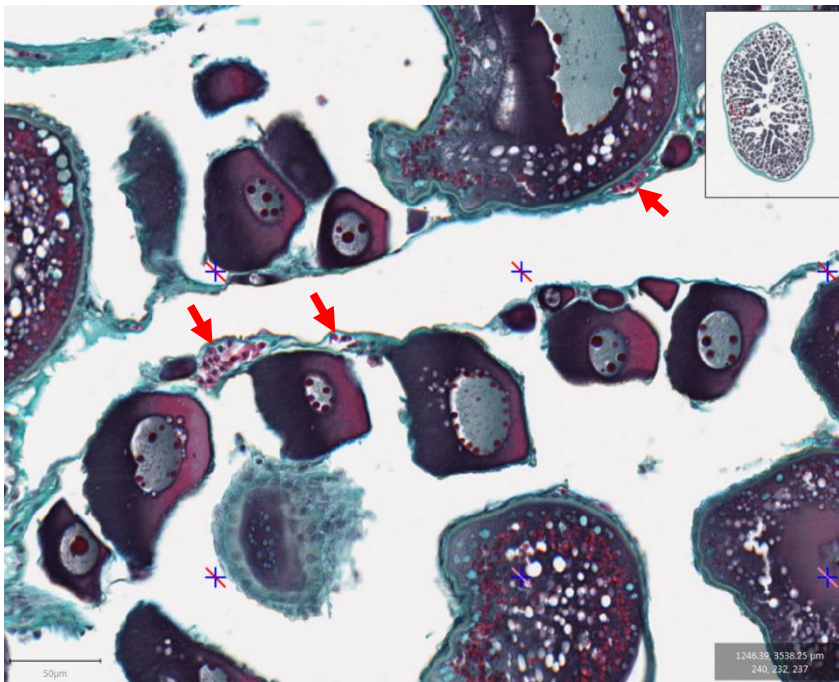
**og**, **po1** and **po2** follicles have a **theca**. **cao** and vitellogenic follicles have a **zp** and a **theca**. Put this cross in the **ct** category



These crosses ( ) are on **ct**, near blood cells. If a cross had fallen on a blood cell, the category **bv** would have been selected. Here this is not the case, put these crosses in the **ct** category



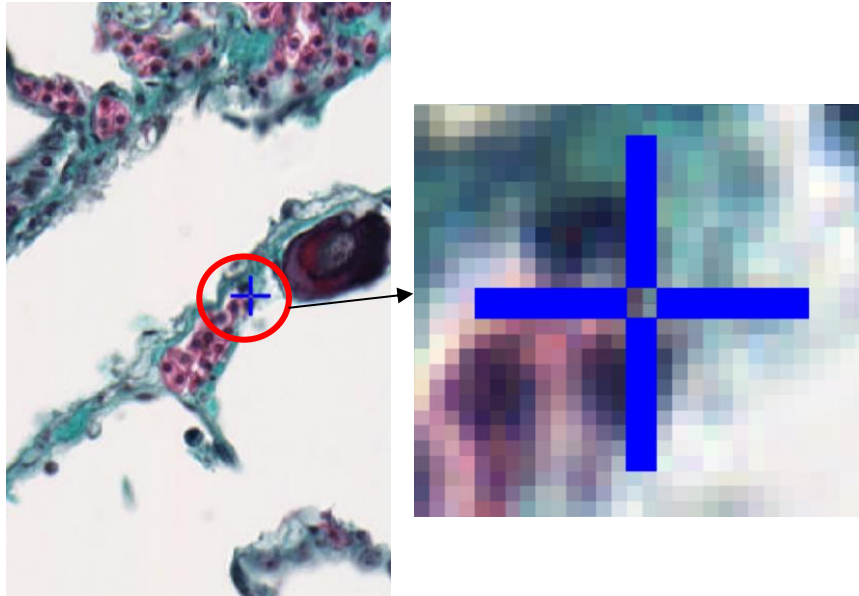
The cross (↯) is on **ct**, between blood cells. The blood cells seem to have been scattered within the lamella during the slide mount. If the cross had fallen on a blood cell, the category **bv** would have been selected. Here this is not the case, put this cross in the **ct** category



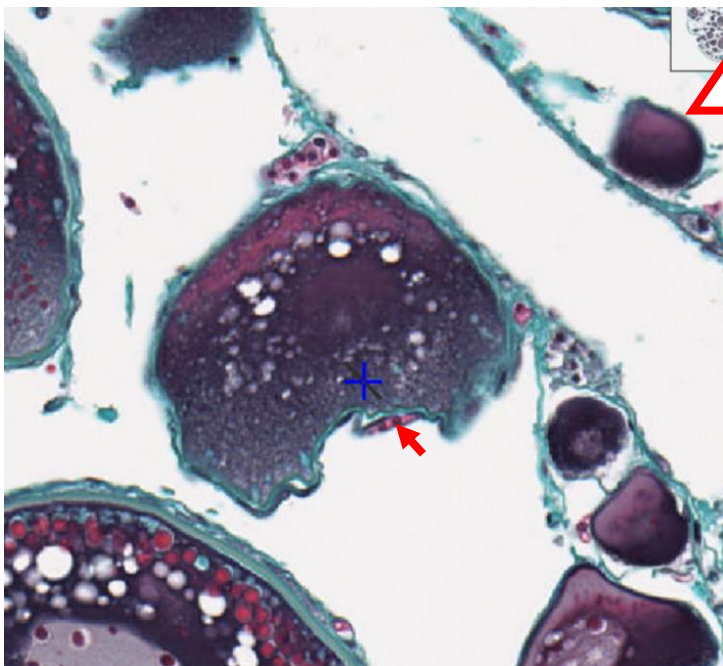
Blood vessels in **ct** (↯). If a cross falls on these, put it in the **ct** category

## 4.6. Blood vessels (bv)

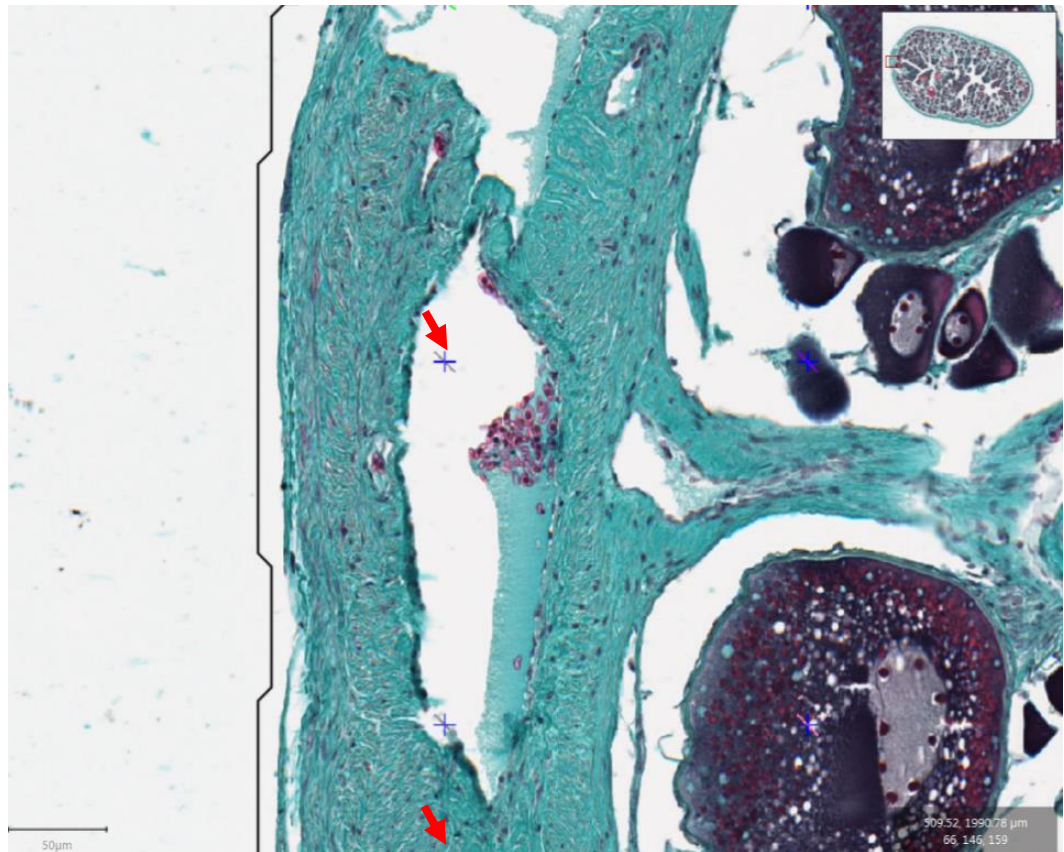
**bv** = blood capillaries and/or blood vessels and/or blood cells



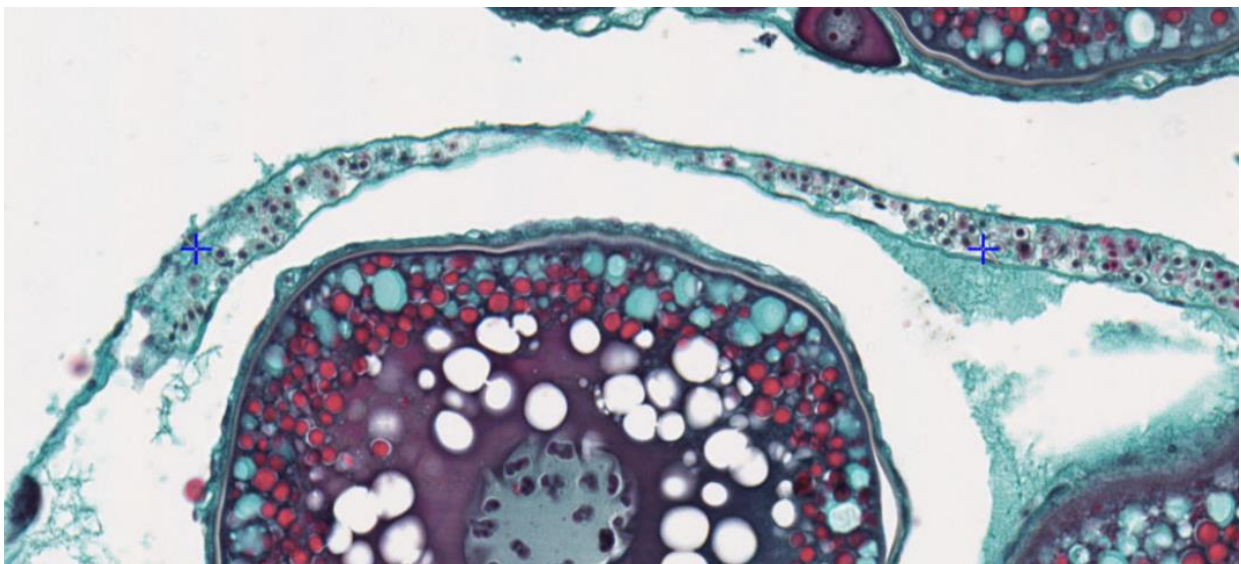
Cross on the edge of a blood vessel.  
When zooming in, the cross has 50% red and 50% green.  
Because the green can be the cell tissue forming the capillary vessel, it is considered part of the **bv** structure, not **ct**. Put this cross under the **bv** category



Blood vessel in the theca of an oocyte (↘). If a cross falls on this **bv**, classify it under the germinal cell's (**cao**) category



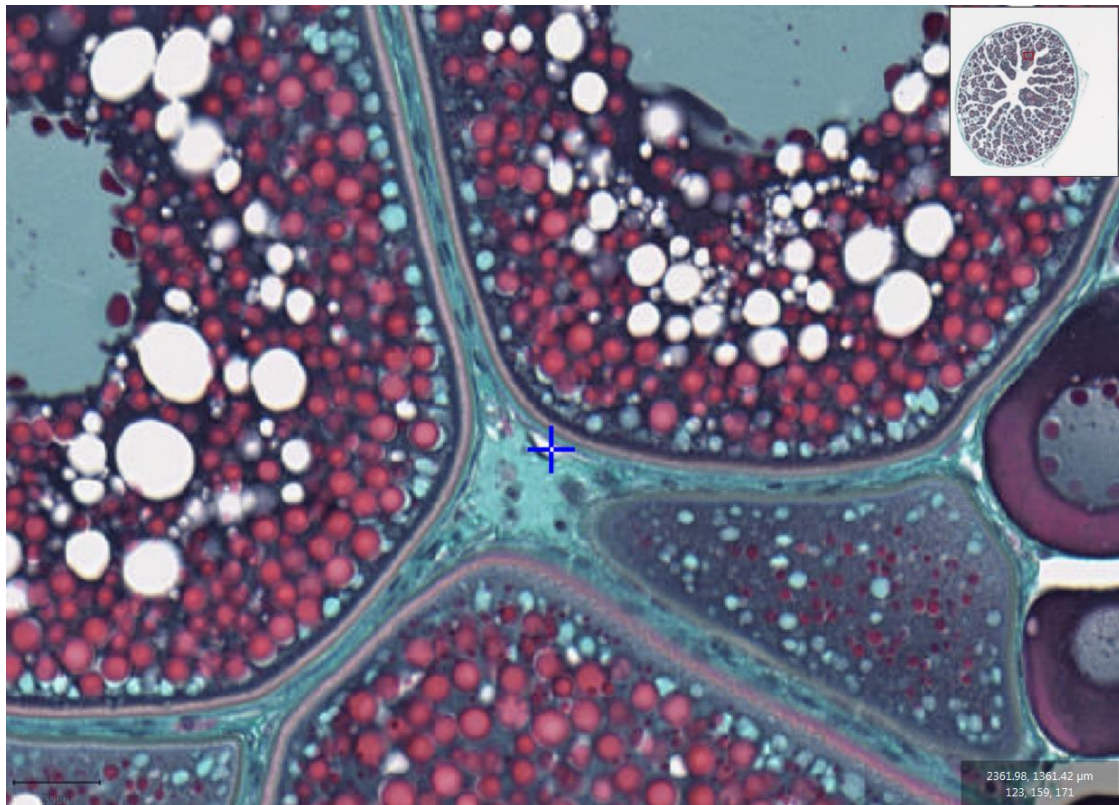
Crosses (↘) in a blood vessel in the **gw**. Put these crosses in the **bv** category



Two crosses in a blood vessel in **ct**, with blood cells that were partly colored. Put this cross in the **bv** category. If there is a doubt, these crosses may also be placed under the **U** category



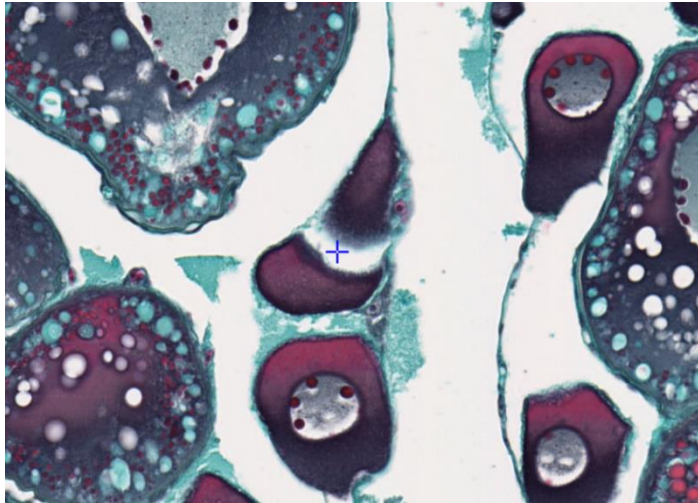
## 4.7. Intercellular space (Is)



Refer to the center of the cross (4 colored pixels) to determine if the cross is over **ct** or **Is**.

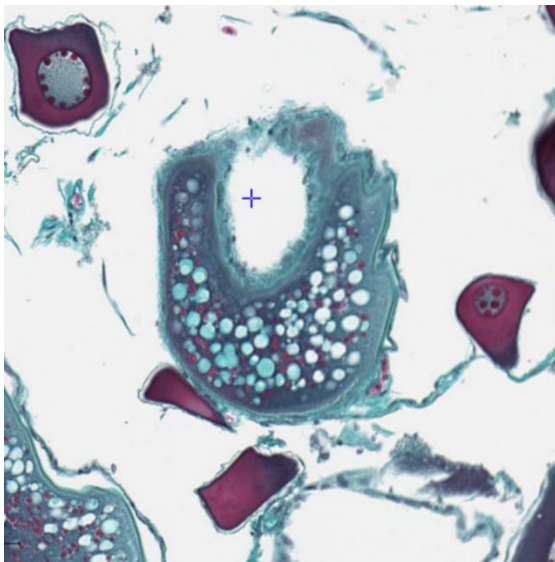
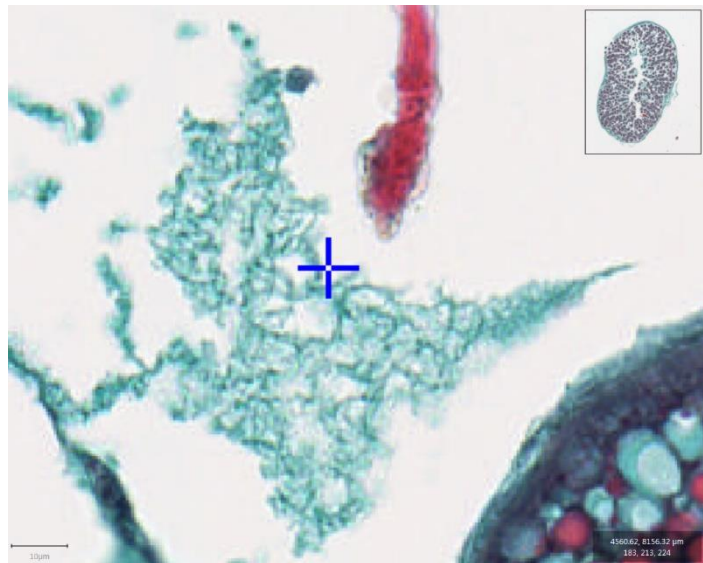


The cross seems surrounded by the **theca** and **ct**, but the **ct** is distorted and the cross falls on a “white zone”. Put this cross in the **Is** category. If there is a doubt, place the cross in the **U** category



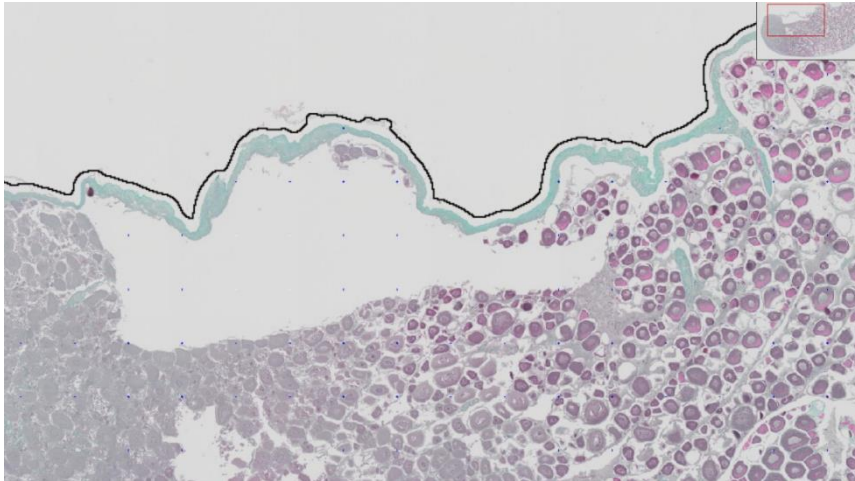
The cross lands between two structures. Put it in the **Is** category

Refer to the center of the cross (4 colored pixels) to determine if the cross is over **ct** or **Is**. Put this cross in the **Is** category

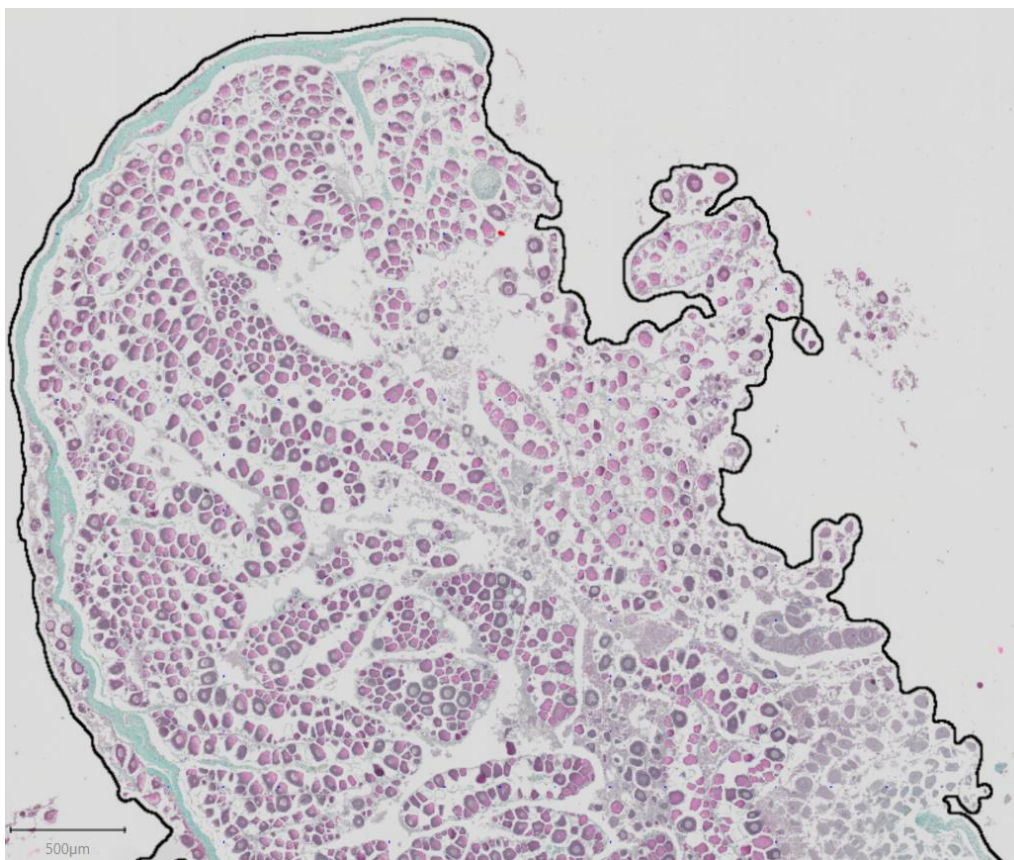


The cross seems surrounded by the theca, but the oocyte is distorted and the “white zone” the cross is in is not an area where the organelle have been ripped away, it is over **Is**. Put this cross in the **Is** category. If there is a doubt, this cross may be placed in the **U** category





The **gw** has been disconnected from the rest of the inner structures. All crosses that fall in this 'white area' that is still inside the gonad are to be classified under the **Is** category



When the **gw** is broken and cellular structures are 'pouring out':

- Classify all follicles, even the ones that are on the other side of the **gw**.
- All 'white space' between the follicles and gw are to be classified under **Is**. There will be no **E** category in this case

When the **gw** is broken but there are no follicles between the sample boundary and the **gw**:

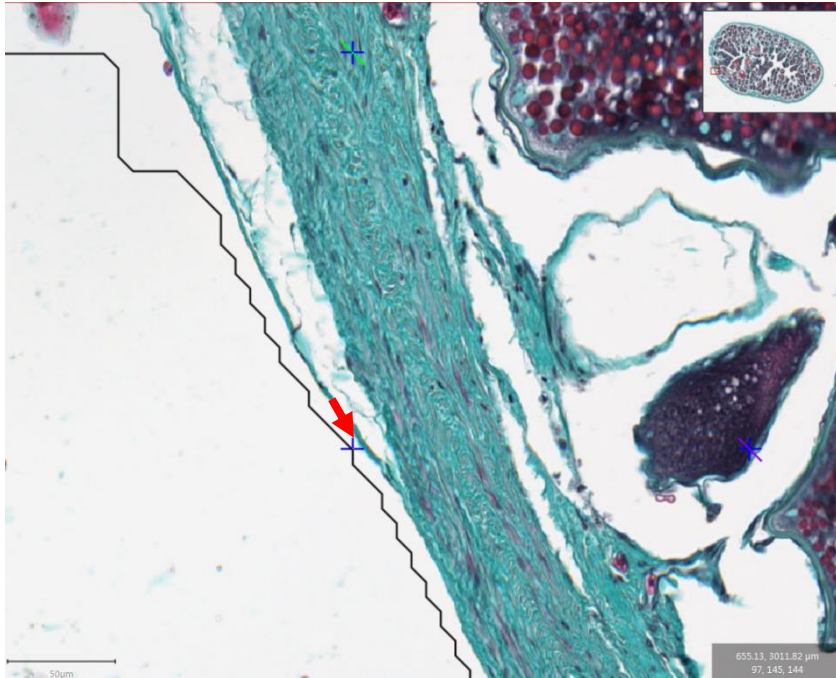
- Crosses found between the outer most cell layer of the **gw** and the sampling area (black line) are to be categorized under **E**

When the **gw** is complete:

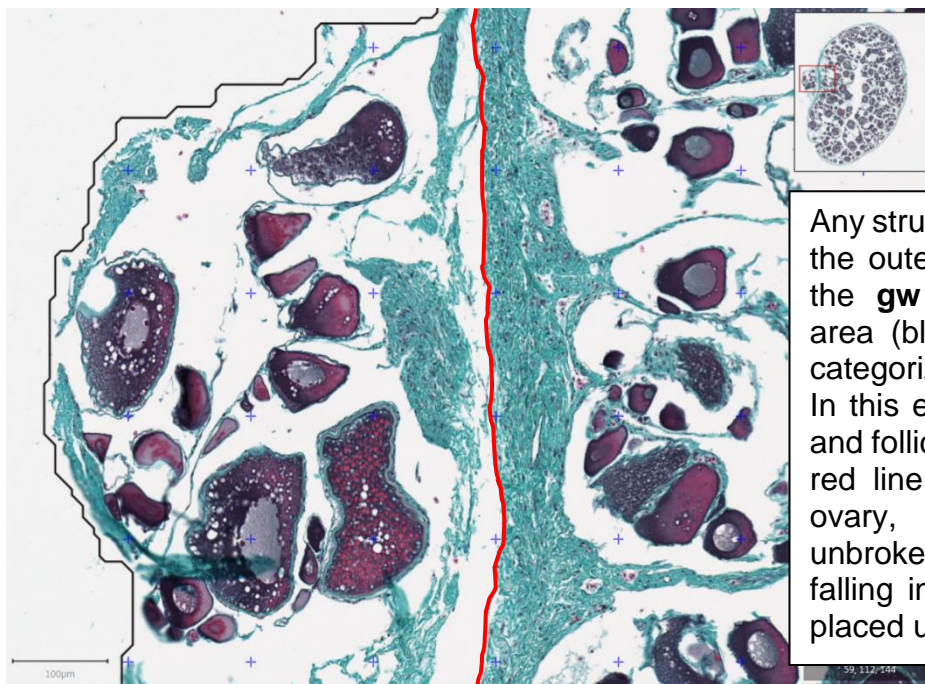
- Any structure found between the outer most cell layer of the **gw** and the sampling area (black line) are to be categorized under **E**.

### 4.8. Unnatural emptiness (E)

E = All zones outside of the ovarian boundary (i.e. gw)

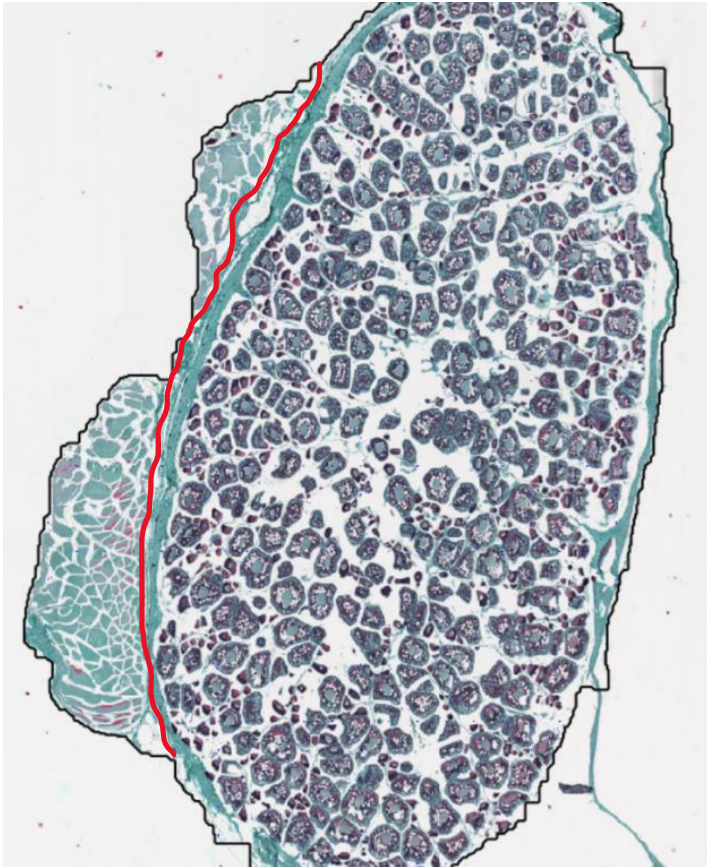


Cross (↯) outside of the outer layer of gw cells. Put this cross in the E category

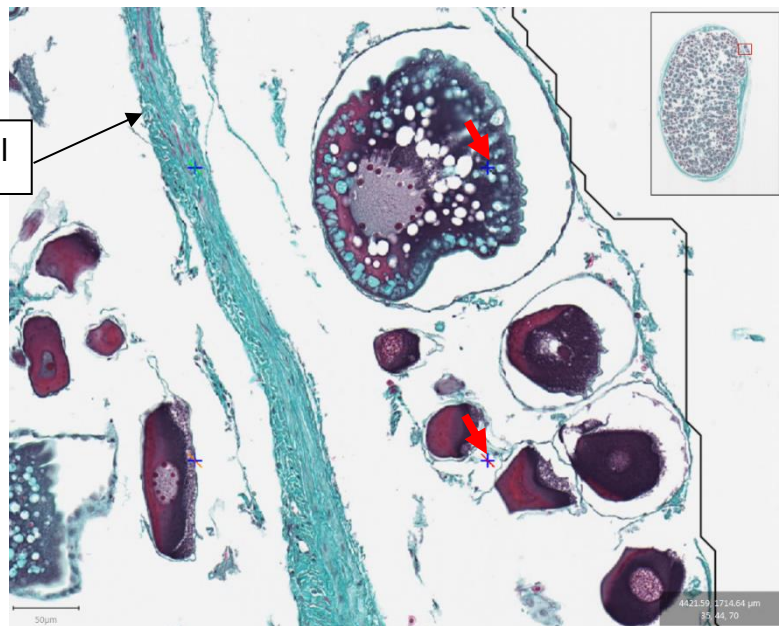


Any structure found between the outer most cell layer of the gw and the sampling area (black line) are to be categorized under E. In this example, the tissues and follicles on the left of the red line are outside of the ovary, delimited by the unbroken gw. Any cross falling in that area must be placed under the E category

Gonadal wall



Any structure found between the outer most cell layer of the **gw** and the sampling area (black line) are to be categorized under **E**.  
 In this example, the tissue on the left of the red line is the mesovarium (tissue that connects the ovary to the coelomic cavity), and it is outside of the ovary, delimited by the unbroken **gw**. Any cross falling in that area must be placed under the **E** category



Gonadal wall

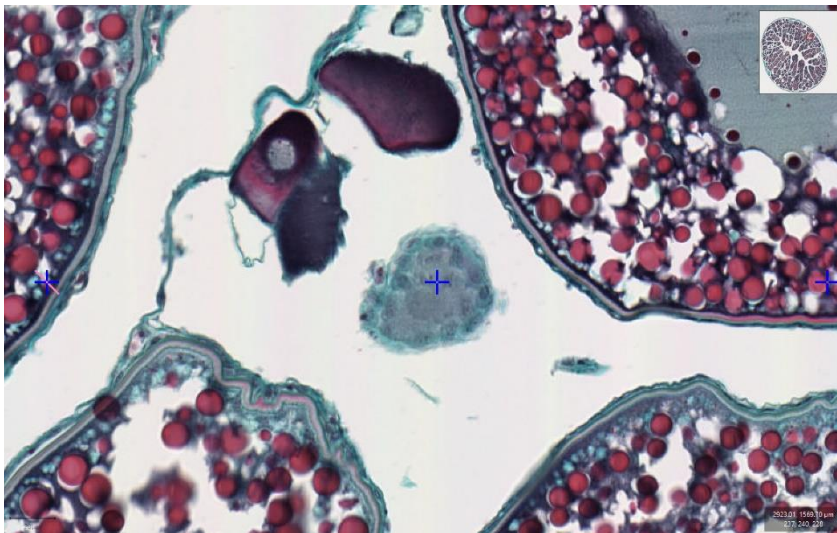
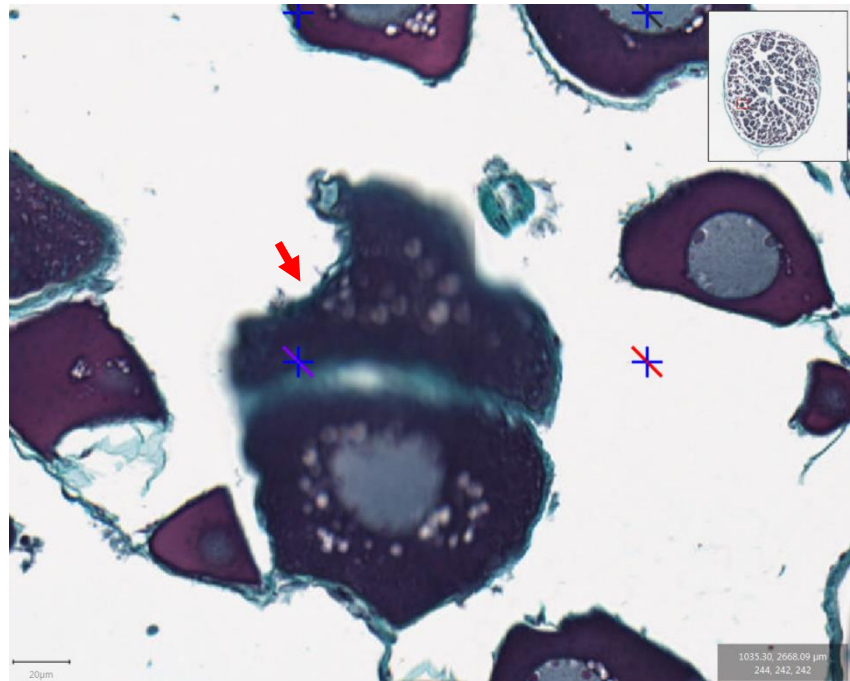
The follicles are outside of the ovary and the **gw** is intact, put these crosses (↘) in the **E** category.

If the follicles are outside of the ovary and the **gw** is intact, put them in the **E** category. If the follicles are outside the ovary but the **gw** is missing, select the cell type

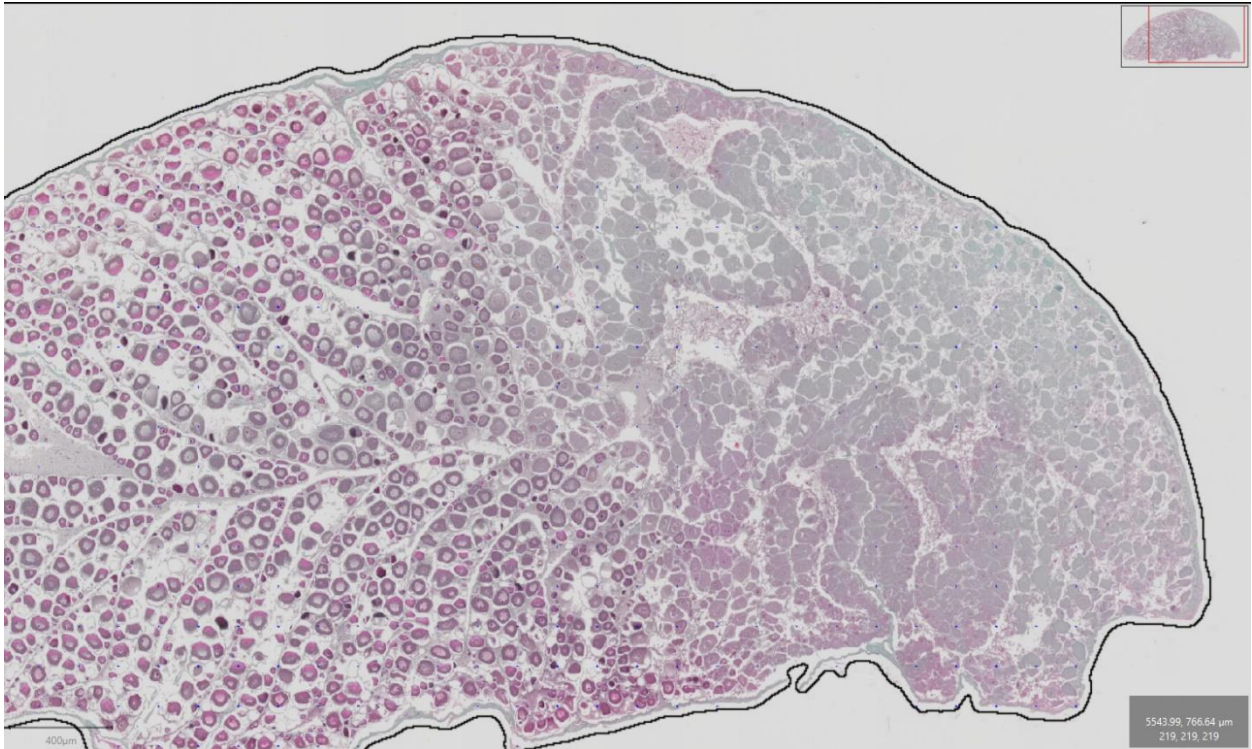


## 4.9. Undetermined (U)

Cross (↯) on a blurry structure. Put it in the **U** category



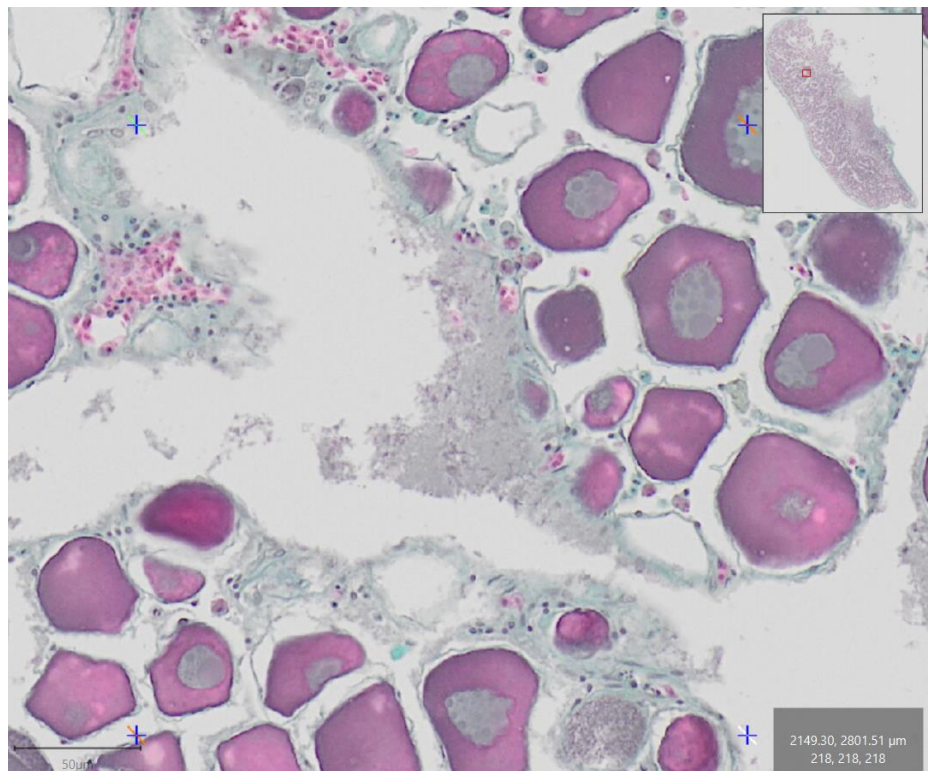
Structures that have not been identified in the protocol or in the lexicon must be placed in the **U** category



Discolored area : Some structures might be mislabeled without the help of coloration. If a structure is discolored, place it in the **U** category



Some discolored structures that can be found in the lumen may be confused with lysis. Place these discolored areas in the lumen under the **U** category



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