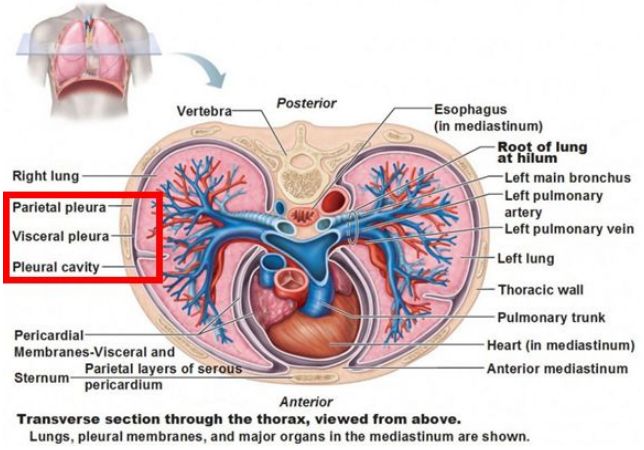
STANDARD OPERATING PROCEDURE

1. Scope

The primary objective of this protocol is to describe a method for the image acquisition from the chest wall tissue blocks.

2. Field of view: general description

a. The desired Field Of View (FOV) includes the pleural cavity plus the two pulmonary pleura (visceral and parietal). In the image below, the anatomy of a transverse section through the thorax is shown. The three main elements of the FOV are highlighted in red.

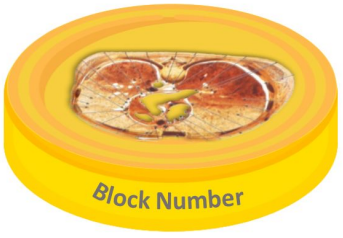


Transverse section through the thorax, viewed from above.
Lungs, pleural membranes, and major organs in the mediastinum are shown.

b. **Interesting features:** macrophages, neutrophils, inflammatory cells, foreign bodies (fibers) in the pleura and adhesions.

3. Chest wall block

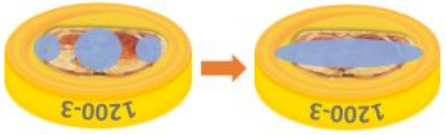
a. The view from above of the chest wall blocks used for the image acquisition corresponds to a transversal section of the rat thorax. Therefore, the chest wall anatomy described in the previous figure can be used as a reference to locate the desired FOVs to be obtained.



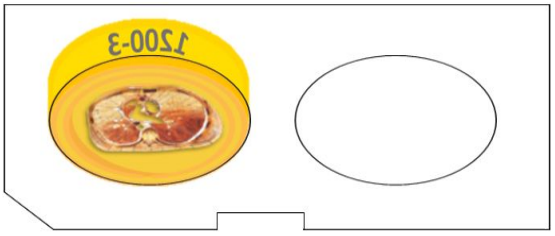
b. Each block has a *Block Number*. This number describes the sample itself as it contains the following information: animal number, treatment group, study time-point, and block number. This information is needed for the naming of the raw data files at the end of the scanning process.

1200-3 → Animal number (i.e. animal #1200)
1200-3 → Treatment group (i.e. group 1)
12**0**0-3 → Time point (i.e. 45 day)*
1200-**3** → Block number (i.e. block 3)
**The equivalent time-point to a given number is available in the study plan table*

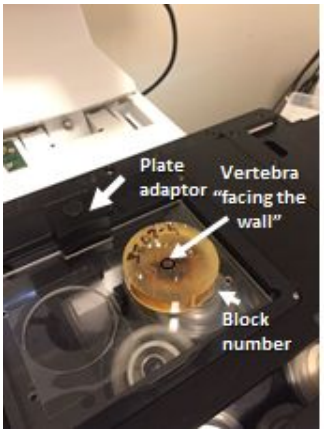
4. **Set the template on stage**
   - **a.** Turn on the microscope including the step of turning on the X-Cite light source (described in other protocol).
   - **b.** Move the stage of the microscope until the lower limit is reached. This will prevent damaging the objective while placing the sample in the stage.
   - **c.** Apply a drop of Zeiss oil on to the 63x microscope oil immersion objective without physically touching it. Make sure you are not placing the oil on to the water immersion objective.
   - **d.** Take a block from the *To be scanned* box.
   - **e.** Using a fine-point black Sharpie, place a small circle over the back side of the spinal cord.
   - **f.** Clean the block by placing two drops of oil on to the surface of the slab and gently wipe them off with a tissue paper. This step fills tiny surface irregularities with oil to allow an efficient coupling to the coverslip.
   - **g.** Place a big drop of oil at the center of the block and two small drops of oil at each side of the chest wall.
   - **h.** Connect the three drops of oil dragging the plastic applicator across



   - **i.** Place the block on to coverslip (gently) and make sure not to get bubbles in the chest wall area. The block shall be oriented in a way that the thorax vertebra is in the same side of the cut corner of the plexiglass sample holder and the block number is facing you, as represented in the figure.
     View from below of the plexiglass sample holder:



   - **j.** Apply oil on the coverslip base that corresponds to the area of the block to be scanned
   - **k.** Set the plexiglass sample holder with the block on the microscope stage. Make sure that the stage is securely positioned with the plate adaptor.



l. Move the stage of the microscope with the joystick and center the objective around the vertebra.
   i. Since the search consists of only traversing the pleura perimeter, the vertebra works as a point of reference to facilitate the search of FOVs.
   ii. In case you get lost in the search of the pleura, return to the vertebra and start the search again. This is useful when going from one half of the block to the other one.

5. Process to scan

a. Start Zen Black Program
b. Click on "**Start the system**" and wait until it loads.

Search FOVs

c. Go to **LOCATE** window → click on ***"fluorescent488"***
d. Turn 3 clicks up on the X-Cite illuminator
e. Look through the eyepieces and with the fine focus and coarse focus on the side of the microscope, go through the block until the tissue is apparent.
f. Travel through the perimeter of the block, using the vertebra as a reference.
g. Locate a FOV that encompasses the pleura cavity plus the two pulmonary pleura.
If possible, try to locate a FOV where the visceral pleura is smooth. This will facilitate the data processing.
When looking for a FOV, take into account the interesting features to be found in the tissue.
h. After finding a FOV that meets the criteria, turn off the fluorescence.

Establish plane of focus and set the depth:

i. Go to the **ACQUISITION** window
j. Load the configuration "***RIC_chestwall_Z-stack***" at the *Experiment Manager Menu*
k. On the acquisition tab, make sure that **only** [Z-stack] is checked at the *Experiment Manager Menu*
l. Click **CONTINUOUS** mode to see the image in the computer screen
m. Click on **SPLIT** to see both channels.
n. Adjust the gain to channel 1 and channel 2
   i. Channel 1 → Reflective light (will be mostly black and show fibers or foreign bodies if present)
   ii. Channel 2 → Tissue
o. Using the knob of focus of the microscope look on the screen the top of the block. The top of the block should be set right under the speckles of channel 1.
p. Click **MANUALLY** on the ***Focus Menu*** to set the Z-position to 0.
q. Move around 14 microns deep on the Z-position and keep going until 30 (to check how deep and the quality of the image). Go back to 14 microns in depth and click **MANUALLY** again. This will set to 0 the Z-stack again.
r. Click **STOP**
s. Go to the ***Z-stack Menu*** and click on **CENTER**. It should be 0.00

Tile Scan

t. Uncheck Z-Stack from the acquisition tab.
u. Check ***Tile Scan*** (this is the only box that needs to be checked).
v. The Tile Scan shall be done with the 63x oil objective and consist of 5x5 tiles.
w. Modify the zoom to **0.6** (at the *Scan Area Menu*).
x. Click on **START EXPERIMENT**

i. The Tile Scan shall contain both pleura.
ii. Remount if there's any air bubble that interferes with the scan region of interest.

y. Uncheck ***Tile Scan***.

Positions Selection

z. Check the ***Positions*** box and make sure that the list of positions is empty. If not, remove the positions by clicking on "Remove all".
aa. Modify the zoom at **1.00**
bb. Click on ***Stage***. Use the stage square as a reference to select the FOVs to be scanned. This helps to avoid overlapping of FOVs. The cross in the square indicates the center of the FOV to be scanned and helps to discriminate whether the FOV will encompass both pleurae or not.
cc. Once a FOV has been elected, click on **ADD** located at the ***Positions Menu***.
dd. Move to another FOV and click **ADD**. Keep moving and adding as needed to get all of the possible desired FOVs from the Tile Scan.
ee. Click on ***Positions*** button. The FOVs selected will appear in the Tile Scan enumerated from 1-X.
ff. Click on the 2D Channel view

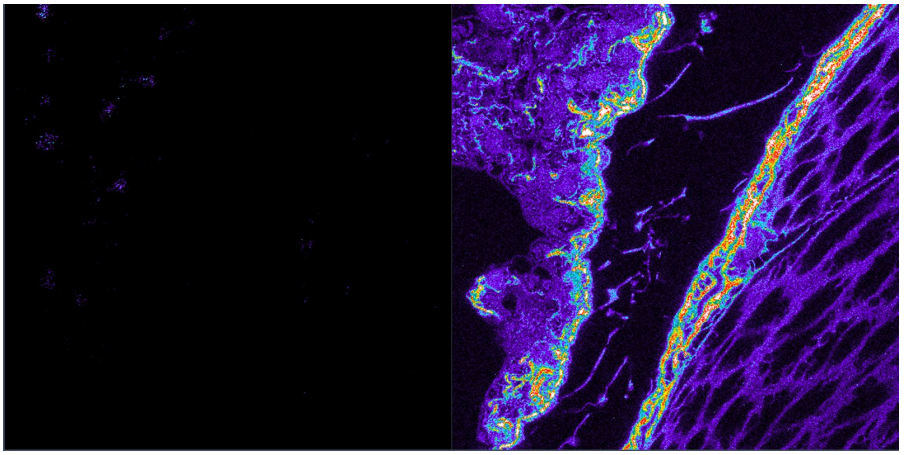
Save the Tile Scan

gg. Save the Tile Scan as a **JPEG**:
i. Go to [File] → Export → Click on the "Select name of file and save" button
ii. The general path for saving the Raw Data acquired for Chestwall blocks is: Cloud Station → Citox → Chestwall → [Time Point] → Raw Data → Chestwall Random.
iii. Choose the proper folder from the Cloud Station Drive according to the Time Point scanned and save the file.
iv. An example of a Tile Scan file name is 1200CTS3A_1thru8, where:
- **1200** = Animal number
- **C** = Chestwall (Tissue)
- **TS** = Tile Scan
- **3** = Slab number used for the acquisition
- **A** = Each TS is assigned a letter of the alphabet according to the order in which they are obtained (i.e. this TS was the first one scanned)
- **_1thru8** = number of FOVs taken from the TS. Notice the underscore sign is included.

Z-stack acquisition

hh. Once the positions have been added and the Tile Scan has been saved, check the ***Z-stack*** box. Make sure that just the **Z-stack** and **Positions** boxes are checked.
ii. Click **START EXPERIMENT.**
jj. Click on **SPLIT** to see both channels.
kk. Adjust the gains of the channels to reduce oversaturation, undersaturation, etc.
i. Channel 1: try to maintain your gain just for the foreign bodies to be seen (if present) and just a little bit of the reflected tissue. But in general, shall be mostly black.
ii. Channel 2 → try to keep the gain so that the mesothelial cells are bluish and connective tissue (CT) is whitish. But, always try to balance the oversaturation over the bluish of the cells.

iii. Example of the gain intensity in both channels for the mid-slice of the Z-stack



ll. As the sections go deeper, you have to increase the gain of both channels.

mm. Save the Z-stack as a **.czi file**:

i. Go to [File] → Export

ii. The general path for saving the Raw Data acquired for Chestwall blocks is: Cloud Station → Citox → Chestwall → [Time Point] → Raw Data →

iii. Chestwall Random.Choose the proper folder from the Cloud Station Drive according to the Time Point scanned and save the file.

iv. An example of a Z-stack file name is 1200CR3A_1thru8, where:

- **1200** = Animal number
- **C** = Chestwall (Tissue)
- **R** = Random search (a “P” is used when the search type is “Purposeful”)
- **3** = Slab number used for the acquisition
- **A** = means that the FOVs were taken from the Tile Scan A from that block
- **_1thru8** = number of FOVs series encompassed in the file. Notice the underscore sign is included.

nn. If any scanned FOV contains any interesting feature, rescan that FOV individually with an Average Number of 4 and 1024x1024. Save the file as a **.czi file**.
An example of a rescanned FOV file name is 1200CR3A_4. Where 4 means, it was the 4th FOV of the Tile Scan the one that was rescanned.

6. Remove the block from the microscope

a. Move the stage of the microscope until the lower limit is reached.

b. Move the plate adaptors and carefully remove the plexiglass template from the stage.

c. Remove the block from the coverslip by twisting gently the block to that the oil seal breaks.

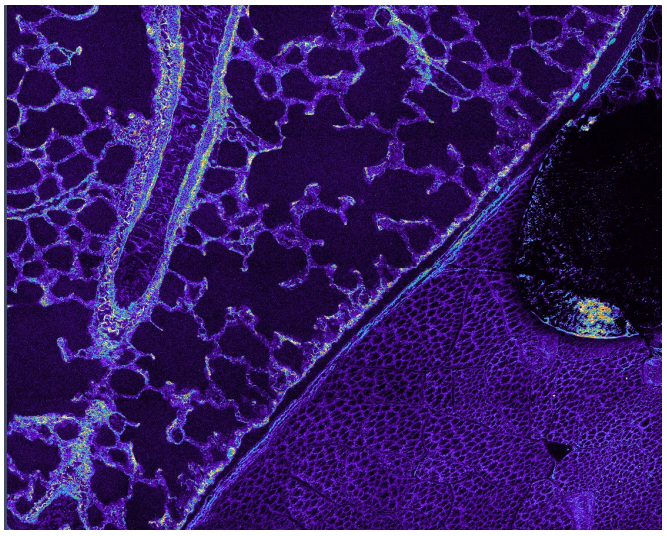
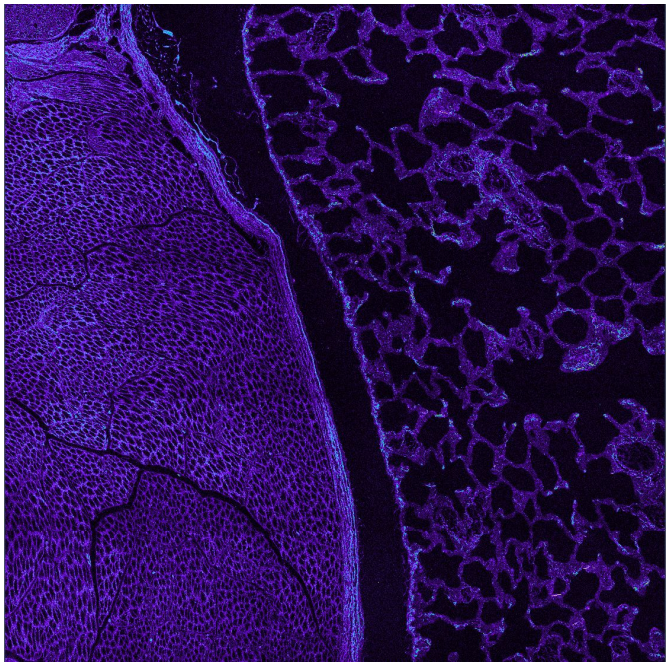
d. Clean the block with a tissue paper and when dry, place it in the storage box for chestwall blocks.

e. Clean the coverslip with a tissue paper (circular movements from the edgevto the center). Do not apply force on the coverslip when cleaning it.

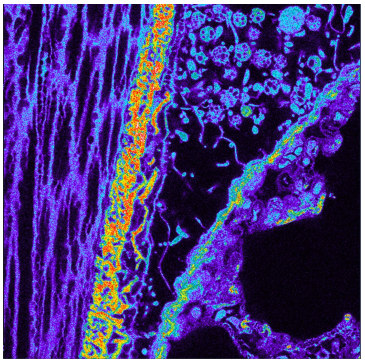
f. Clean the objective for excess oil with the microscope paper only.

7. When the data acquisition goes wrong:

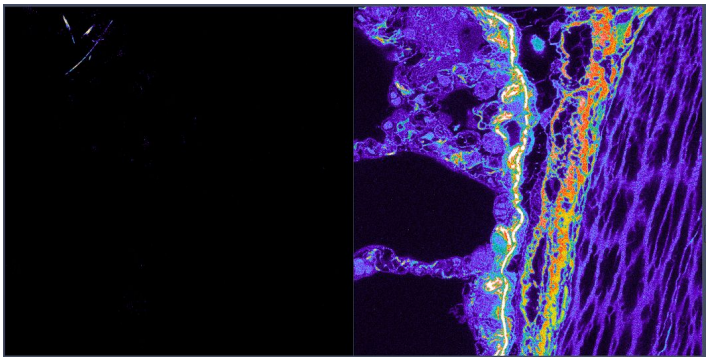
FOV/Z-stack appearance	CAUSE(S)	TROUBLESHOOTING
Difficulty to set depth	● Incomplete coupling with oil ● Failed coverslip seal ● Poor surface polish	Remount the template Recut/repolish
There is no difficulty to set depth but the pleura is in different angles in such a way that affects the quality of the acquired images	Poor surface polish	Recut/repolish the block
Speckles on the first slices	Focal plane too high	Re-center
There is no difficulty to set depth but, even if the gain in both channels is too high, it is hard to get the right gain on each channel	Laser power low	Verify that the laser power at the LASOS box has been set correctly If it is, then try to increase the laser power directly in ZEN Black to 4.0

Tile Scan Case Study	
	**Example 1** ● The TS encompasses both pleura with a close distance between them. ● The TS shows interesting features that could be scanned; i.e. where the cells are in the upper corner of the TS **Nevertheless** ● The TS has an air bubble ● The air bubble interferes with the interesting features to be scanned **Therefore,** remounting is necessary
	**Example 2** ● The TS encompasses both pleura **Nevertheless** ● The gain on Channel 2 was LOW when the TS was done ● We can not select the FOVs properly because we can not identify the block surface behaviour. **Therefore** ● When you establish the center of the Z-stack, determine the appropriate gain for this center Z-position. **Why?** ● Because the Tile Scan gives you a 5x5 landscape from the center Z-position you chose. ● This way, you can distinguish how the surface of the block behaves and will help you identify where to select FOVs positions that will not compromise one or both pleura acquisition.

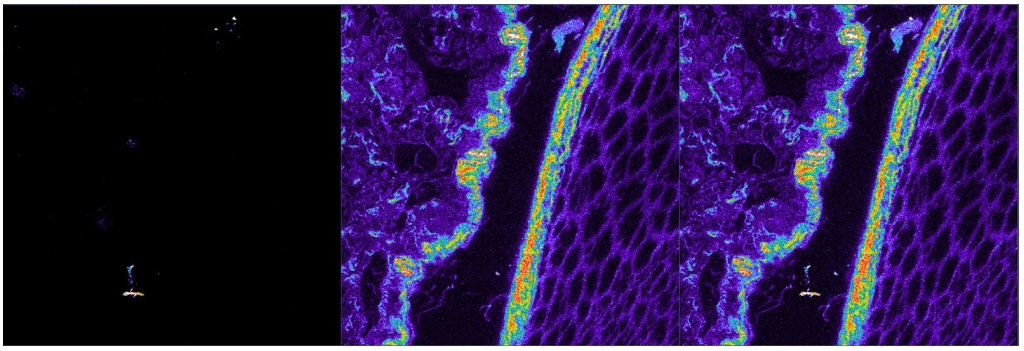
Interesting features examples:



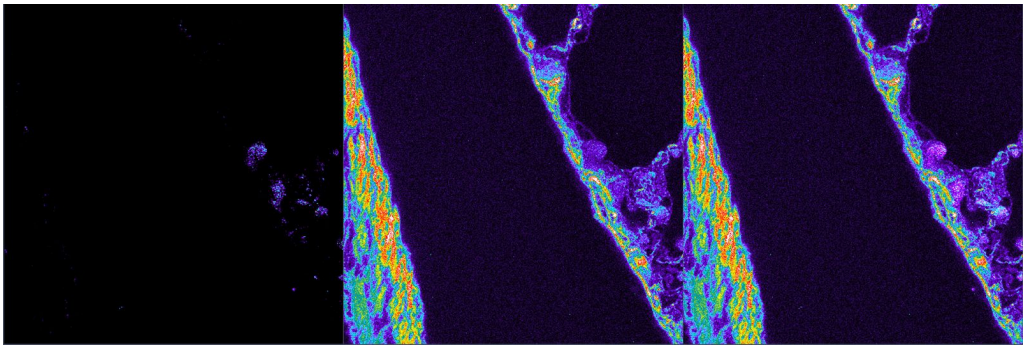
Example 1: Inflammation



Example 2: Fibers and thick mesothelial cells layer



Example 3: fiber and cell with foreign bodies in pleural space



Example 4: foreign bodies (optimal gain for Channel 1)