Imaging and Flow Cytometry Core

Akoya Vectra Polaris

Standard Operating Protocol

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A. Preparation of slides

Glass slide: 1 mm thick, good quality (could withstand heating and cooling cycles)
Coverslip: No. 1.5 thickness (170 µm)

<table>
<tr><th>Types of slides</th><th>AR, MWT</th><th>Fluorophores (excluding DAPI)</th><th>DAPI</th><th>Purpose</th></tr>
<tr><td colspan="4">A. Library slides*</td><td rowspan="4">Collect spectrum of each fluorophore and autofluorescence for creating a spectral unmixing library</td></tr>
<tr><td>Unstained</td><td>+</td><td>-</td><td>-</td></tr>
<tr><td rowspan="2">Single stained#</td><td>-</td><td>+
(1 slide for each fluorophore)</td><td>-</td></tr>
<tr><td>-</td><td>-</td><td>+</td></tr>
<tr><td>B. Monoplex slides^</td><td>+</td><td>+
(1 slide for each fluorophore)</td><td>+</td><td>Assess staining performance</td></tr>
<tr><td>C. Multiplex slide@</td><td>+</td><td>+</td><td>+</td><td>Study samples</td></tr>
</table>

* Use study tissue type
# Omitted for opal fluorophores or fluorophores with established spectrum in InForm; recommend to use an antibody marking an abundant epitope for each fluorophore (e.g., CD20)
^ e.g., monoplex slide for 3rd biomarker in sequence of a 7-color multiplex assay should experience AR, two MWT before the addition of antibody, secondary, and fluorophore, and four MWT after staining; only needs to be completed once per study, or every 6 months.
@Optimize staining conditions with monoplex slides before preparing multiplex slides
Abbreviations: AR, antigen retrieval; MWT, microwave treatment

For example, a 7-color multiplex assay should involve 15 slides:

- 8 library slides (1 unstained, 7 single stained slides)
- 6 monoplex slides
- 1 optimized 7-color multiplex slide

Protocol of Opal staining: [Opal Multiplex IHC Assay Development Guide and Image Acquisition Information - Phenoptics Research Solutions]

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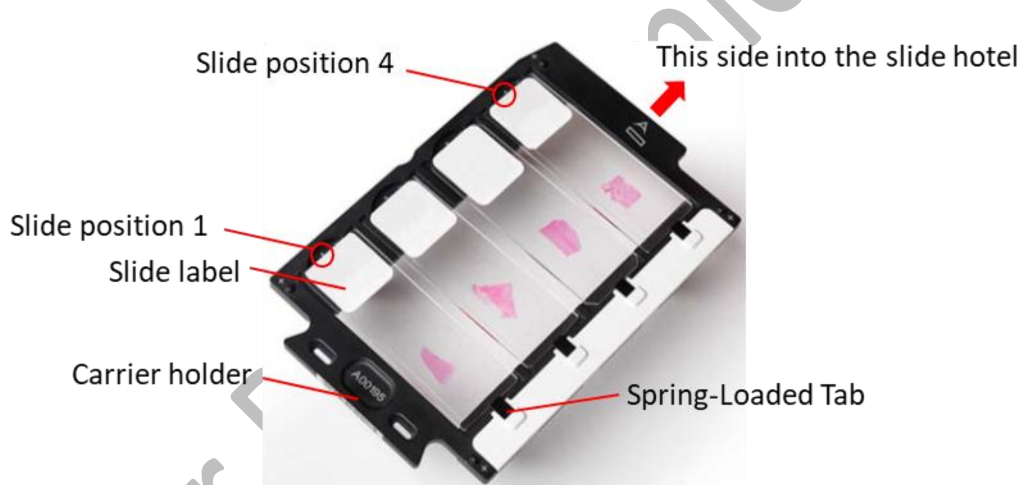
B. Initialization of Vectra Polaris

1. Fill in the log book. Write **actual** start time.
2. Turn on Vectra Polaris.
3. Turn on the computer (account: User; password: (at the left bottom of monitor)).
4. Launch Vectra Polaris software .
5. **Cool down** the slides to room temperature.
6. **Clean** the slides with 100% ethanol.

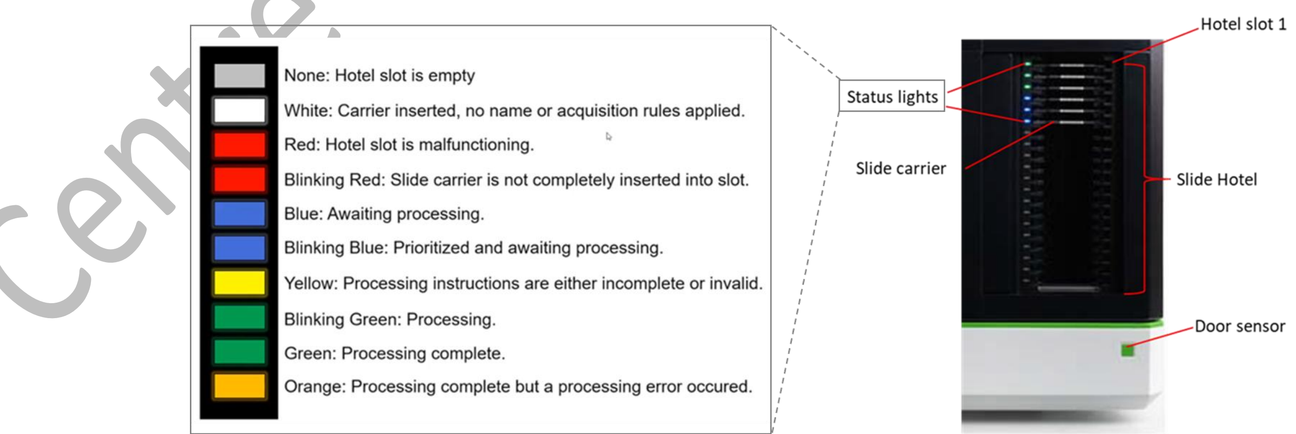
⚠ Always clean your slides with 100% ethanol before scanning because there is dirt unobservable by eyes but detectable by camera.

7. Place sample slides onto a slide carrier.
8. **Push** all slides to the edge towards the sign.



Slide Carrier

9. Load the slide carriers to any slots of the slide hotel. The status light should become white.



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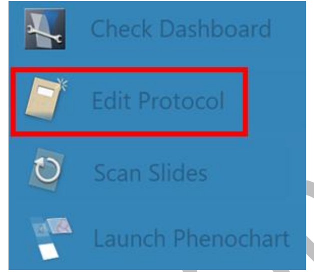
C. Setting Up Scanning Protocols

i. <u>Bright-field scanning</u>

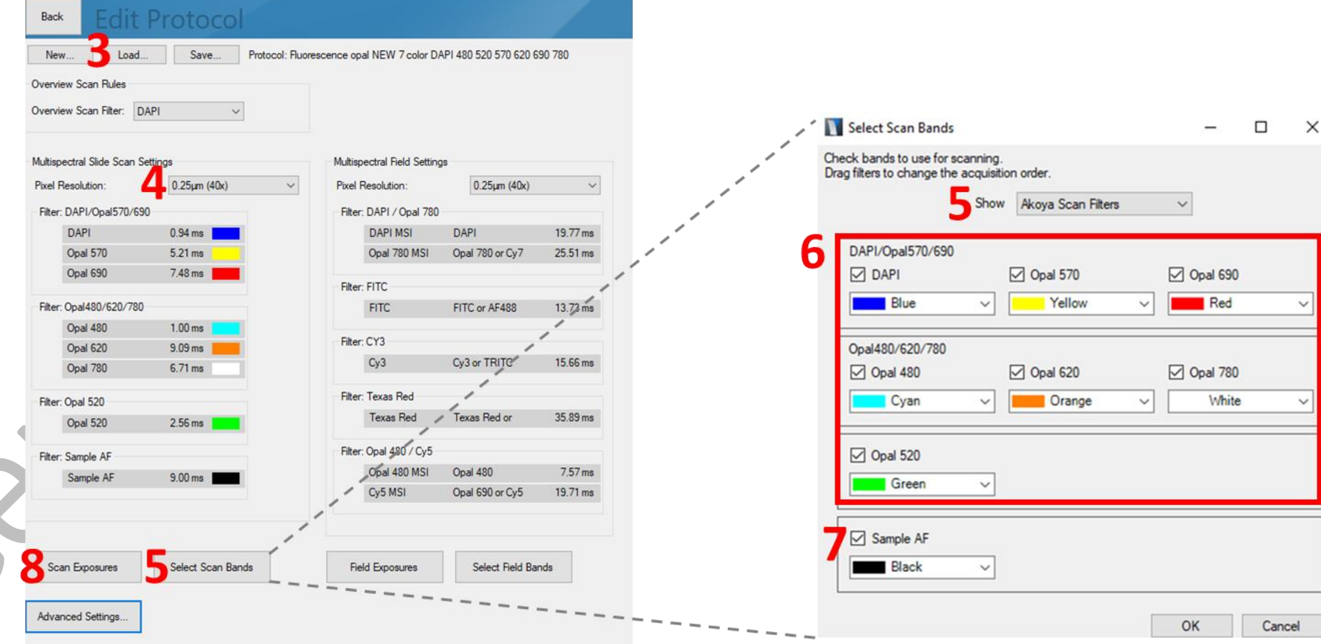
1. Copy **Bright Field 10X/20X/40X.ppr** from E**:\Data\VectraPolaris_CPOS_standard protocol** to your own folder.
2. Go to Part D.

ii. <u>Fluorescence scanning (using opal fluorophores, excluding opal 540 and opal 650)</u>

1. Copy **Fluorescence opal polaris 7 color….ppr** from E**:\Data\Vectra Polaris_CPOS_standard protocol** to your own folder.
2. On the **Home** Page of Vectra Polaris software, click **"Edit Protocol"**.



3. **Load** the protocol template.
4. In **"Multispectral Slide Scan Settings",** choose the **pixel resolution** of interest.
5. Click **"Select Scan Bands" -> Show "Akoya Scan Filters".**
6. Select the filters for opal fluorophores used and <u>de</u>select those unused.
7. Select **"Sample AF"** filter to allow unmixing of autofluorescence and sample in Phenochart software.



8. Click **"Scan Exposures" -> "Load Carrier" -> "Take Overview"**.
9. **Drag** the focus bar to bring the specimen closer to the focus **->** click **"Auto Focus"**.

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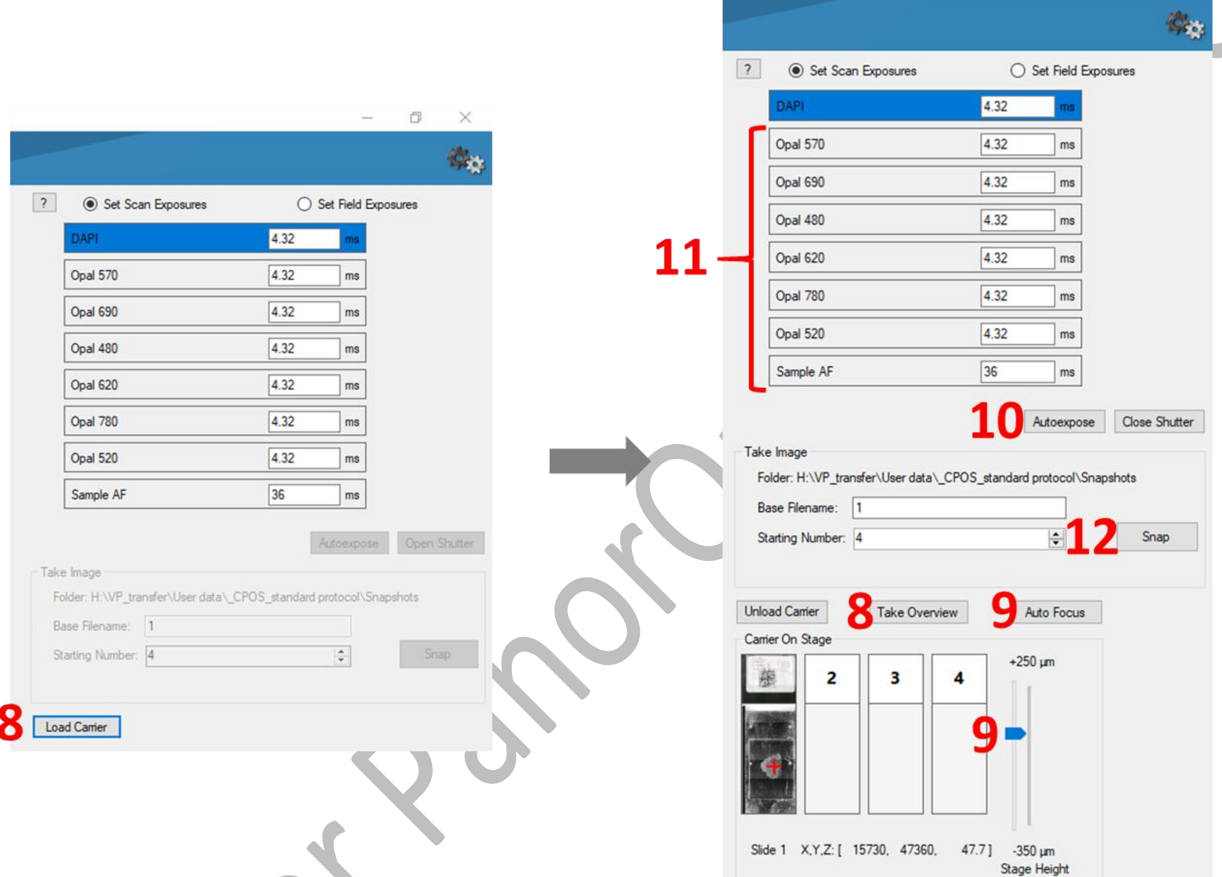
10. **Drag** the slide to region with fluorescence signal -> click **"Autoexposure"**

⚠ "Auto Focus" in protocol setting is only for adjusting exposure time but not related to where to focus during image scan.

11. Repeat step 10 with each fluorescence filter.
12. Click **"Snap"** to take an example image of the slide.



13. Go to **"snapshots" folder** in your folder to see the snapped image. You can always manually adjust the exposure time if you are not satisfied with the image as long as it does not overexposure.
14. Click **"Back"** -> **"Save"** to save the protocol.
15. Click **"Back"** to return to Home Page.
16. Go to Part D.

iii. <u>Fluorescence scanning (using non-opal, opal 540 or opal 650)</u>

Copy **Fluorescence NON-opal or opal 520 opal 650.ppr** from E**:\Data\Vectra Polaris_CPOS_standard protocol** to your own folder.

1. Follow steps 2-5 in Part C ii.
2. Select appropriate filters that allow you to select fields for multispectral imaging (MSI), e.g., if DAPI signal is enough for you to choose the fields for MSI, then only turn on DAPI. Turning on fewer filters could shorten scanning time.
3. In **"Multispectral Field Settings",** choose the **pixel resolution** of interest.

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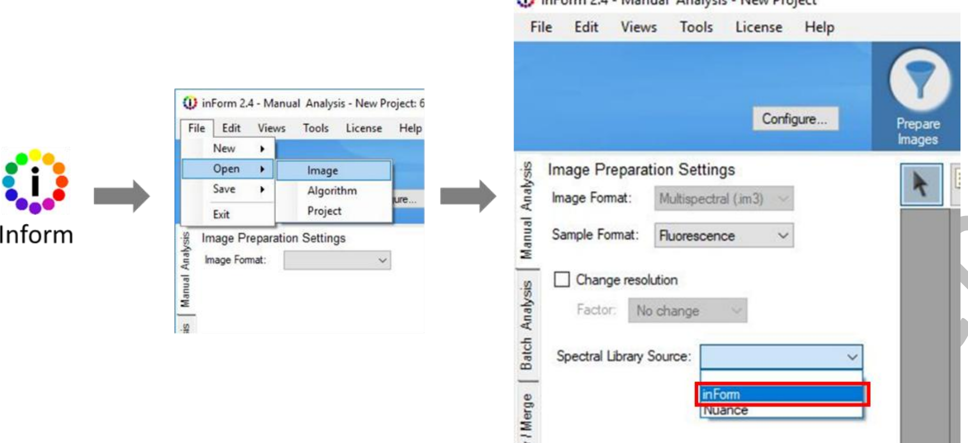
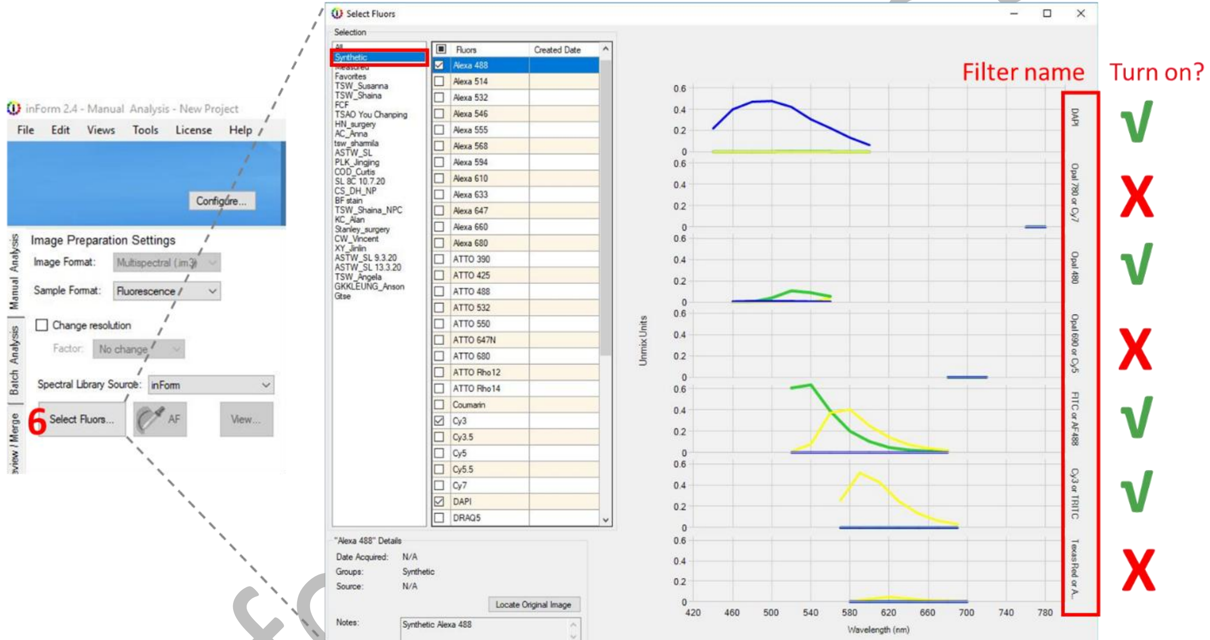
4. Open InForm software, click **“File” -> “Open” -> “Image” -> “example.im3** **” at E:\Data\Vectra Polaris_CPOS_standard protocol -> choose “inForm” as Spectral Library Source**.



5. Click **“Select Flours” -> “Synthetic” -> select used fluorophores**.



6. Click **“Select Field Bands” -> Show “Akoya MSI Filters” -> turn on filters selected from step 6.**

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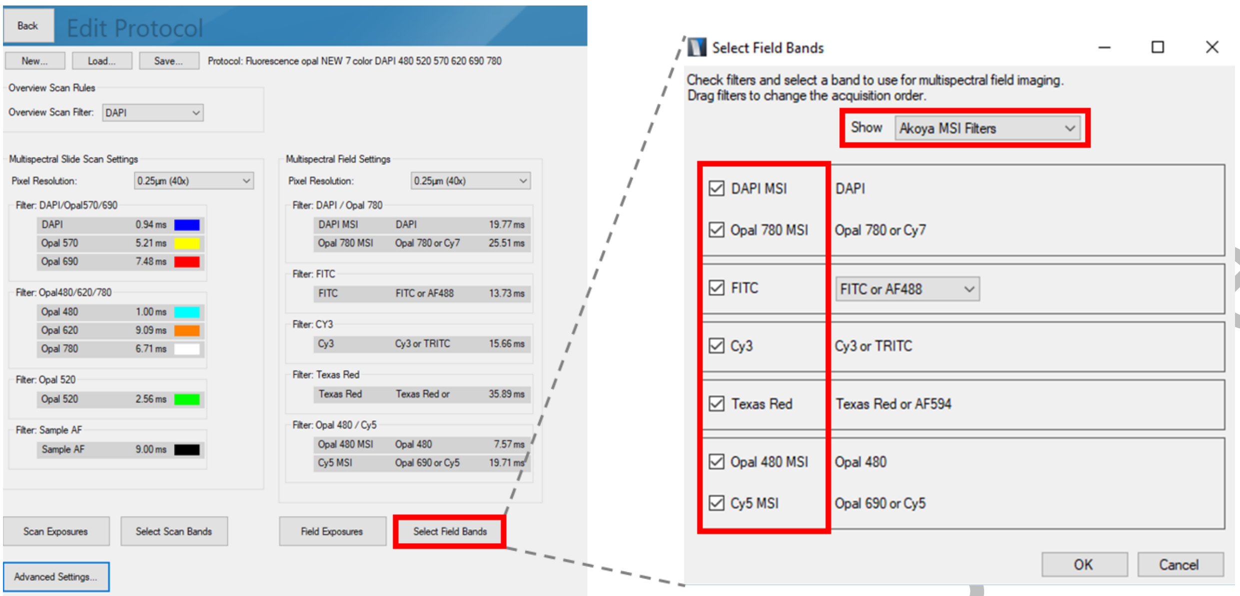
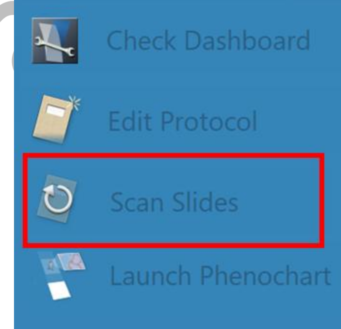


7. Follow steps 8 – 11 in Part C ii.
8. Select **"Set Field Exposures"** -> follow steps 8 – 12 in Part C ii.
9. Click **"Back"** -> **"Save"** to save the protocol.
10. Click **"Back"** to return to Home Page.

D. Whole Slide Scan

1. On the **Home** Page of Vectra Polaris software, click **"Scan Slides"**.



2. Click slide **status icon -> Task: Scan Slides -> Study: your folder -> Protocol: protocol set up in Part C -> Slide ID: sample name -> "Scan"** to start scanning.

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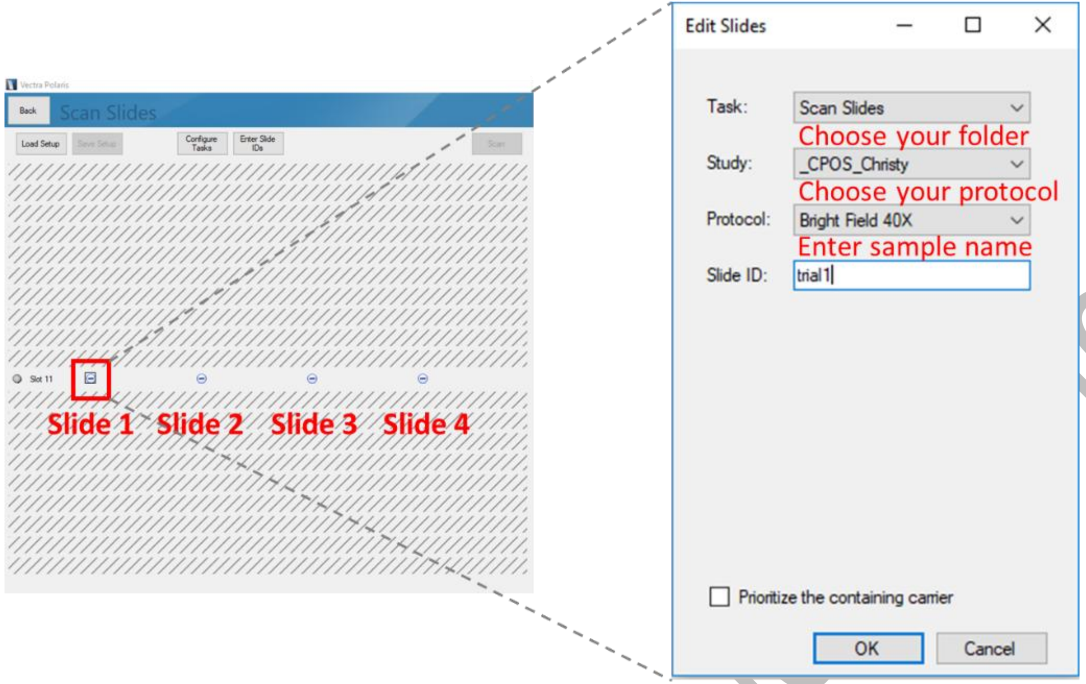

3. For setting the same rules for whole slot/more slots, click **"Configure Tasks" -> slot x -> apply the same rule for all slides -> follow step 2.**

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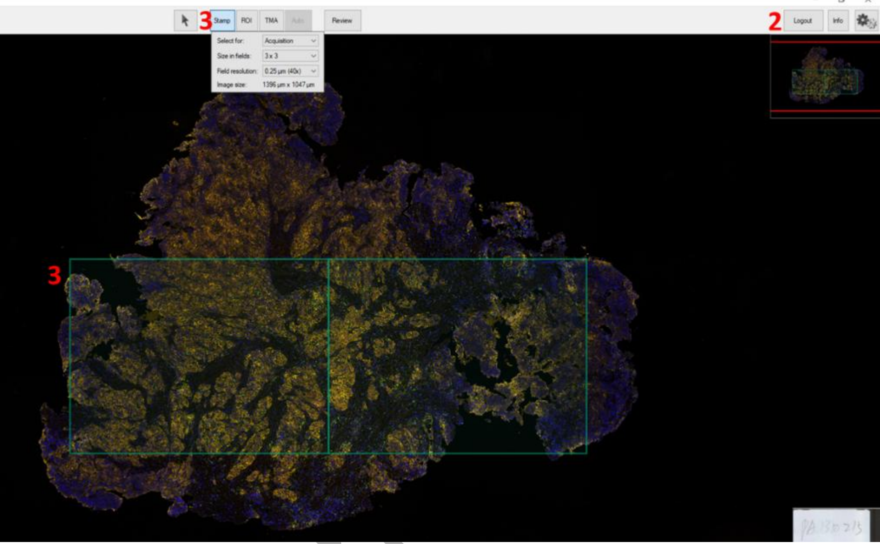
E. Multispectral Field Imaging (MSI)

1. After whole slide scan, open the **.qptiff** file .
2. **Click "Login" -> type anything -> "OK"**.
3. **Annotate** MSI fields on the image:
   - click **"Stamp" -> select for: "Acquisition" -> choose "size in fields" of interest -> choose "field resolution" set in step 3 of Part C iii**; or
   - click **"ROI" -> choose "field resolution" set in step 3 of Part C iii -> draw areas** composing of multiple MSI fields



4. **Delete** unwanted annotation(s) by pressing "Ctrl" while clicking on them.
5. Follow step 2 in Part D, select **Task: Acquire Fields instead, while other settings remain unchanged.**

> ⚠ You must assign the same slide ID as the whole slide scan; otherwise, annotations could not be found.

6. Click **"Scan"** to start multispectral **field** scanning.
7. View the scanned image in the "MSI" folder.
8. Write **actual** finish time on logbook.

F. Transfer Data

Access your data at E drive through **Vectra Polaris offline station** (computer on the left of Vectra Polaris). Transfer your data by inserting your own **USB drive** to Vectra Polaris offline station or uploading to **data transfer server** of Imaging and Flow Cytometry Core **(required registration)**.

> ⚠ If you concern data confidentiality, transfer and remove your data from the computer a.s.a.p. Users storing data in computers in core facility should bear the risk of data leakage.

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G. System shutdown

1. Take back your slides and return the carriers.
2. Close the door of Vectra Polaris.

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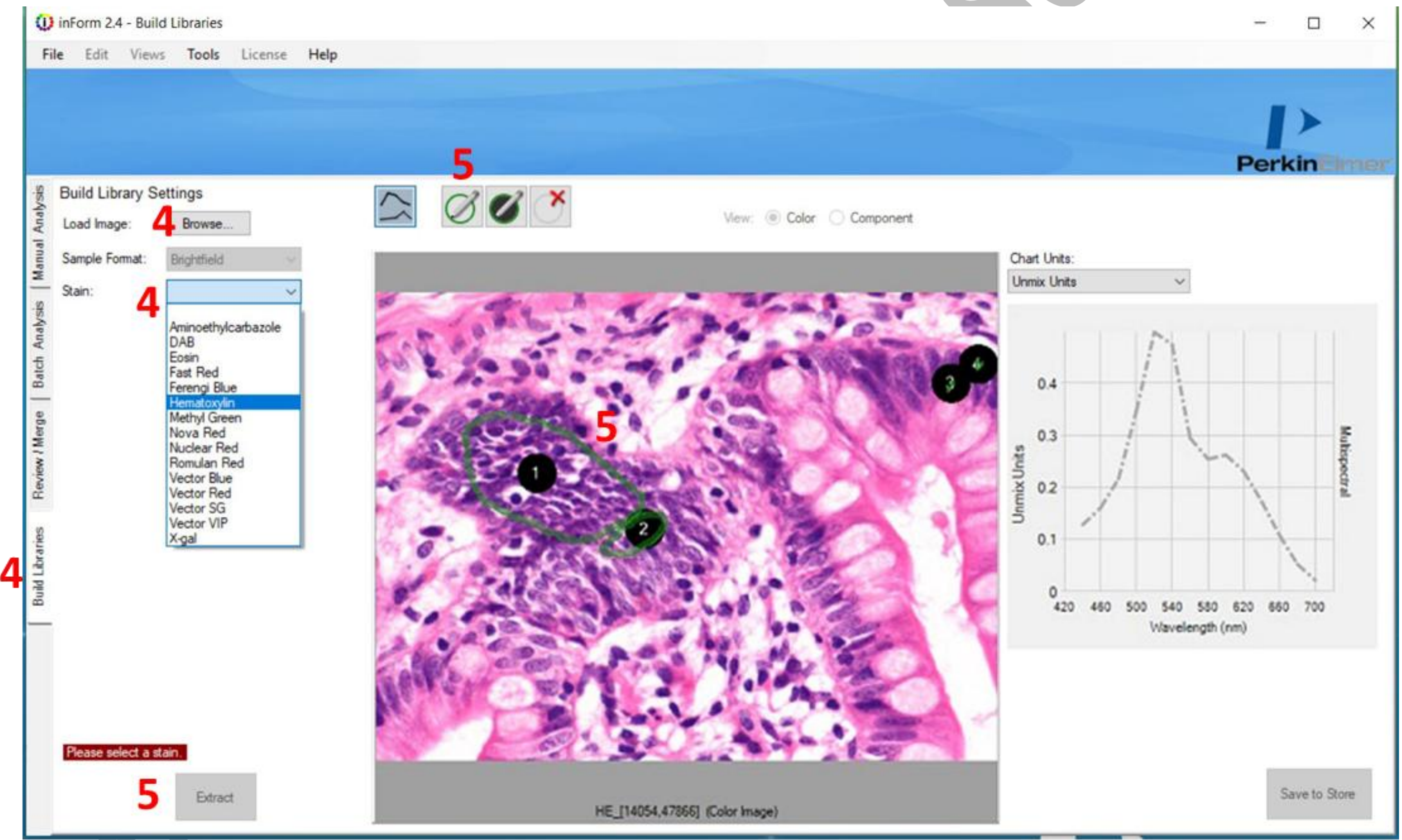
H. Library preparation

i. Library preparation

1. Launch inForm software .
2. Click **"Tools" -> "Stain Store Manager" -> "Choose Which Types of Stains and Fluors Your Lab Uses" -> check if a stain profile fits your need**.
3. If no, click "**Other (User Specified)" -> "New" -> enter the name of new stain -> choose brightfield or fluorescence -> select if it is a nuclear counterstain -> "OK"**.
4. Click **"Build Libraries" -> "Browse" -> open monoplex image of your stain** (**.im3**).
5. For **Brightfield** sample, **select stain ->** **-> highlight the stain on image -> "Extract" -> "Save to Store"** under your group.



6. For **Fluorescent** sample, select stain -> -> highlight the fluor on image -> choose a **signal band** (where the fluor most strongly expresses) and a **dark band** (where the fluor does not express at all) -> **"Extract"** -> **"Save to Store"** under your own Group.

I. unmixing

i. Whole slide unmixing

1. Open the scanned image (.qptiff).
2. Click **“Login” -> type anything -> “OK”.**
3. Click **“Unmixing” -> “Opal + AF”**.

ii. MSI field unmixing

1. In the inForm software , select **File -> Open -> Image -> select unstained image & multiplex images**.
2. Click **“Manual Analysis”**.
3. Select **Spectral Library Source”: “inform” -> “Select Fluors” -> “Synthetic” for officially established fluor spectrum -> select the fluors to be unmixed.**
4. To remove autofluorescence, click **“AF” -> highlight the brightest part on unstained image -> “Prepare All”** to unmix.
5. Click **“View Editor”** **-> data displayed: composite image ->** select the stain to be displayed.

J. Quantification

1. For image with **qptiff** format , **open it -> login -> stamp -> inForm project -> choose area (s) for quantification**.
2. In inForm software , select **“File” -> “New” -> “Image” (.im3 or .qptiff from step 1)**.
3. Select **“Configure” -> “Trainable Tissue Segmentation”, “Adaptive Cell Segmentation”** and **“Phenotyping”**.

All the steps are optional, except that cell segmentation is required for phenotyping.

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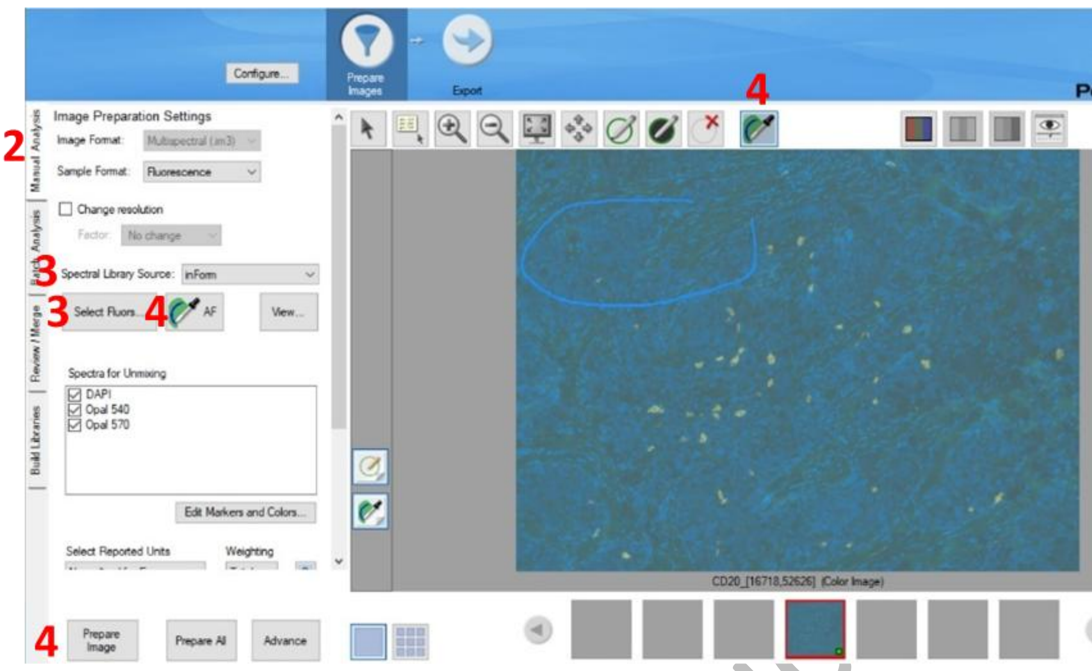

i. Tissue segmentation

1. Click **"Segment Tissue"** .
2. In **"Tissue Categories",** click **"new" -> type in tissue name -> assign annotation color -> "Draw" -> ->draw the regions of tissue on image.**
3. To delete an annotated region, click -> click on the unwanted region.
4. Select the components for training -> **"Train Tissue Segmenter"** to start training until segmentation accuracy **>= 95%.**

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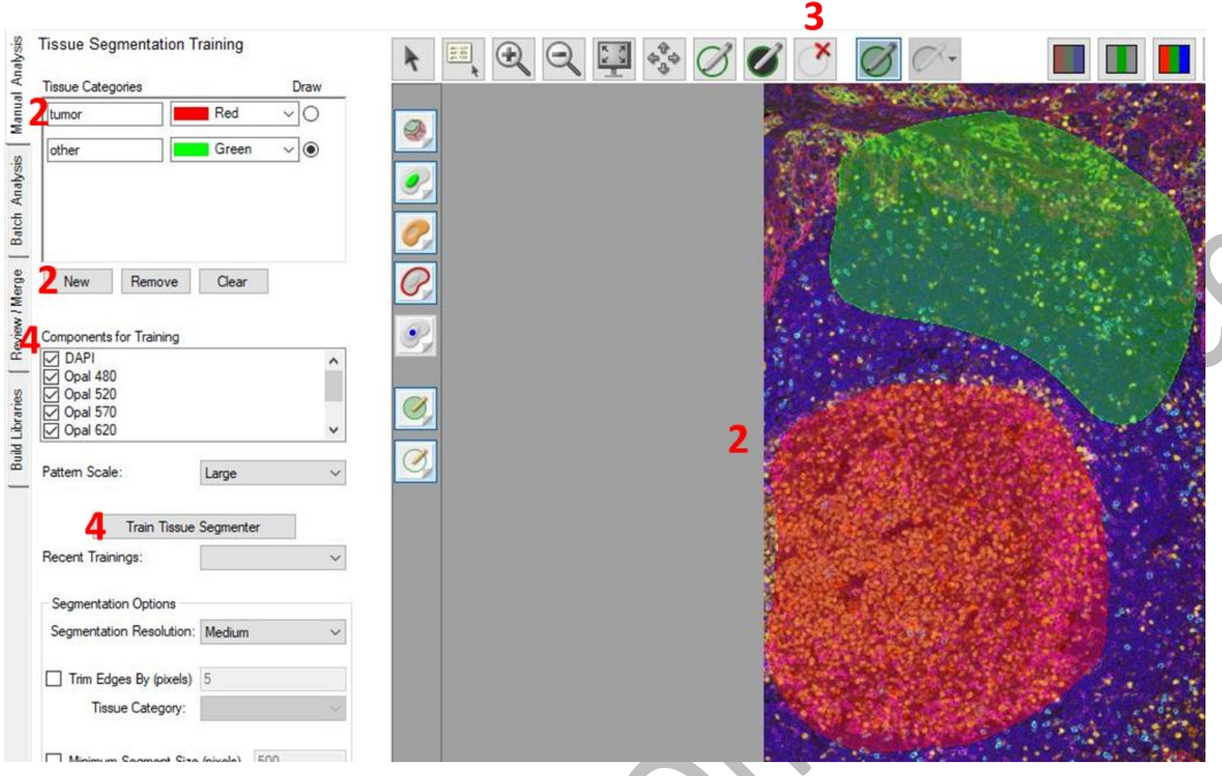

ii. Cell segmentation

7. Click **Segment Cells** .
8. In the panel of **"Segment"**, select the cellular compartments you want to segment: Nuclei, Cytoplasm, and/or Membrane.
9. In the panel of **"Components"**, click **"Add" -> choose component -> "..." -> nuclei/cytoplasm/membrane -> adjust typical intensity.**
10. If a component belongs to the cytoplasm or membrane, select **"Use this signal to assist in nuclear splitting"**.
11. Adjust the **"Splitting Sensitivity"** and **"Minimum Nuclear Size"**.
12. Increase the **"Fill Holes Smaller Than"** value until the holes in nuclei are filled.
13. Click **"Prepare All"** to segment the cells in all images.

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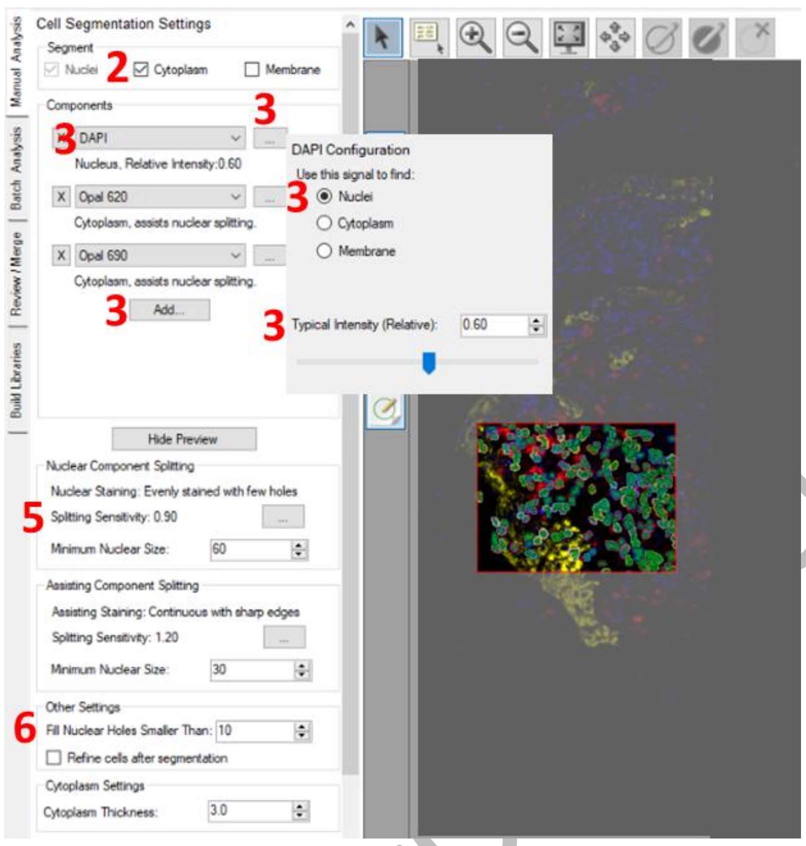
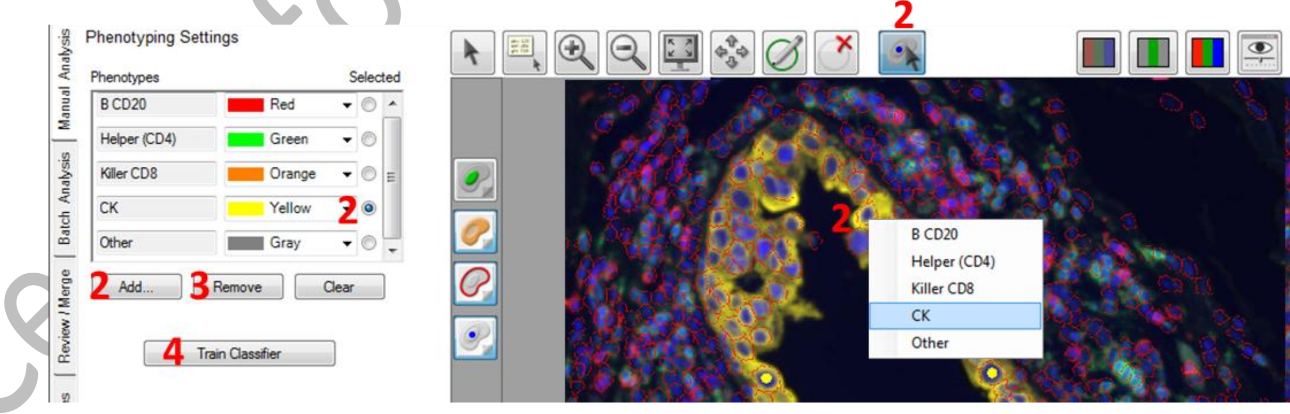


iii. Phenotyping cells

1. Click **Phenotyping** .

2. Click **"Add" -> name the phenotypes -> assign annotation color -> -> cells with phenotype on image (at least 5 cells for each phenotype).**
3. To delete an unwanted annotated cell, click **"Remove"** -> unwanted cell(s).
4. Click **"Train Classifier"** to start training.
5. Review the results of training. **"Add"** and **"Remove"** cells if necessary.



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iv. Export data

1. Click "**Export**" .
2. Click **"Browse" ->** select an empty folder at **"E:\USER_DATA\(PI_User name)"**.
3. Select the required output files:
   - Composite Image for the unmixed image.
   - Component Images (multi-image TIFF) for analysis using third party analysis software, e.g. ImageJ.
   - Tissue Segmentation Data, Cell Segmentation Data and Phenotyping Data for quantitative analysis.

v. Batch analysis

1. For image with **qptiff** format , **open it -> login -> stamp/ROI -> inForm batch -> choose area(s) for batch analysis.**
2. Click **"Add images" or "Add slides" -> .im3 or .qptiff from step 1 -> "Run"**.

vi. Addition of scale bar

1. In inForm , click **"tools" -> "show image info" -> find "Image Size"**.
2. Insert your exported image in Microsoft Powerpoint with **5 cm of transverse**, add **1 cm of scale bar**.
3. Calculate the **proportional expression of the scale bar x** with the example below:

   e.g., Image size from step 1: **0.69 mm** x 0.52 mm

   The proportional expression of a 1 cm scale bar: **0.69 mm** / **5 cm** = **x mm** / **1 cm**

   So, 1 cm scale bar means 138 µm

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