# Standard Operating Protocol

# of

# Akoya Vectra Polaris™

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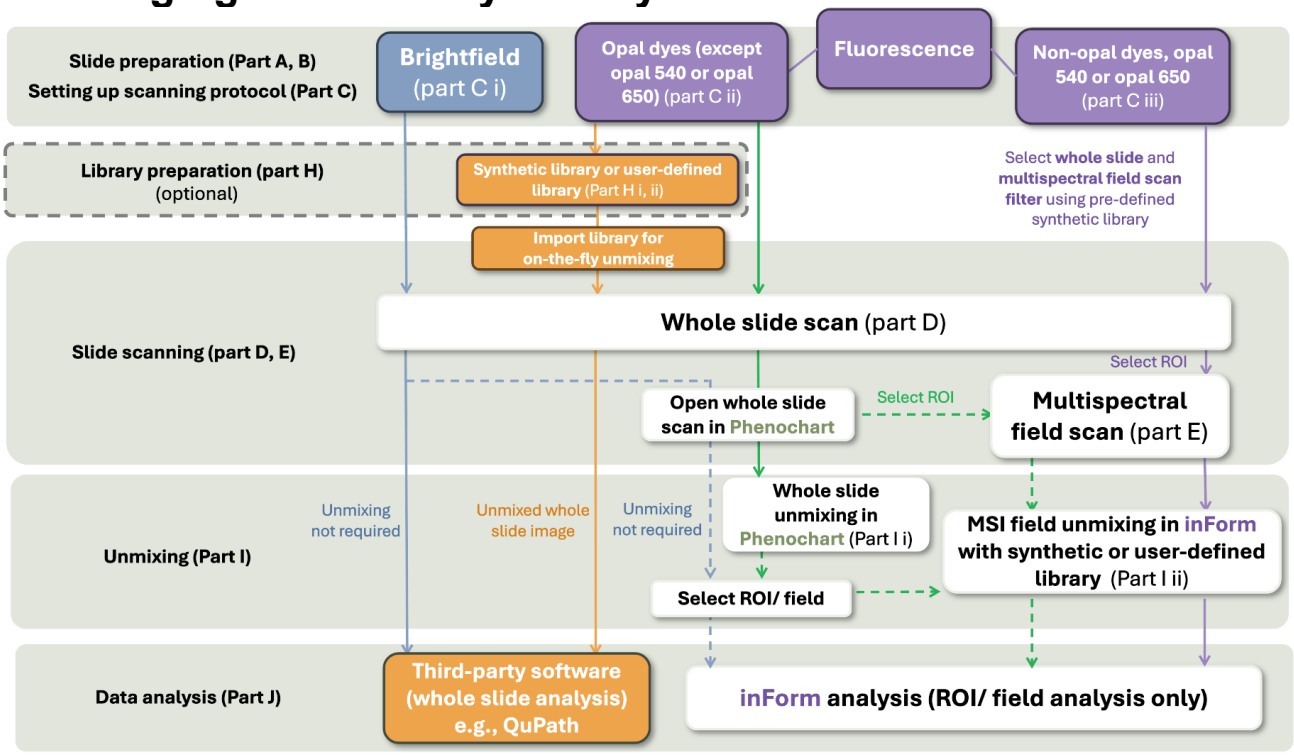



## A. Preparation of slides

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

| Types of slides | AR, MWT | Fluorophores (excluding DAPI) | DAPI | Purpose |
|---|---|---|---|---|
| **A. Library slides*** | | | | Collect the spectrum of each fluorophore and autofluorescence for creating a spectral unmixing library. |
| Unstained | + | - | - | |
| Single-stained # | - | + (1 slide for each fluorophore) | - | |
| | - | - | + | |
| **B. Monoplex slides^** | + | + (1 slide for each fluorophore) | + | Assess staining performance |
| **C. Multiplex slide@** | + | + | + | Study samples |

* Use study tissue type
# Omitted for opal fluorophores or fluorophores with established spectra 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

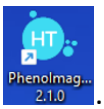
For example, a 7-color multiplex assay should involve 15 slides:

- 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

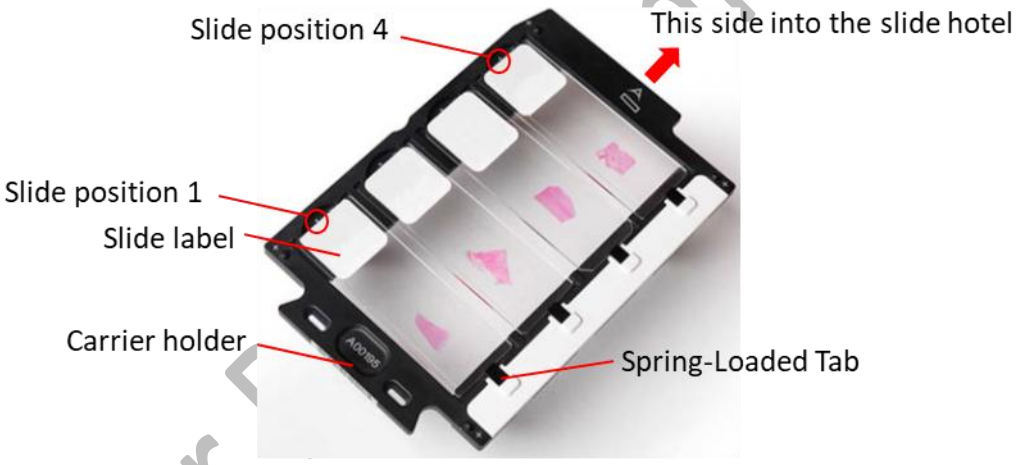
## B. Initialization of Vectra Polaris

1. Fill in the logbook. Write the **actual** start time.
2. Turn on Vectra Polaris.
3. Turn on the computer (account: User; password: (at the bottom left of the monitor)).
4. Launch PhenoImager HT2.1.0 software 

.
5. **Equilibrate** the slides at room temperature.
6. **Clean** the slides with 100% ethanol.

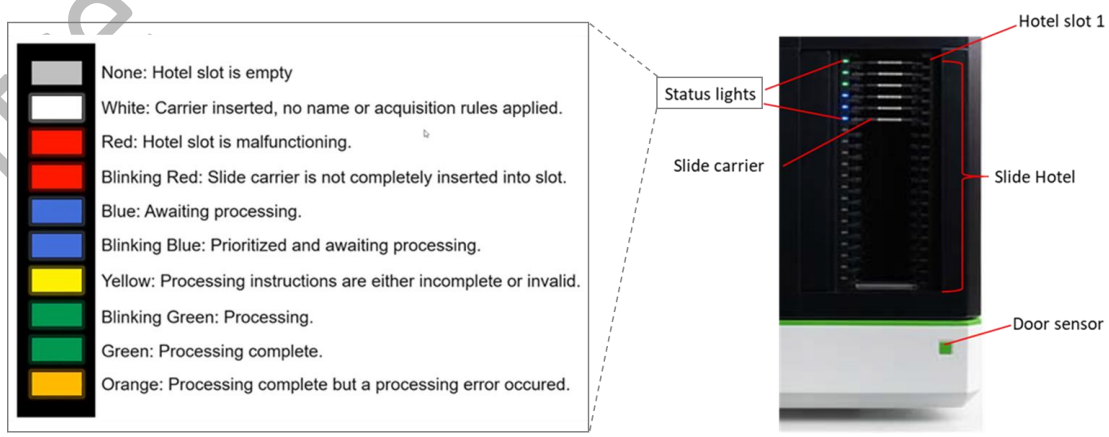
> ⚠ Always clean your slides with 100% ethanol before scanning because there is dirt unobservable by eyes but detectable by camera.

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.



# C. Setting Up Scanning Protocols

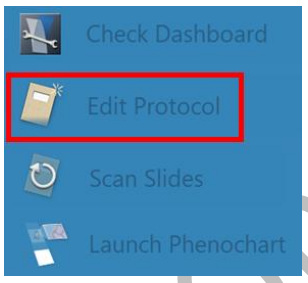
## i. Bright-field scanning

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

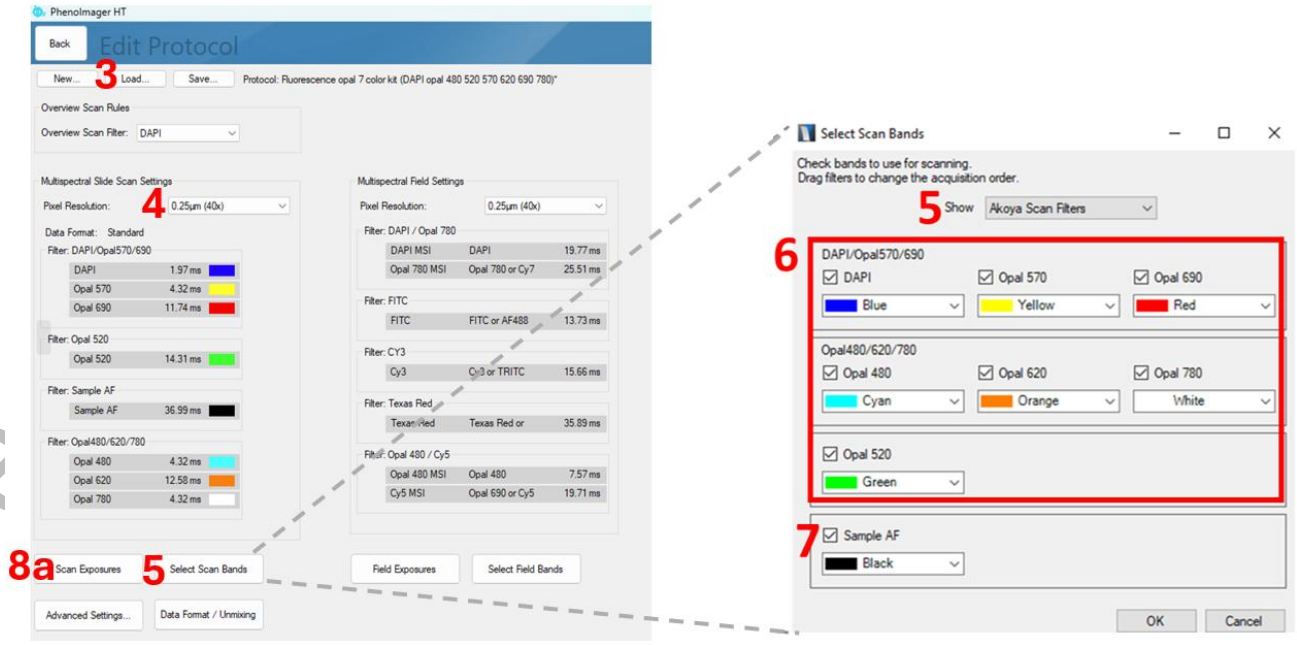
## ii. Opal fluorescence scanning without unmixing (excluding opal 540 and opal 650)

1. Copy **Fluorescence opal 7 color kit (DAPI opal 480 520 570 620 690 780).ppr** from **D:\Data\PhenoImagerHT_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 deselect those unused.
7. Select the **"Sample AF"** filter to allow unmixing of autofluorescence and sample in Phenochart software.

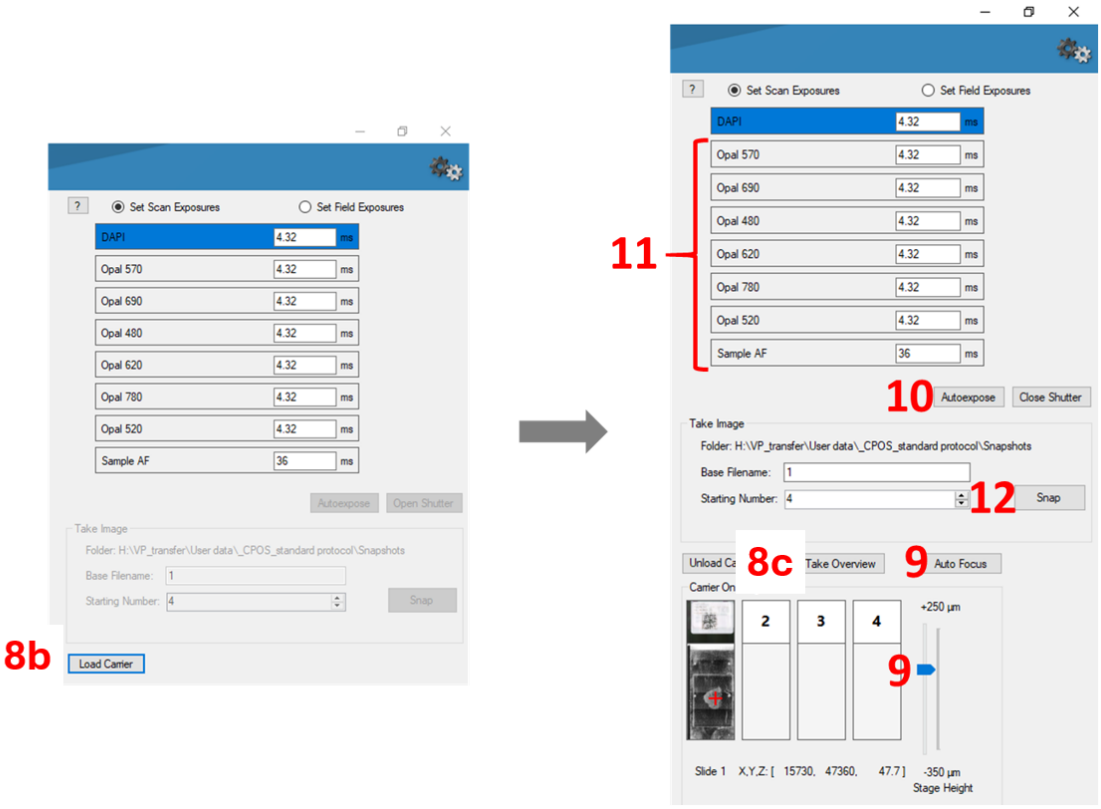



8. a. Click "**Scan Exposures**" b. Click"**Load Carrier**" c. Click "**Take Overview**"
9. **Drag** the focus bar to bring the specimen closer to the focus **->** click **"Auto Focus"**.

10. **Drag** the slide to the 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.

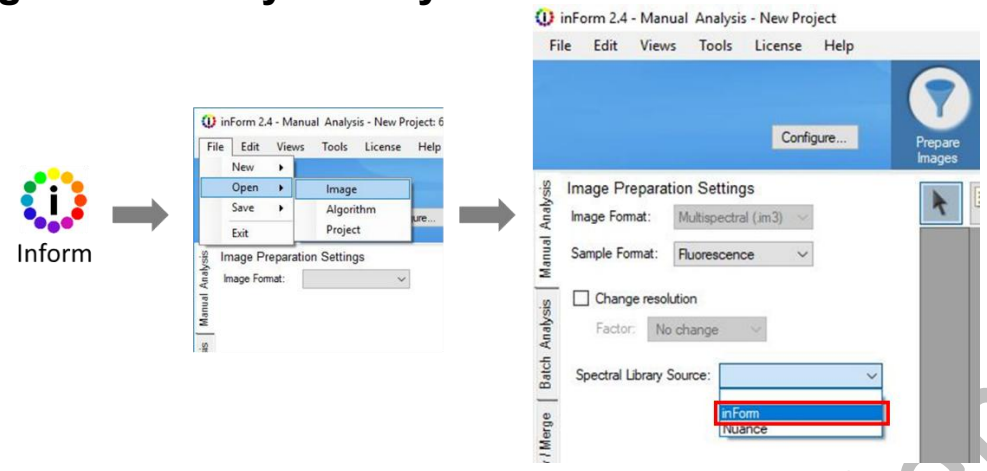
11. Repeat step 10 with each fluorescence filter.
12. Click **"Snap"** to take an example image of the slide.



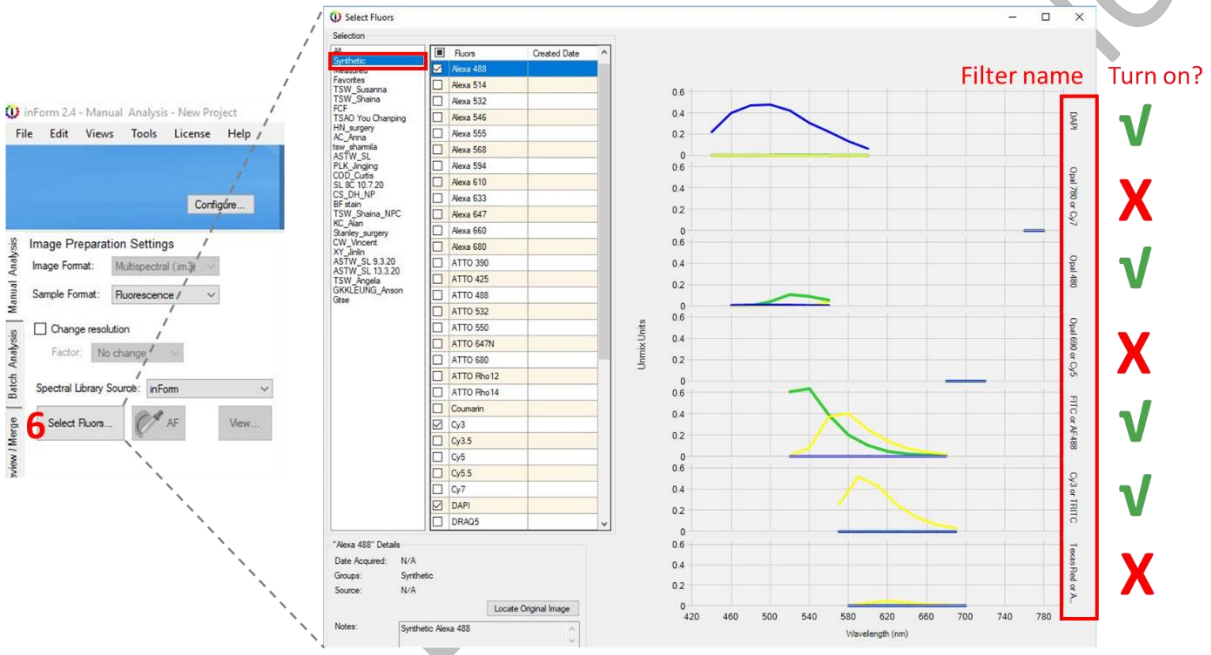

13. Go to **"snapshots" folder** in your folder to see the snapped image. You can always manually adjust the exposure time if you are not satisfied with the image as long as it does not overexpose.
14. Click **"Back"** -> **"Save"** to save the protocol.
15. Click **"Back"** to return to the Home Page.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

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

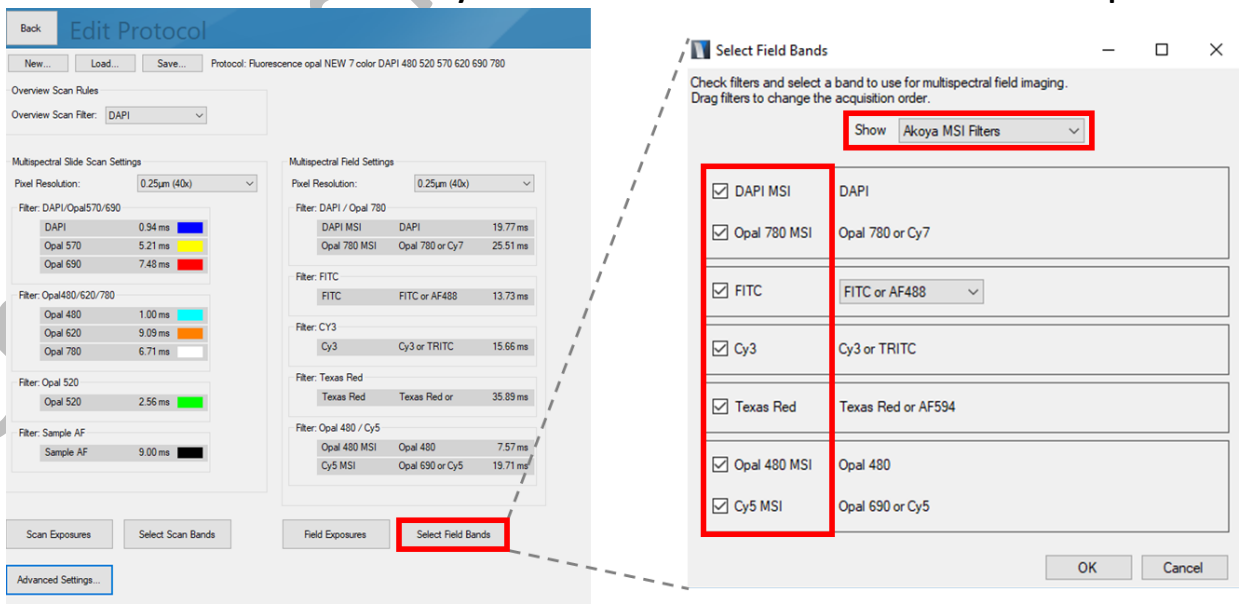
1. Copy **Fluorescence NON-opal or opal 520 opal 650.ppr** from **D:\Data\PhenoImagerHT_CPOS_standard protocol** to your own folder.
2. Follow **steps 2-7 in Part C ii** to select appropriate resolution & filters for first scanning (Multispectral Slide Scan). If your first scanning data is ONLY for selecting fields to do second scanning (multispectral field), you could select "pixel resolution" as 10X(1um) and filters "DAPI" & "AF" for the first scanning, which could shorten scanning time.
3. In **"Multispectral Field Settings",** choose the **pixel resolution** of interest.
4. Open InForm software, click **"File" -> "Open" -> "Image" -> "example.im3**  **" D:\Data\PhenoImagerHT_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 5.**



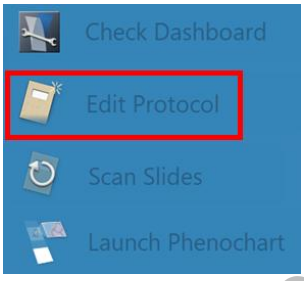

7. Follow **steps 8 – 11 in Part C ii** to set the exposure for the first scanning (slide scan)

8. Select **"Set Field Exposures"** -> follow **steps 9 – 12 in Part C ii** to set the exposure for the second scanning (field scan).
9. Click **"Back"** -> **"Save"** to save the protocol.
10. Click **"Back"** to return to the Home Page.

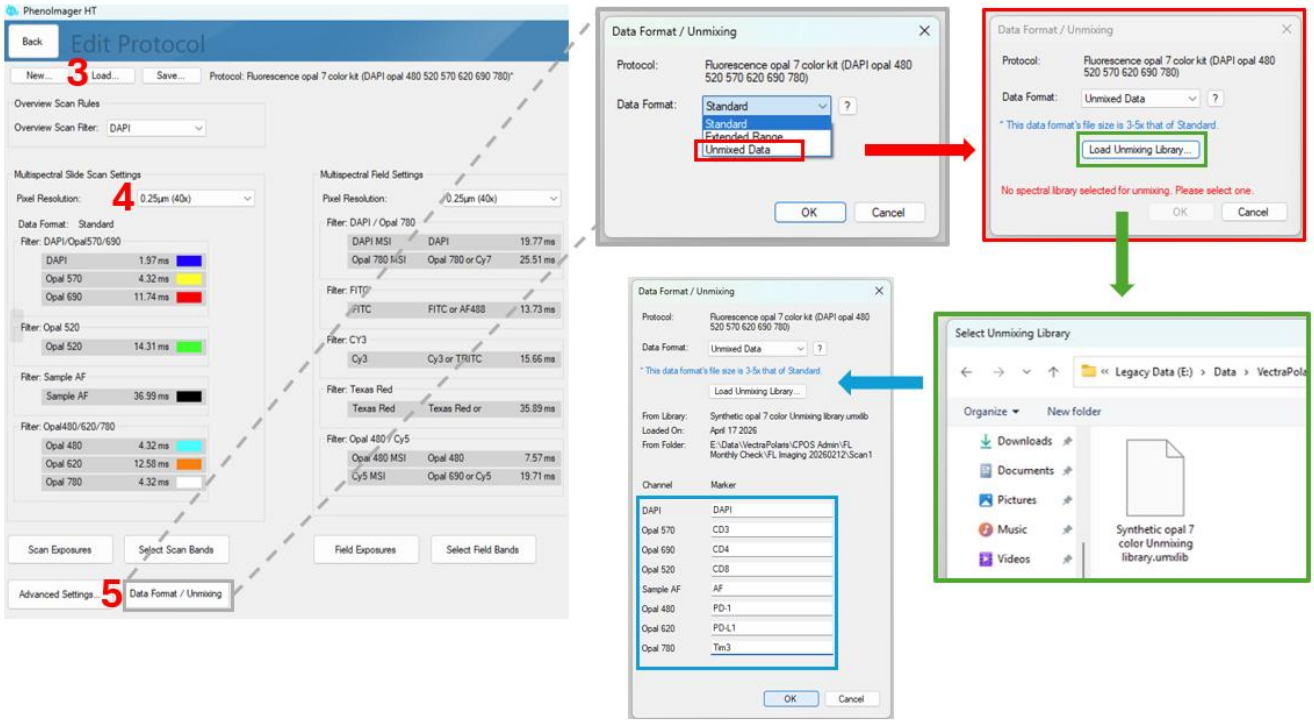
**iv. <u>Opal fluorescence scanning with on-the-fly unmixing (excluding opal 540 or opal 650)</u>**

1. Copy **Unmixing Fluorescence opal 7 color kit (DAPI opal 480 520 570 620 690 780).ppr** from **D:\Data\PhenoImagerHT_CPOS_standard protocol** to your own folder.
2. On the **Home** Page of Vectra Polaris software 

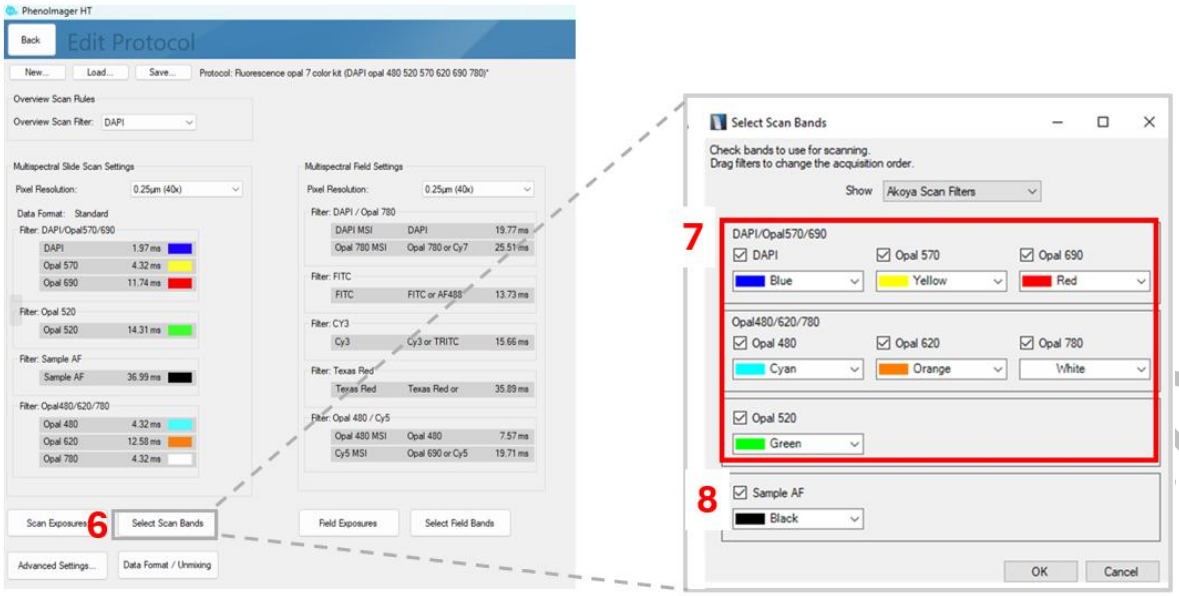
, click **"Edit Protocol"**.




3. **Load** the protocol template.
4. In **"Multispectral Slide Scan Settings",** choose the **pixel resolution** of interest.
5. Click " **Data Format/Unmixing**" **->** Select "**Unmixed Data**" **->** Click "**Load Unmixing Library**" you set **->** Name the Markers you paired with Opal dyes.
   (Notes: Please refer to part "**H. part i & H part ii**" in this protocol to prepare your unmixing library.)



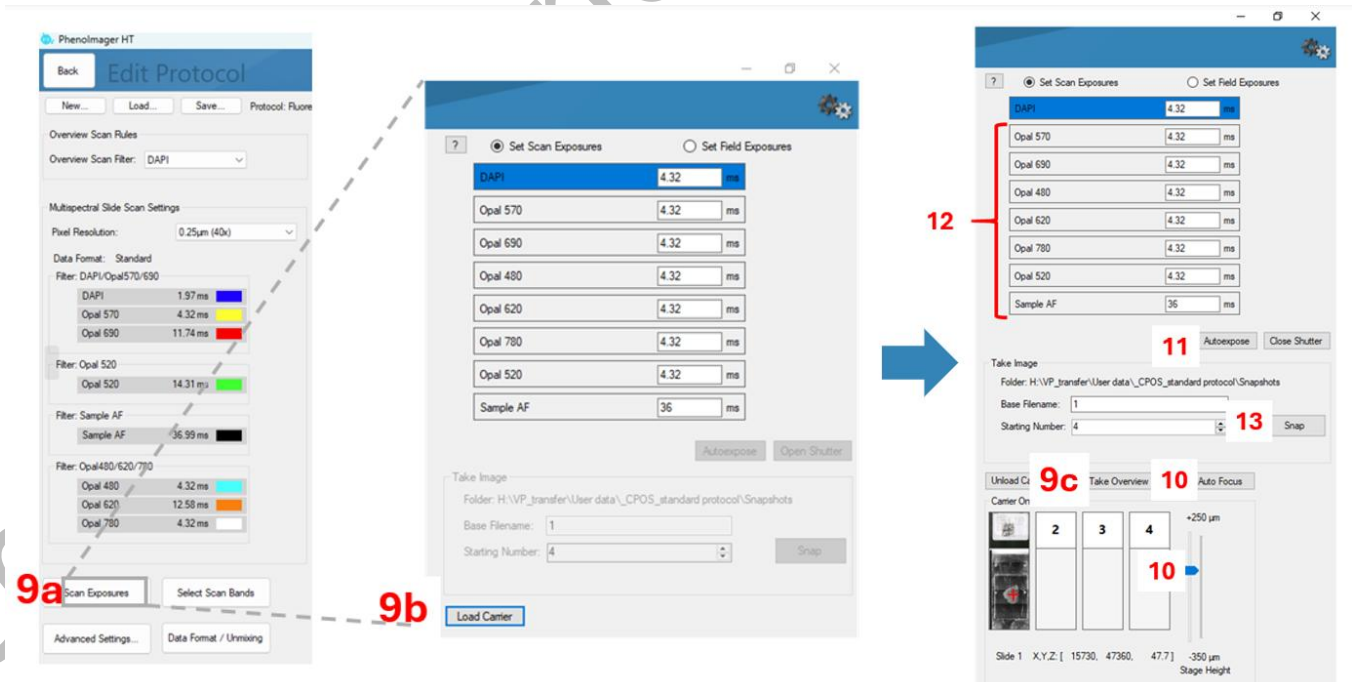

6. Click **"Select Scan Bands" -> Show "Akoya Scan Filters".**
7. Select the filters for opal fluorophores used and <u>de</u>select those unused.
8. Select the **"Sample AF"** filter to allow unmixing of autofluorescence and sample in Phenochart software.




9. a. Click "**Scan Exposures**" b. Click"**Load Carrier**" c. Click "**Take Overview**"
10. **Drag** the focus bar to bring the specimen closer to the focus **->** click **"Auto Focus"**.
11. Drag the slide to the 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.

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

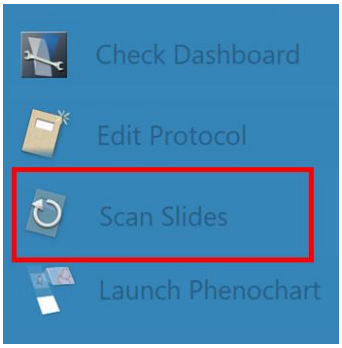



14. Go to the **"snapshots" folder** in your folder to see the snapped image. You can always manually adjust the exposure time if you are not satisfied with the image as long as it does not overexpose.
15. Click **"Back"** -> **"Save"** to save the protocol.
16. Click **"Back"** to return to the Home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

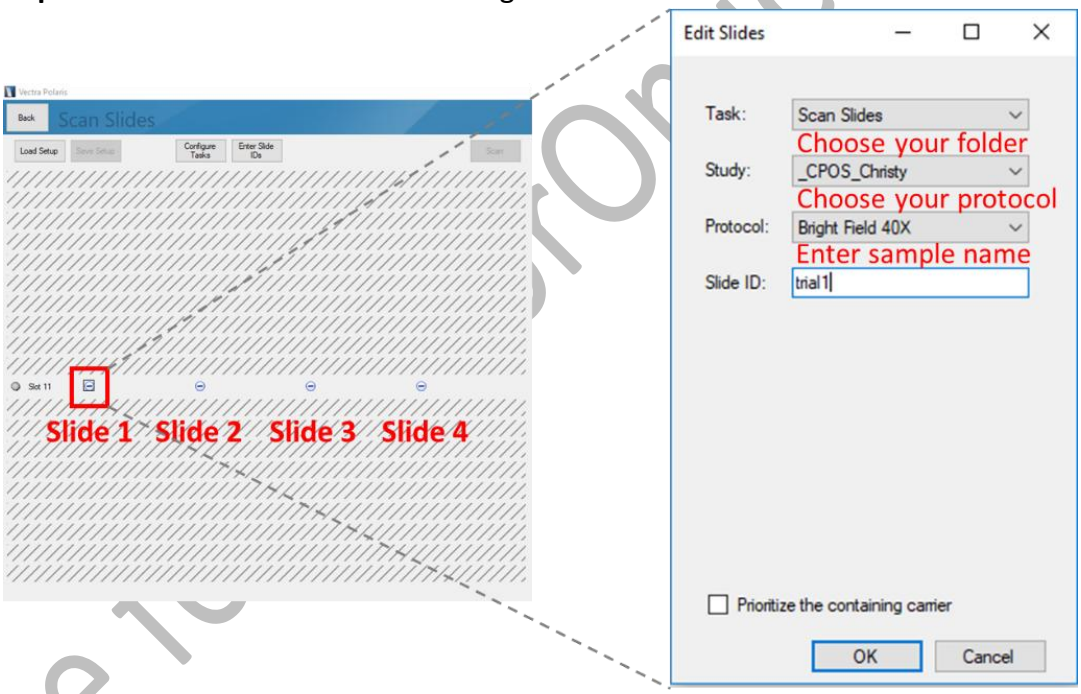
17. Go to Part D.

## D. Whole Slide Scan

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



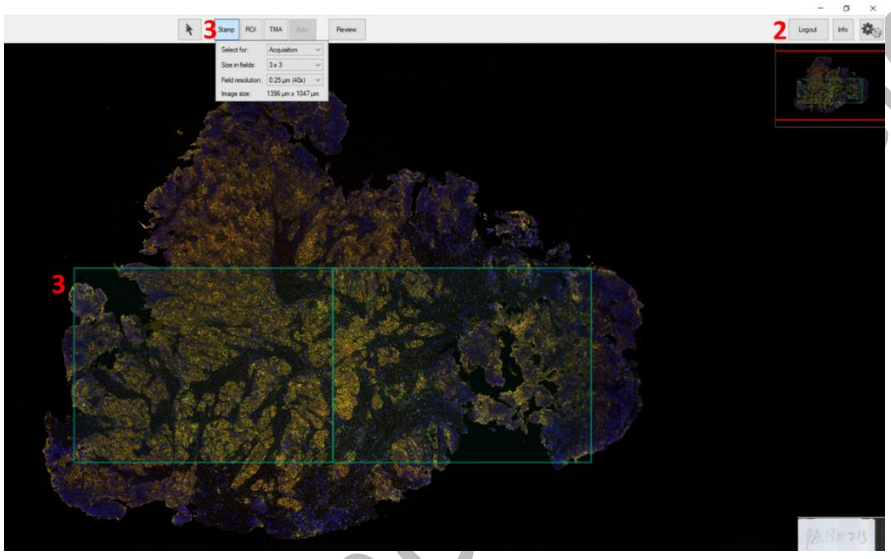

2. Click slide **status icon -> Task: Scan Slides -> Study: your folder -> Protocol: protocol set up in Part C -> Slide ID: sample name -> "Scan"** to start scanning.




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

## 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** composed 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.

## F. Transfer Data

> ⚠ 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.

Transfer your data to your data transfer server by either the Vectra Polaris online PC or the Vectra Polaris offline station. A **USB drive** is not allowed to be inserted into either the Vectra Polaris online or offline PC.

## G. System shutdown

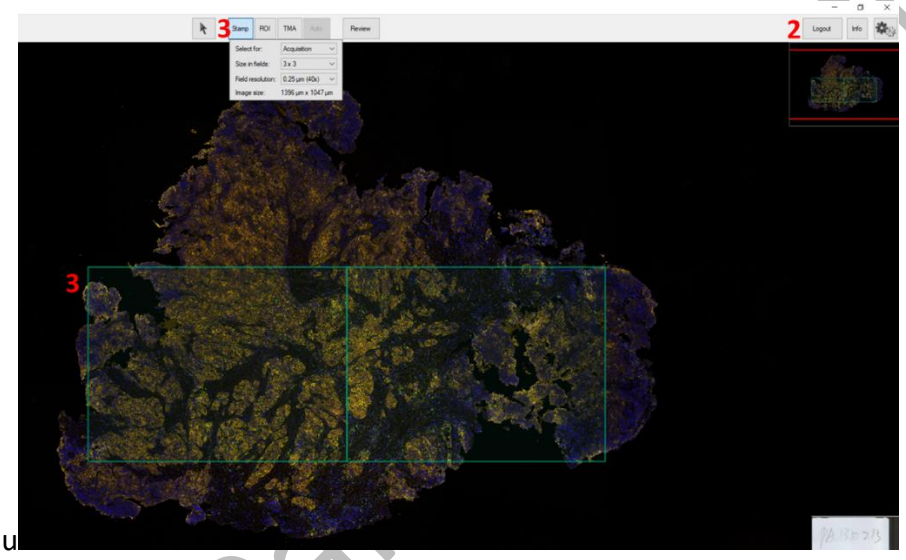
1. Take back your slides and return the carriers.
2. Close the door of Vectra Polaris.
3. Log off your PPMS Tracker.
   (4-6 **ONLY** for experienced users during non-office hours)
4. Exit PhenoImager software.
5. Shut down the computer.
6. Shutdown Vectra Polaris.

# H. Library preparation

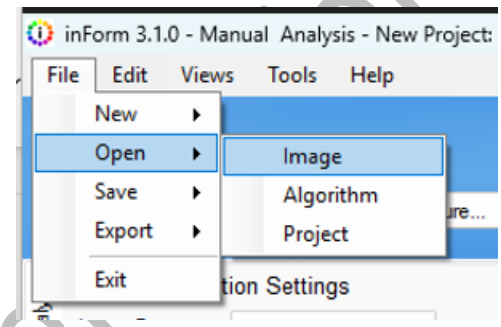
## i. Synthetic Unmixing library Build

(If monoplex-stained slides of Opal dyes are not available, a synthetic unmixing library can still be constructed. However, its performance will be suboptimal compared with a user-defined library, as synthetic spectra cannot fully account for batch-specific staining variations and intrinsic tissue autofluorescence background.)

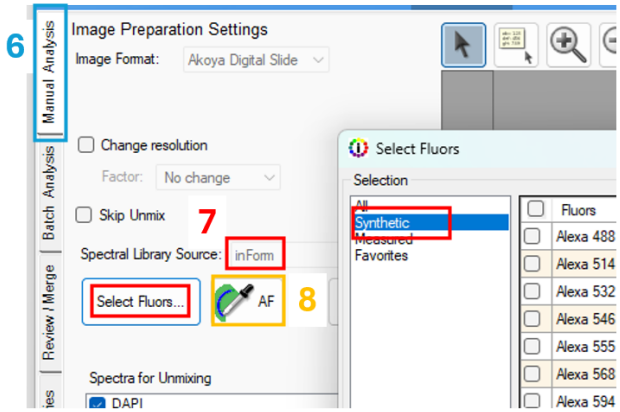
1. Open the standard **.qptiff** file of your unstained sample with **PhenoChart**.
2. **Click "Login" -> type anything -> "OK"**.
3. **Annotate** "**Inform Project**" Fields on the image:
   - Click **"Stamp" -> select for: "Inform Project" ->** choose fields of interest



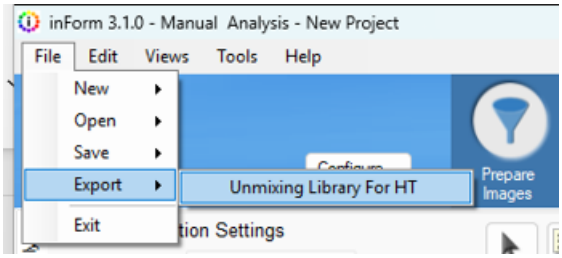

4. **Delete** unwanted annotation(s) by pressing "Ctrl" while clicking on them.
5. In the inForm software, select **File -> Open -> Image -> select the** qptiff data you annotated at step 3.




6. Click **"Manual Analysis"**.
7. Select **Spectral Library Source": "inform" -> "Select Fluors" -> "Synthetic" for officially established fluor spectrum -> select the fluors to be unmixed.**
8. To remove autofluorescence, click **"AF" -> highlight the brightest part on the unstained image -> "Prepare All"** to unmix.




9. Click **“File” -> “Export” -> ”Unmixing Library For HT” to export the synthetic unmixing library.**



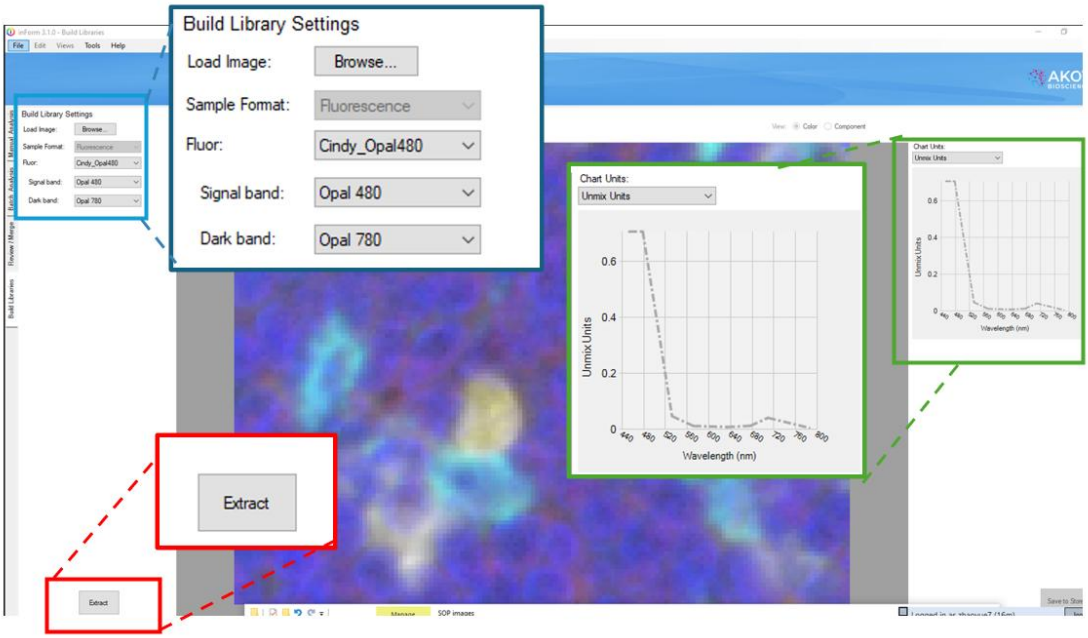

## ii. <u>User-defined Unmixing library Build</u>

1. Launch inForm software .
2. Click **“Tools” -> “Stain Store Manager” -> “Choose Which Types of Stains and Fluors Your Lab Uses” ->** Click “**Other (User Specified)” -> “New” -> enter the name of the new stain -> choose fluorescence -> select if it is a nuclear counterstain -> “OK”**.

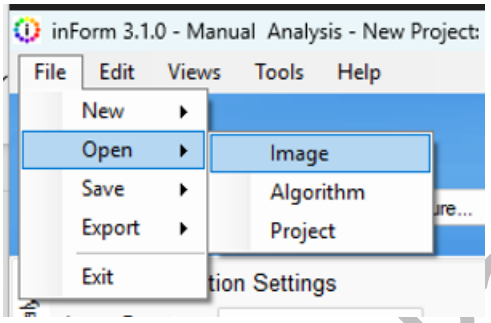
3. Open the standard **.qptiff** file of your monoplex image of your stain and unstained with **PhenoChart** . Follow **steps 2- 4 in H part i** to annotate the region.

4. Click **“Build Libraries” -> “Browse” -> open monoplex image of your stain** ( ).

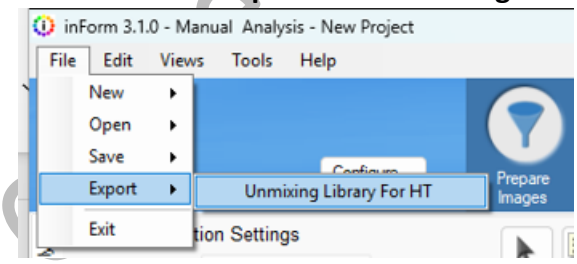
5. For the **Fluorescent** sample, select stain -> -> highlight the fluor on image -> choose a **signal band** (where the fluor most strongly expresses) and a **dark band** (where the fluor does not express at all) -> **“Extract”** -> **“Save to Store”** under your own Group.




6. Open whole slide scan data (standard qptiff data) you annotated with **PhenoChart**. In the inForm software, select **File -> Open -> Image -> select the** qptiff data of the unstained you annotated at step 3.




7. Click **"Manual Analysis"**.
8. Select **Spectral Library Source": "inform" -> "Select Fluors" -> "The group you build" -> select the fluors to be unmixed.**
9. To remove autofluorescence, click **"AF" -> highlight the brightest part on unstained image -> "Prepare All"** to unmix.
10. Click **"File" -> "Export" -> "Unmixing Library For HT" to export the synthetic unmixing library.**

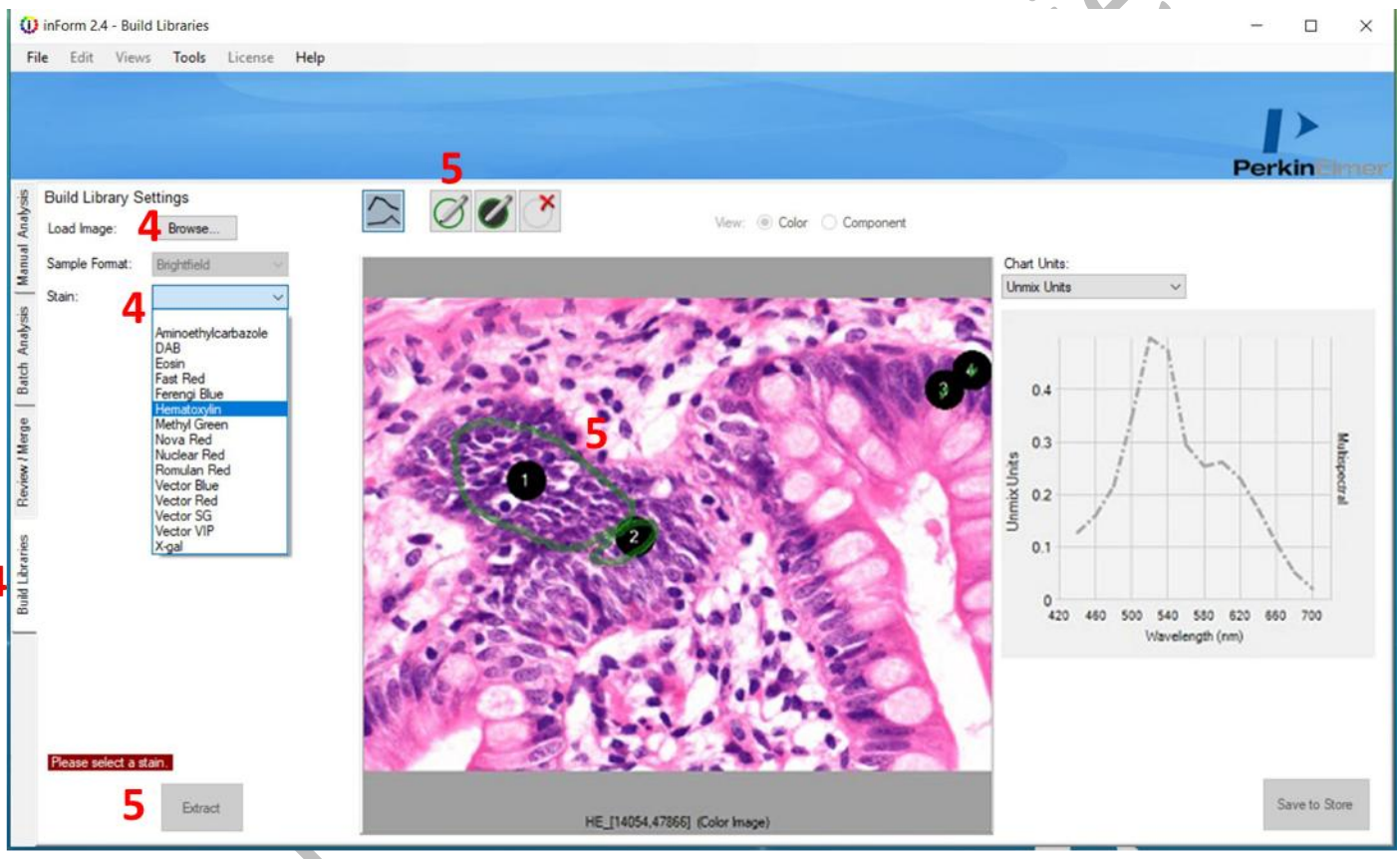



Follow step 2 in Part D, select **Task: Acquire Fields instead, while other settings remain unchanged**

### iii. Library for Brightfield

1. Open the standard **.qptiff** file of the Bright Field scan with **PhenoChart**.

2. **Click "Login" -> type anything -> "OK"**.
3. **Annotate** "**Inform Project**" Fields on the image:
   - Click **"Stamp" -> select for: "Inform Project" ->** choose fields of interest
6. Launch inForm software .
7. Click **"Tools" -> "Stain Store Manager" -> "Choose Which Types of Stains and Fluors Your Lab Uses" ->** click "**Other (User Specified)" -> "New" -> enter the name of the new stain -> choose brightfield or fluorescence -> select if it is a nuclear counterstain -> "OK"**.
8. Click **"Build Libraries" -> "Browse" -> open annotated qptiff**
9. For the **Brightfield** sample, **select stain ->** **-> highlight the stain on image -> "Extract" -> "Save to Store"** under your group.



# I. Unmixing

## i. Whole slide unmixing (Opal dyes only, except opal 540 and opal 650)

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

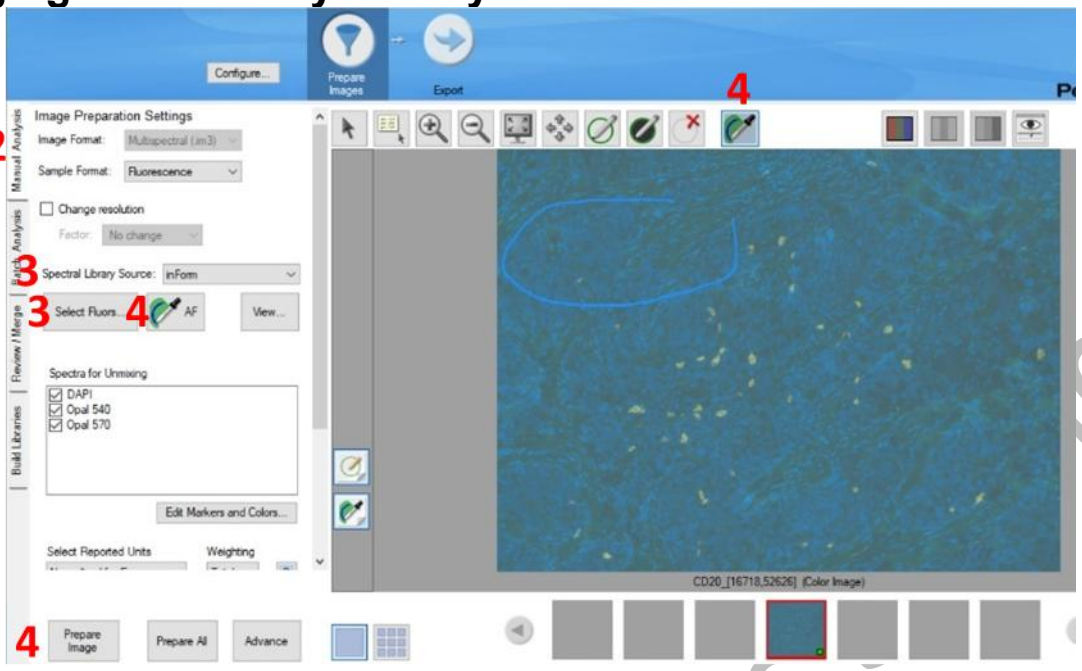
## ii. MSI field unmixing (For opal and non-opal dyes)

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

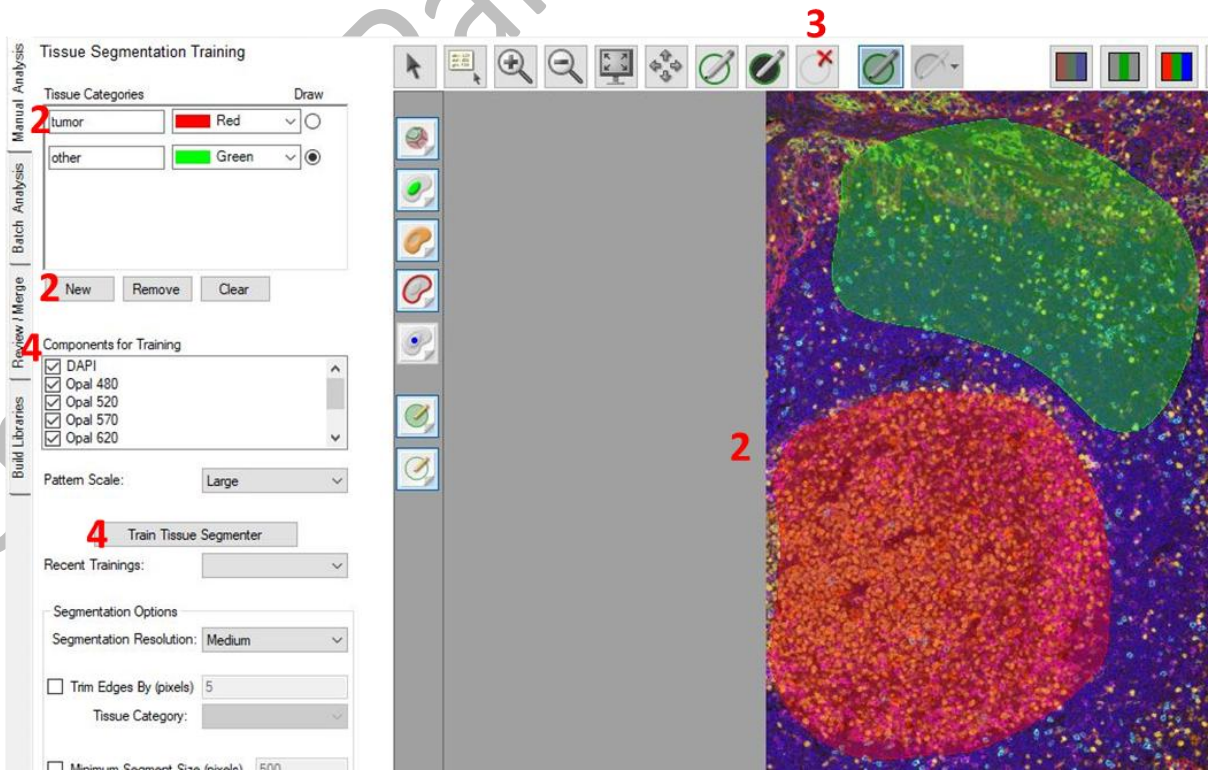
1. For an image with **qptiff** format , **open it -> login -> stamp -> inForm project -> choose area (s) for quantification**.
2. In inForm software , select **"File" -> "New" -> "Image" (.im3 or .qptiff from step 1)**.
3. Select **"Configure" -> "Trainable Tissue Segmentation", "Adaptive Cell Segmentation"**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

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



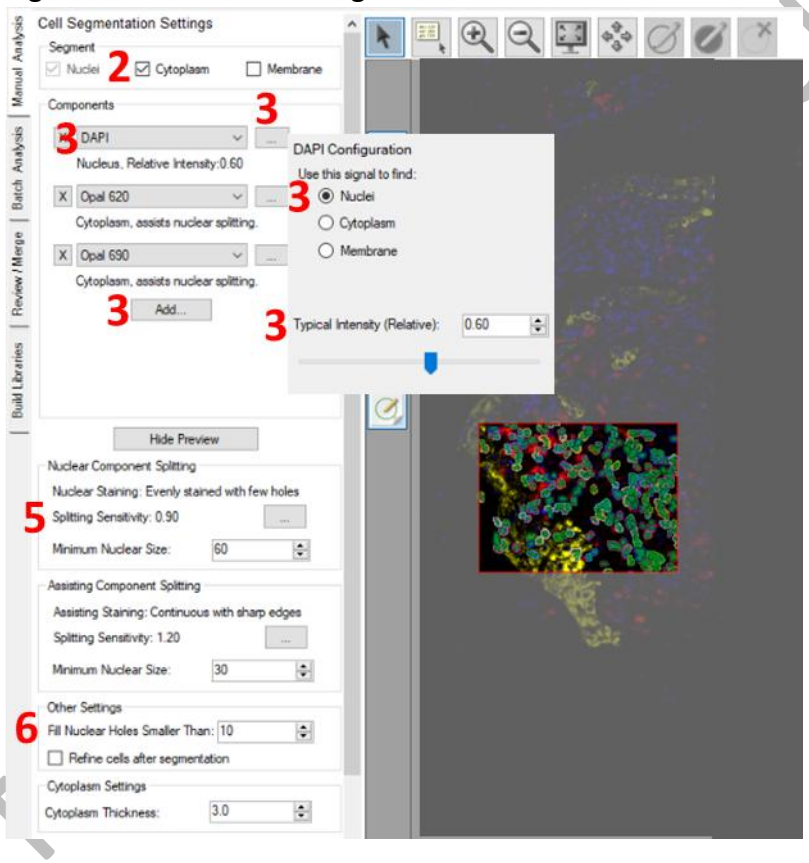

## i. Tissue segmentation

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%.**



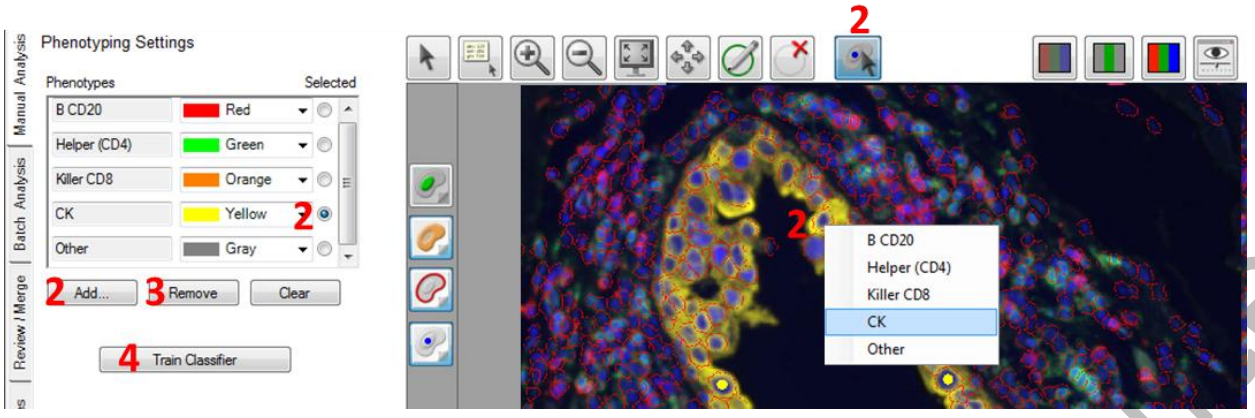
## ii. Cell segmentation

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




## 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.



### 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 an 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