“Flow” Cytometry…
With Tissue?!

Why and How to Use the Canopy ZellScanner Chip Cytometer

WELCOME!

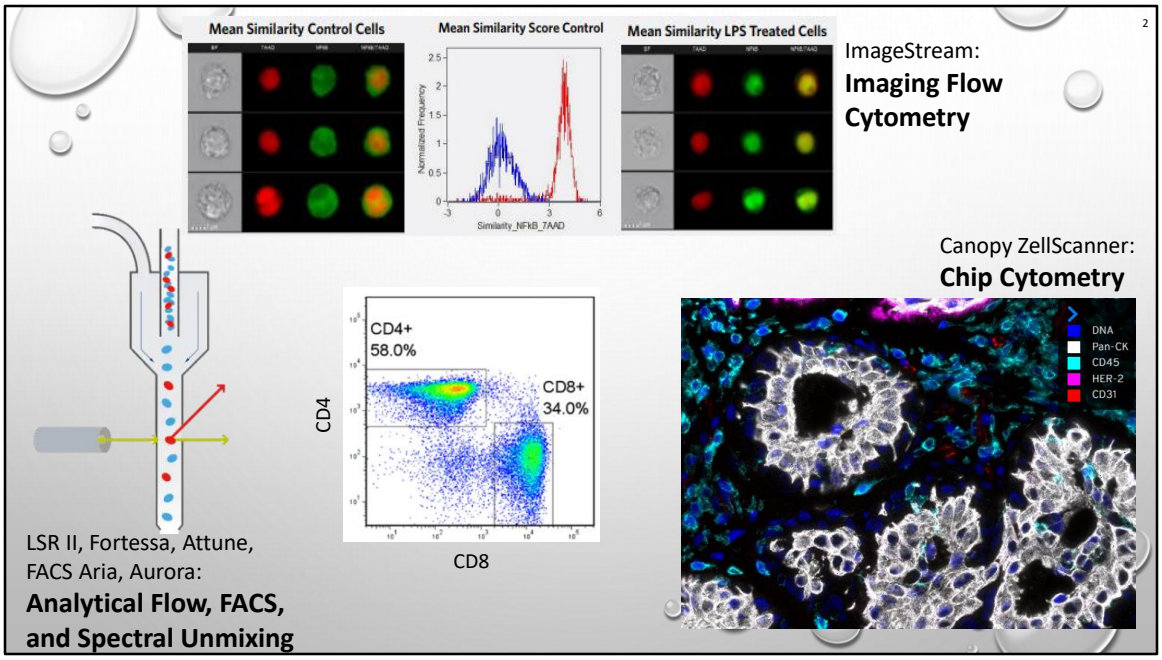
We’ll get started in a moment.

Please MUTE YOUR MICROPHONE unless you’re asking a question.

Feel free to ask questions at any point, via voice or over chat.

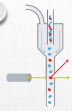
DO NOT FRET ABOUT COPYING EVERYTHING DOWN. **This webinar will be made available** on the Unified Flow Core website by Monday afternoon (2/28) at the latest. (You will likely need access to Pitt’s OneDrive… if you don’t have access, email Nicole at nrb18@pitt.edu and I’ll get you a copy.)

Feb 25, 2022



All 3 can make you a nice dot plot. But they answer different questions. And have different strengths/weaknesses.

What Kinds of Questions Can we Answer?



Analytical & FACS

- Identify/quantify different cell populations relative to each other.
  - E.g. What happens to the relative population of X when comparing test to control?
- (FACS) Sort out your favorite cell population(s).

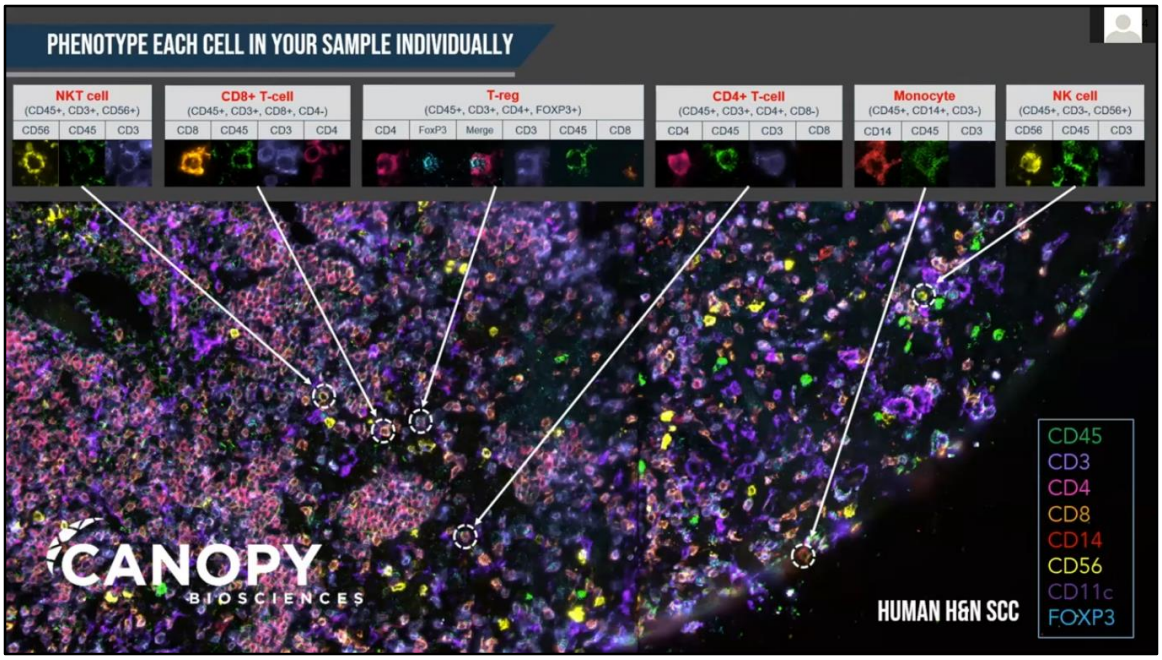
Imaging Cytometry

- Analytical, PLUS
- Answer questions of changes in subcellular:
  - Compartmentalization
  - Morphology
  - Colocalization

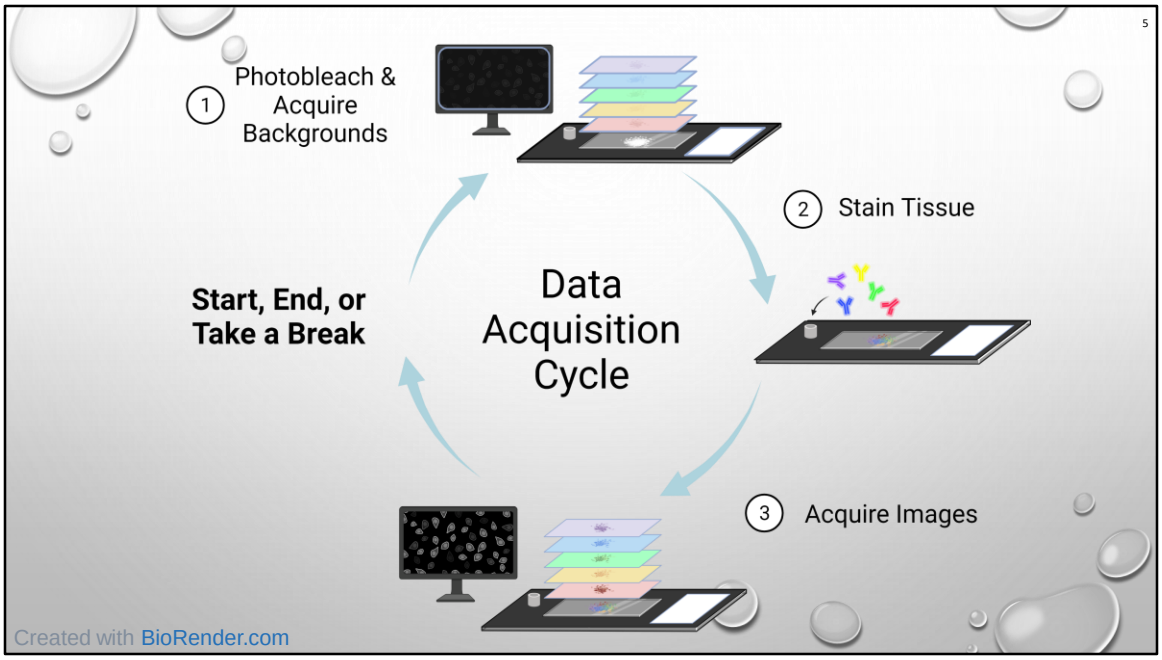
Chip Cytometry

- Analytical, Imaging Cytometry, PLUS
- Answer questions of
  - Cell – cell interaction and proximity
- Can define populations by physical location in tissue.
- Answer histology questions but with more colors (40+).

Imaging cytometry on the chip cytometer is not as advanced as for the ImageStream. If you can run your cells as a flow, do that.



Chip Cytometry is more quantitative than simple microscopy which is limited in the number of simultaneous colors.
Traditional flow cytometric dot plot analysis is more sophisticated than X costains with Y.

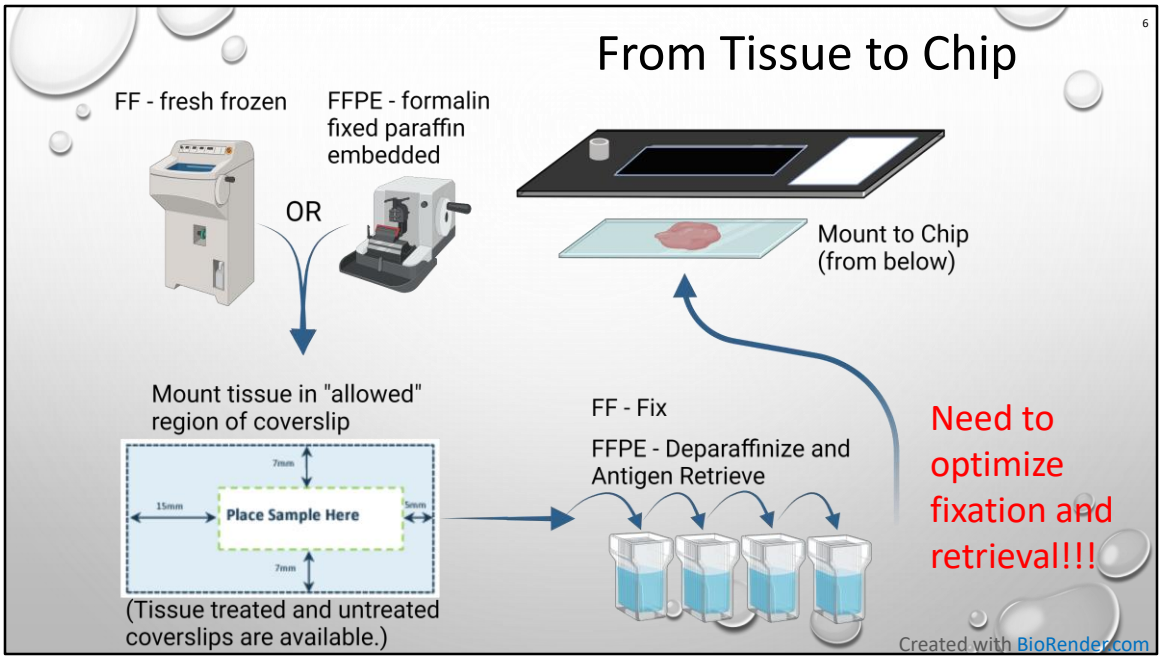


Each cycle can take 2 hours or more depending on staining considerations, number of channels used (don't have to use all 5), and # positions acquired.

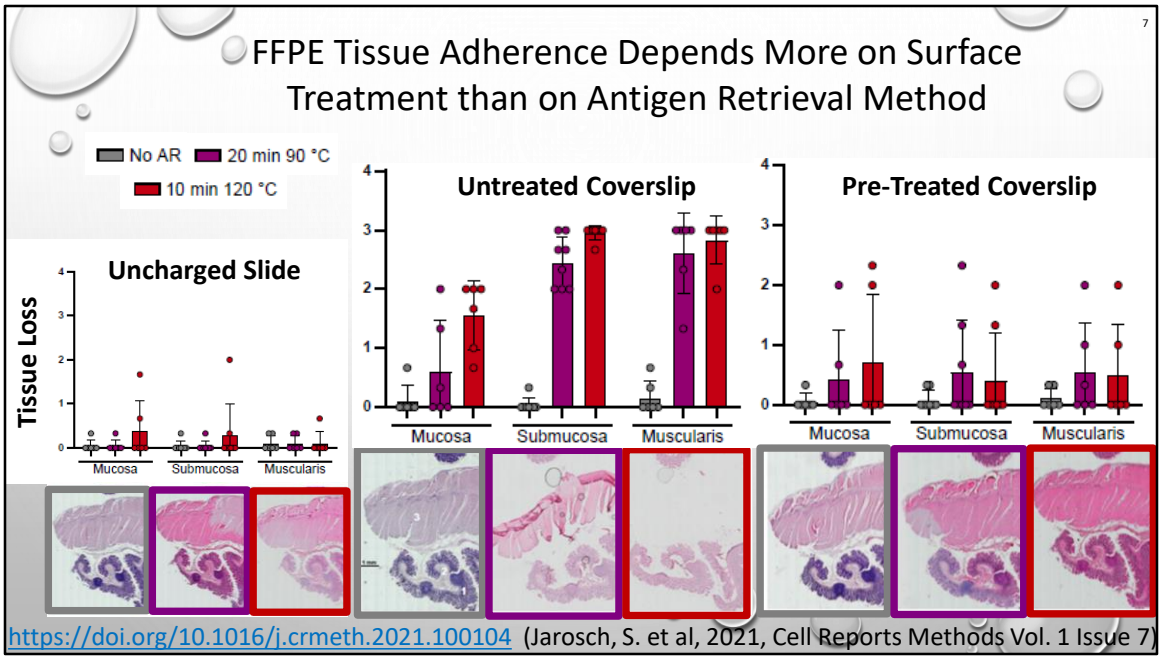
Tissue staining can take as long as it needs to. 15min-overnight. Whatever works. Wash can be automated with pump.

Can interleave two chips in a day once you've got everything figured out. E.g. Chip 1 is acquiring while Chip 2 is staining/washing.

Can photobleach OR chemically bleach, but acquiring BG images afterwards is not optional.



5-7um sections
FF = fresh frozen
FFPE = formalin fixed paraffin embedded. Don't forget to bake!!!
Must use COLD acetone to fix



Tissue Loss (Y-axis) is reduced for both AR conditions (red/purple) when comparing untreated/treated.
Mucosa (top) is pretty robust, so it doesn't really matter. Submucosa & Muscularis (bottom & middle) are more fragile, and benefit from the treatment.
Strangely, no one has any issue with an uncharged slide…

Unfortunately, I just found out this morning, that Canopy won't be selling the tissue treated coverslips anymore.

I emailed Canopy and am waiting to hear back what they suggest.

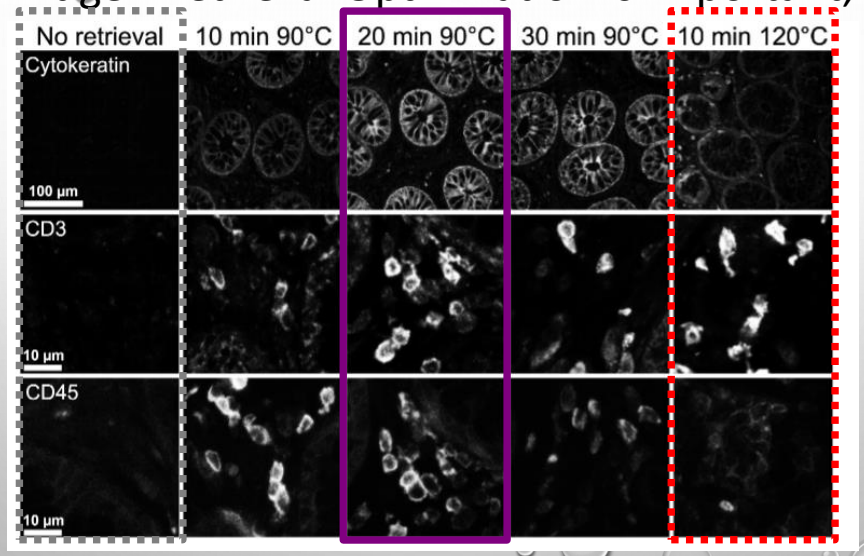
(Worst case, researchers may have to treat the coverslips themselves?)

If so, please let me know what works best for your FF and FFPE tissue:

poly-L Lysine, poly-ornithine, polysilane, or something else??

We essentially want "Superfrost Plus" treated coverslips, which are polysilanized???

Antigen Retrieval Optimization is Important, too



https://doi.org/10.1016/j.crmeth.2021.100104 (Jarosch, S. et al, 2021, Cell Reports Methods Vol. 1 Issue 7)

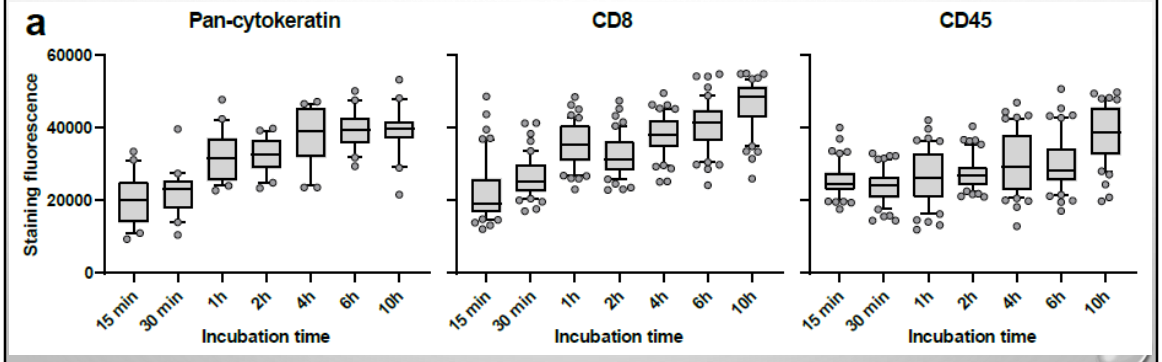
For reference these are the conditions in the previous slides. YMMV based on antigen, but purple seems to give a better overall result with both adherence and fluorescence.

What about Fresh Frozen Tissue (FF) Adherence?

- Allow sections on coverslips to rest at -80°C after sectioning for at least an overnight.
  - Maybe this dries them out a little (like baking FFPE coverslips)?
- Use **VERY COLD** acetone when fixing. Room temperature buffers are okay for subsequent steps, once the tissue is securely fixed. (Says Tony Green of Pitt Biospecimen Core)
  - "Very Cold" means ≤ -20°C.
- Unlike FFPE, you MUST USE pre-treated coverslips with FF tissue (not optional).
- We are still trying to understand which are the critical steps in improving FF tissue adherence for Zell Chips. Please let me know if you discover any tricks or tips.

Remember: Fixation happens BEFORE you mount the coverslip on the chip.

Optimize Antibody Staining Time for Best Results

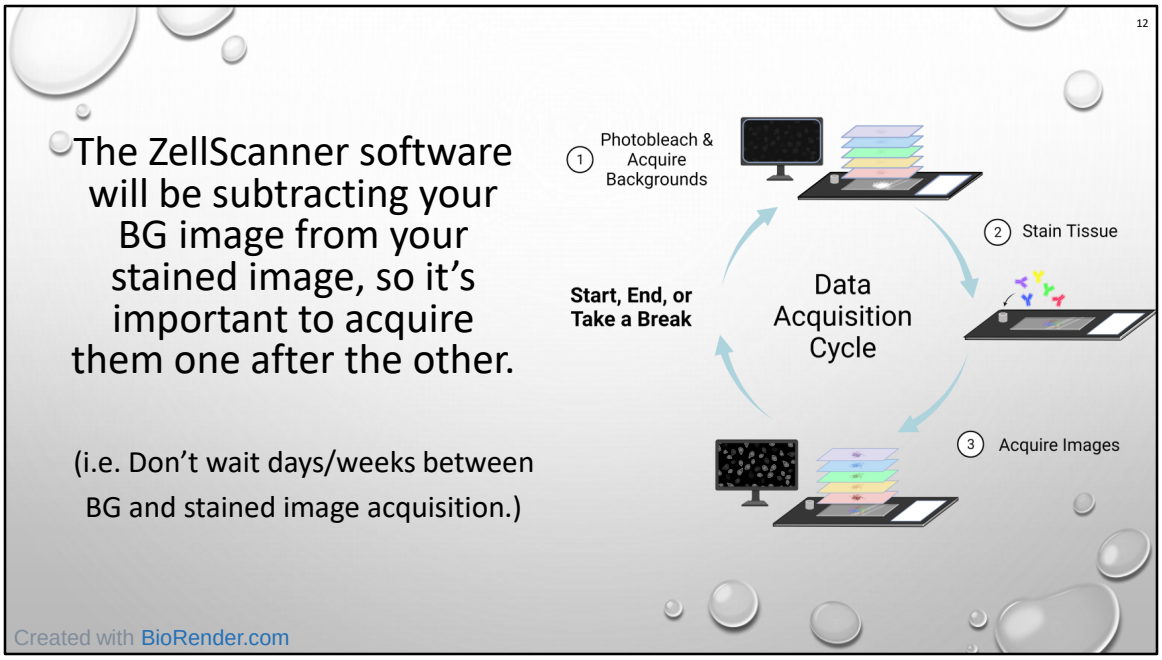


FFPE staining times

Either 1 hour or overnight seems to make sense here…

https://doi.org/10.1016/j.crmeth.2021.100104 (Jarosch, S. et al, 2021, Cell Reports Methods Vol. 1 Issue 7)

Critical times seem to be 1 hr and Overnight.



Each cycle can take 2 hours or more depending on staining considerations, number of channels used (don't have to use all 5), and # positions acquired.

Tissue staining can take as long as it needs to. 15min-overnight. Whatever works. Wash can be automated with pump.

Can interleave two chips in a day once you've got everything figured out. E.g. Chip 1 is acquiring while Chip 2 is staining/washing.

Can photobleach OR chemically bleach, but acquiring BG images afterwards is not optional.

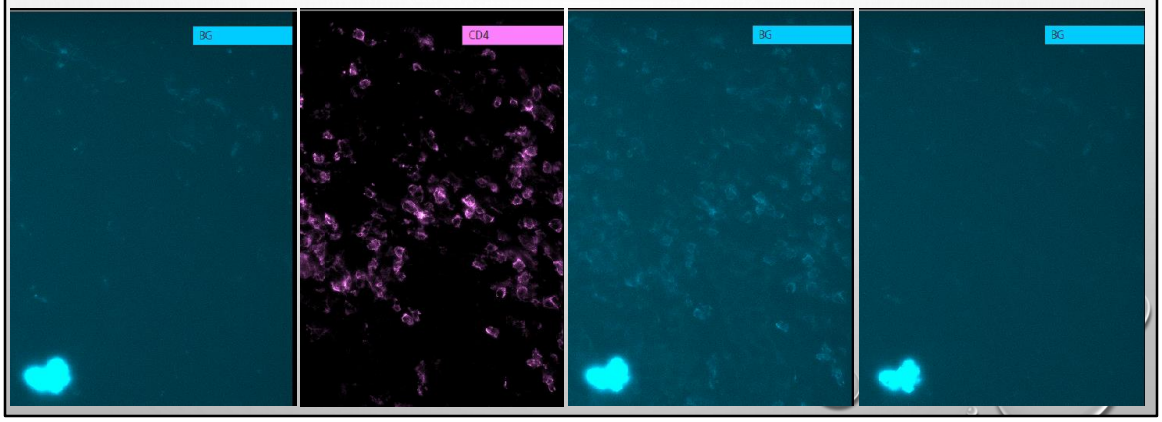
Always Check that the Bleach Round was Effective!!

BG after initial photobleach

Stain, then scan.

BG after first photobleach

BG after second photobleach

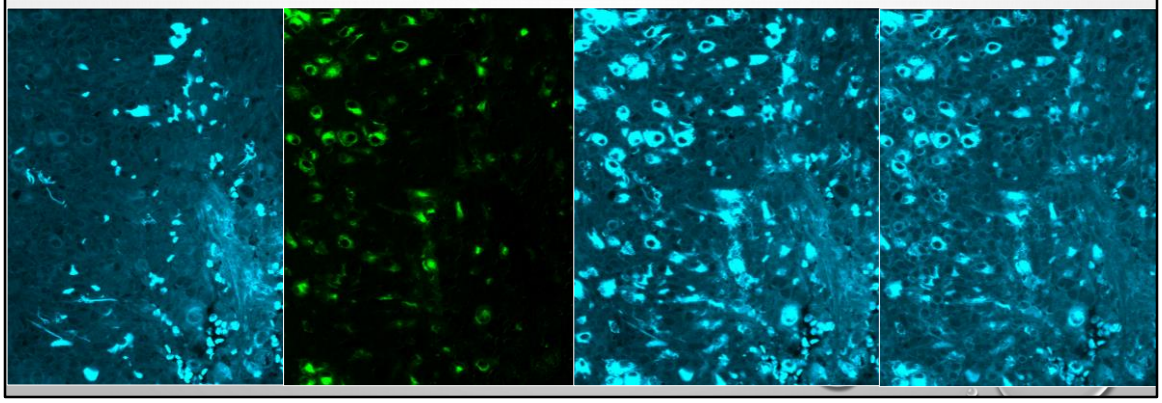


The point of a BG image is to subtract it from the stained/fluorescent issue. Therefore it's important that you don't have ghosts of previous stains.

Bright spot is not a big deal; it will be subtracted away. Since maxed out, no stain will work in that little blob region.

Strong Ab's are Difficult to Bleach, so **Dilute them** and/or **Run them after Weaker Ab's** for the Same Channel

BG after initial photobleach | Stain, then scan | BG after first photobleach | BG after fourth photobleach



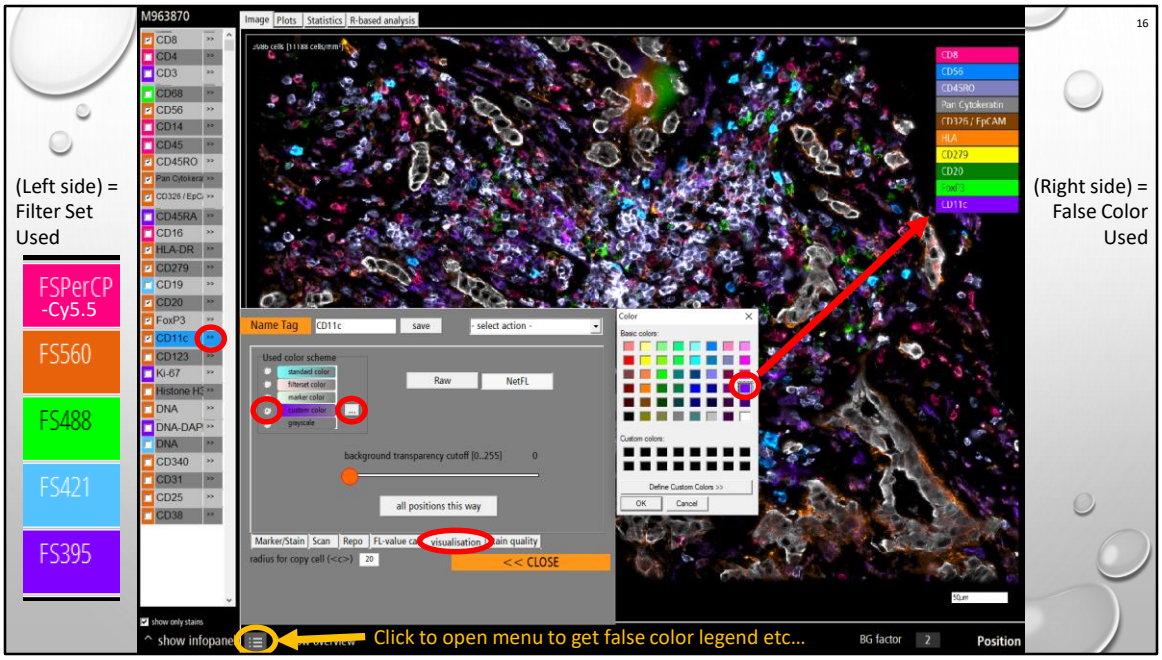
Green (FS488) channel is notorious for autofluorescence in some tissue types esp. if too much Ab retrieval.

Chemical bleaching* is an option, but you have to make sure it doesn't kill your specific epitopes!
PBS+24mM NaOH+4.5%H2O2 Lin et al 2016

Q: So I will be reusing the same 5 colors for 40+ different markers by bleaching and restaining? How can I distinguish CD3-PE from CD45-PE??

A: Yes! The data are stored as co-registered (i.e. aligned) black and white images in separate layers. You can false color and analyze each layer however you like.

The secret to this method is that the images are coregistered/aligned so they can make a nice big stack. This lets you turn layers on/off however you like.

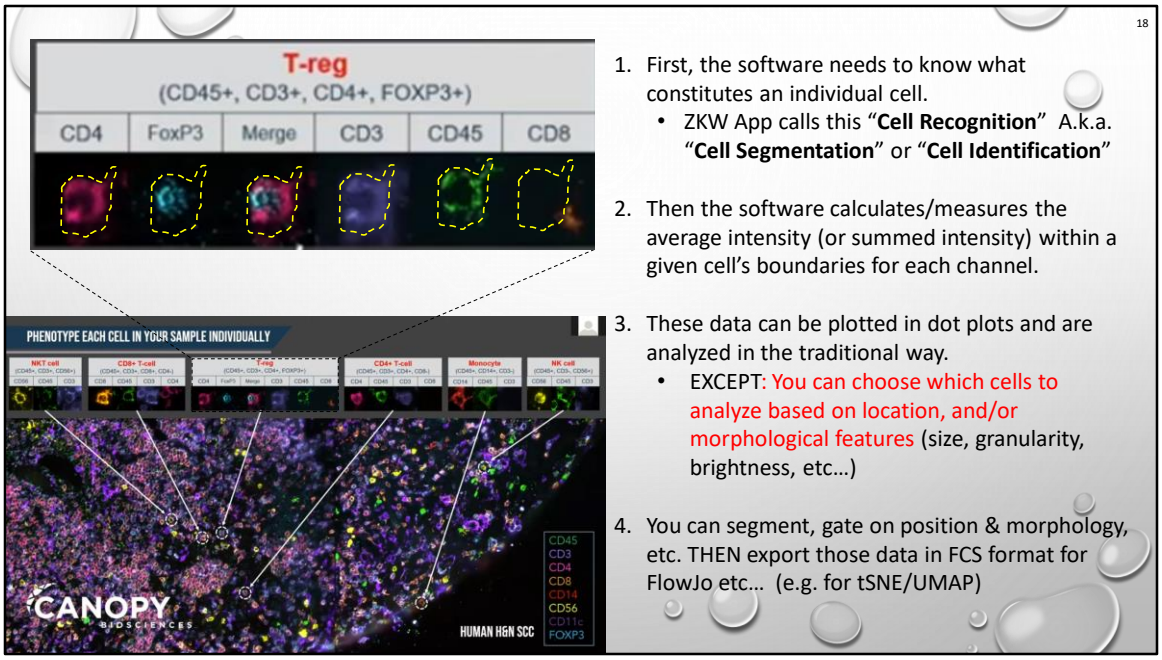


Bleaching is important because you will be using the SAME filterset (color) with DIFFERENT antibodies.

In all, 17 different antibodies conjugated to PE were used on this tissue sample.
As you can see, I ran out of colors to clearly display them before I overlaid them all.
Red circles = how to change the assigned false color
Yellow circle = how to display legend

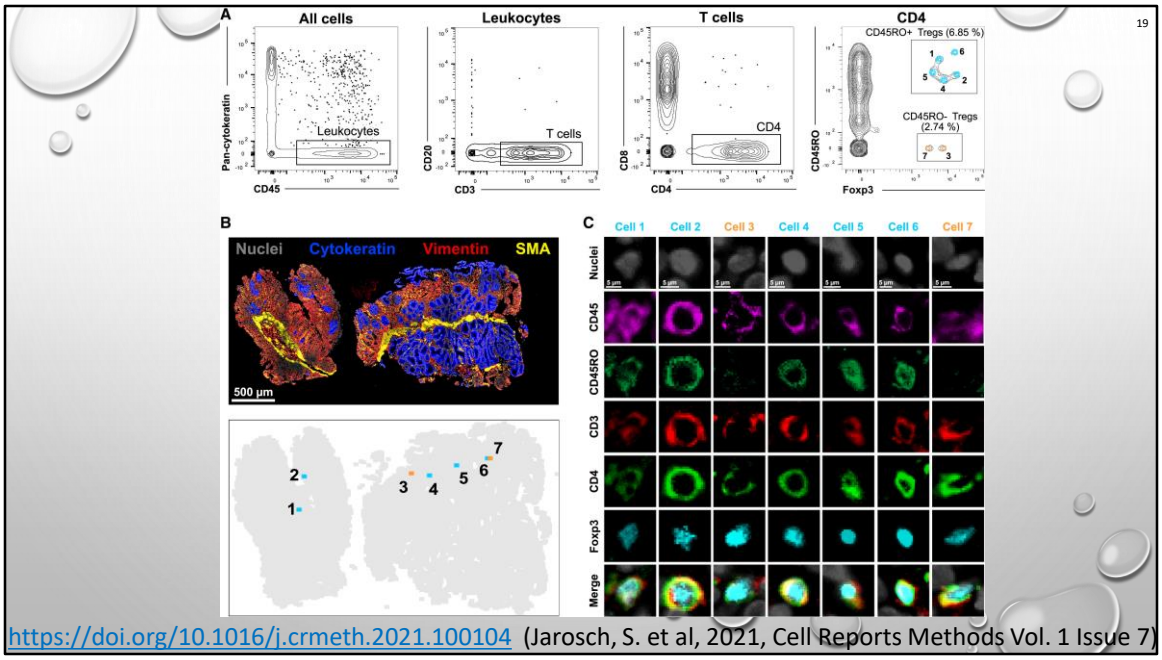
Q: How do I get to a dot plot from an image?

A: Cell Recognition!



Benefit of processing in ZKW App though is that you can interrogate rare cell populations to see if they're just artifacts.

Note: You can do tSNE in the ZKW App, but because software is a little buggy, I tend to trust the version in FlowJo or MatLab as being more mature.



https://doi.org/10.1016/j.crmeth.2021.100104 (Jarosch, S. et al, 2021, Cell Reports Methods Vol. 1 Issue 7)

Figure 5. Detection of rare phenotypically complex cells using gating strategy on automated quantified signal intensities

(A–C) Tissue section from GvHD gut biopsy was stained with 18 markers (CD45, CD45RA, CD45RO, CD3, CD4, CD8, CD14, CD68, CD20, CD25, Foxp3, Gata3, pan-cytokeratin, Ki-67, PD-1, PD-L1, vimentin, SMA). (A) Representative depiction of gating strategy used to navigate into tissue composition. From all segmented cells, Tregs were identified by sequential gating according to CD45+CD3+CD4+Foxp3+ expression and finally discriminated according to CD45RO expression. (B) Example of replotting of a gated population into the original stained tissue, to assess its spatial distribution/location. (C) Original staining images of the seven Tregs identified in (B).

Hardest part: So you found your cells. Now what? What does it mean????

Clearly, the quality of your subsequent analysis is highly dependent on the quality of your segmentation (i.e. your individual “cell recognition”).

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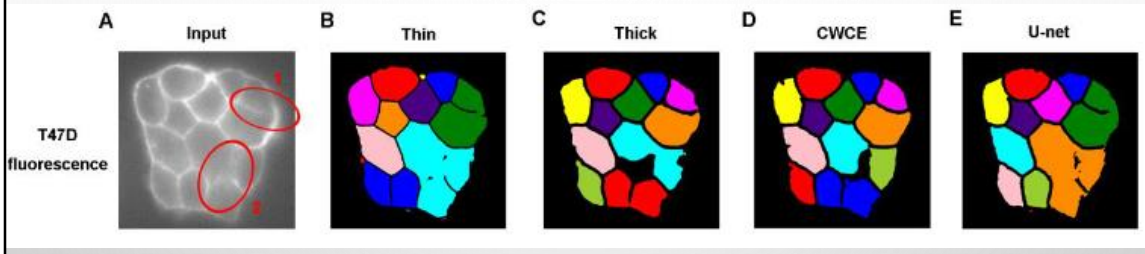
Identifying individual cells in tissue is not trivial for software!



Here, different segmenting algorithms try to segment the grayscale picture on the left. For illustration, each unique cell identified by the segmentation algorithm is given a different color.

https://arxiv.org/ftp/arxiv/papers/1803/1803.10829.pdf

Input is practically ideal and algorithms still have trouble!

Instead of FSC and SSC, gate on **cell diameter**, and other morphological features.

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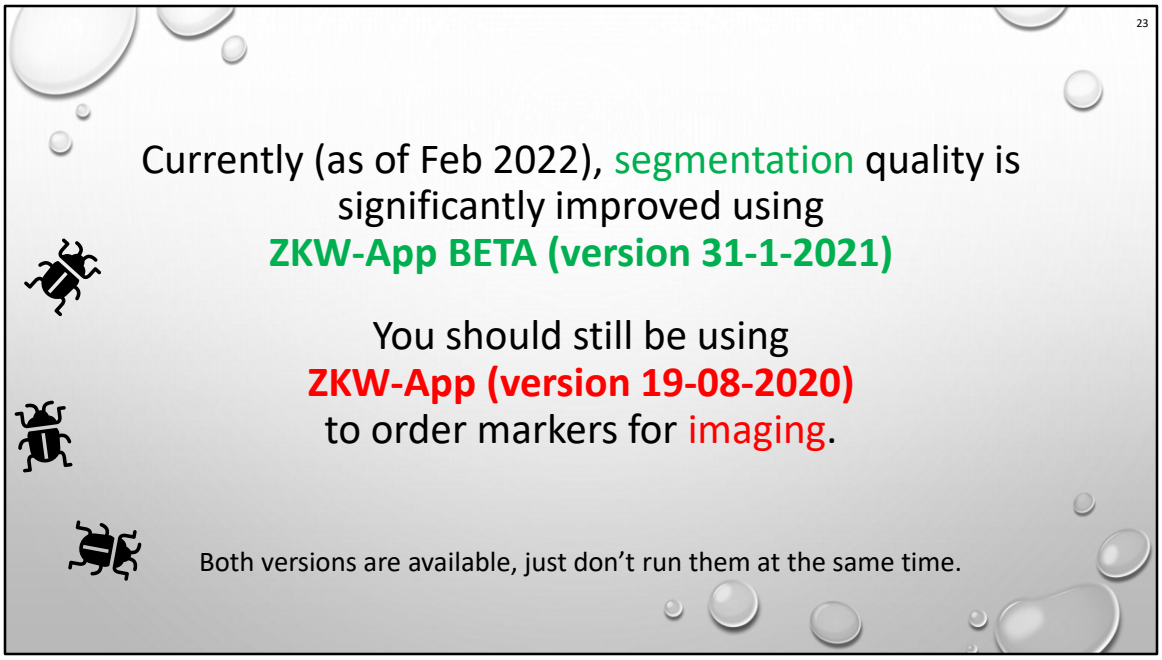
Segmentation Overview
(also see §7.2.2-7.4 of Handbook ZKW-Ap DataWizard*)

1. **Optimize your display settings** so the software can distinguish between cells and background.
2. Plan to **run this analysis iteratively** on different cell subsets.
   - **Restrict calculations to the active position** (using TESTMODE) until you figure out your settings.
   - **Adjust your segmentation parameters** until most of the "cells" drawn contain more or less one cell.
   - Let go of perfection. It doesn't exist.
3. **Edit the segmentation to remove artifacts** (e.g. doublets) via the Cell Removal tab.
   - Can also restrict cell sementation to a specific region.
4. Finally, **(re)run FL-Value Calc** to "measure" the fluorescence value for each cell.
5. Now you can **analyze dot plots** and start tracking down your cells of interest.
   - Consider **deleting specific gates from your segmentation** to further narrow down your population.
6. **TAKE GOOD NOTES**.
7. Only now can you apply your work flow to the whole chip.

*https://canopybiosciences.com/zs-support/software-handbooks/

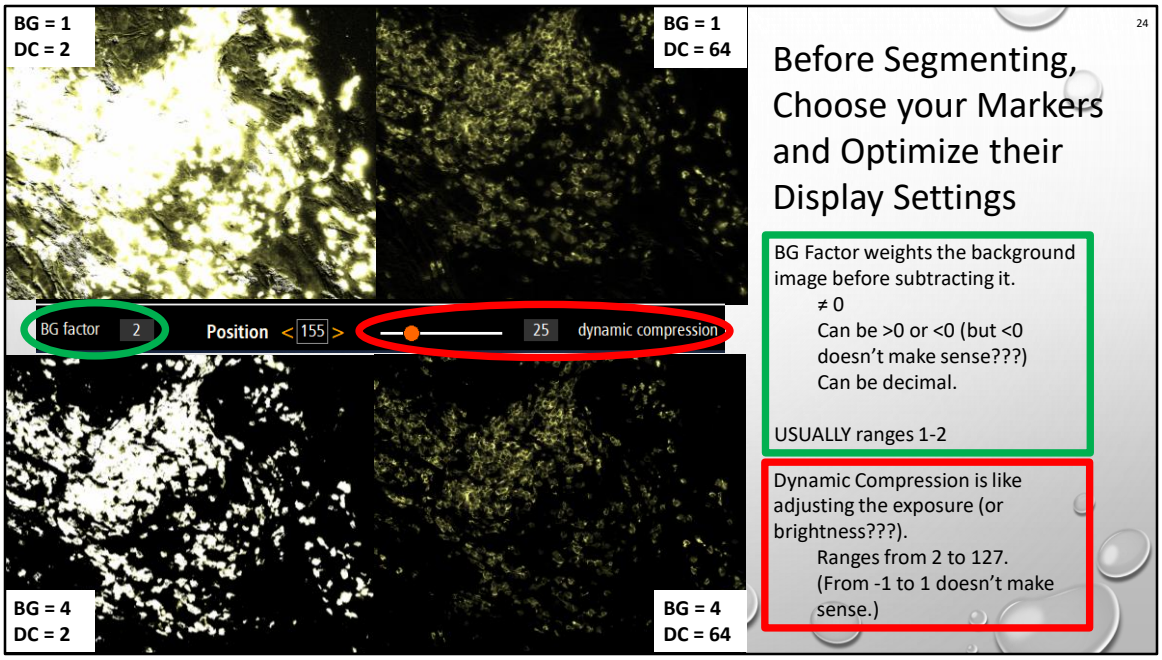
Your segmentation routine will be iterative. Expect this.

DNA DAPI/Hoechst/PI are not photobleachable!



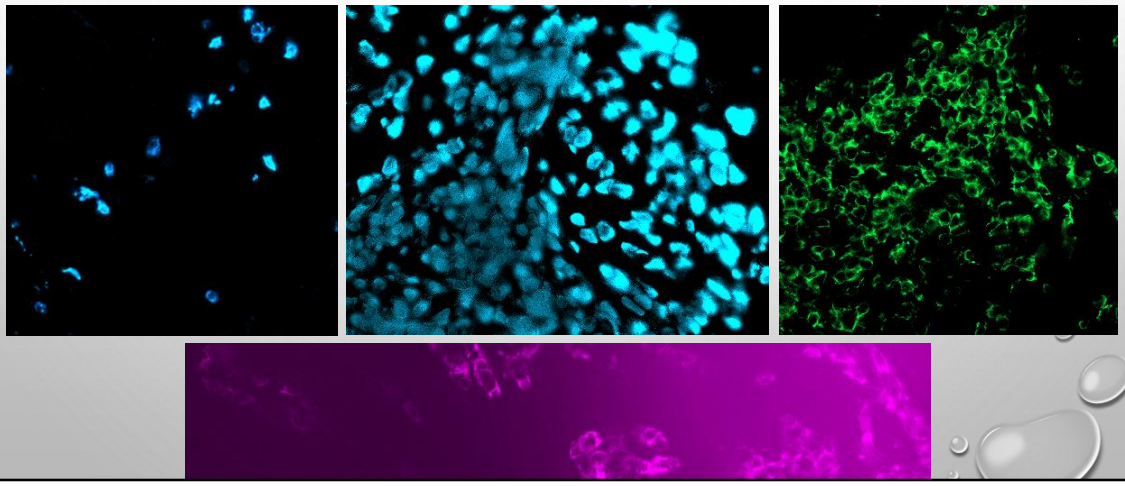
Due to software bugs, (known)

Beta = Data analysis

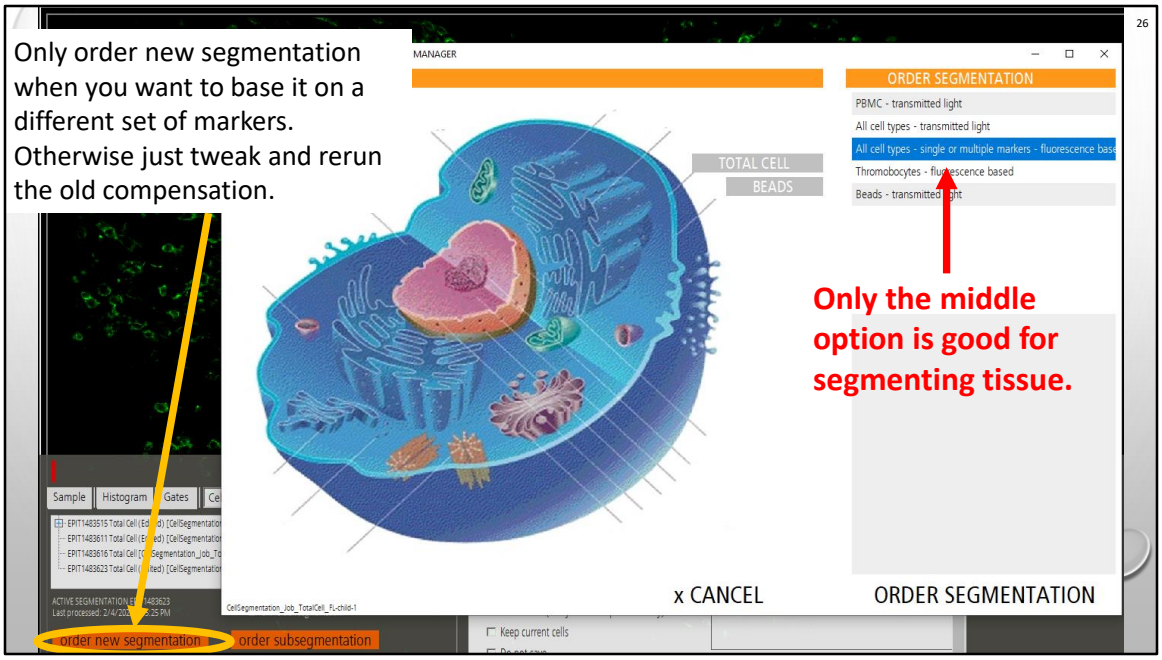


Trying to understand the relationship between contrast, brightness, DC and BG Factor.

Display Features to Try for: Strong Signal:Noise, Well Separated Cells of Uniform Intensity, Even Black Background



Easiest segmentation would be like polka dots:
False color doesn't matter.

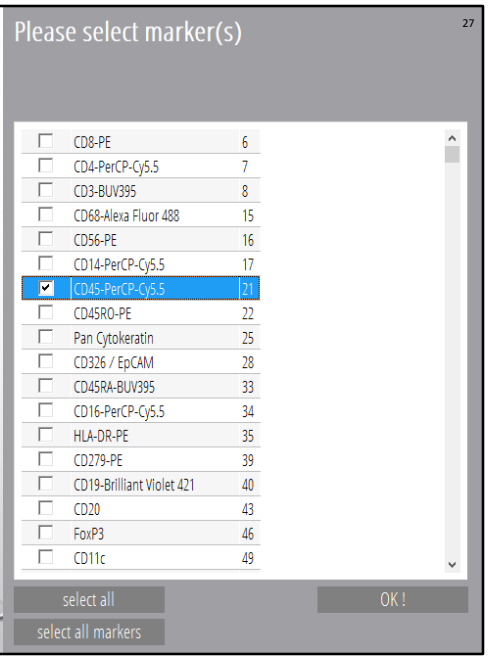


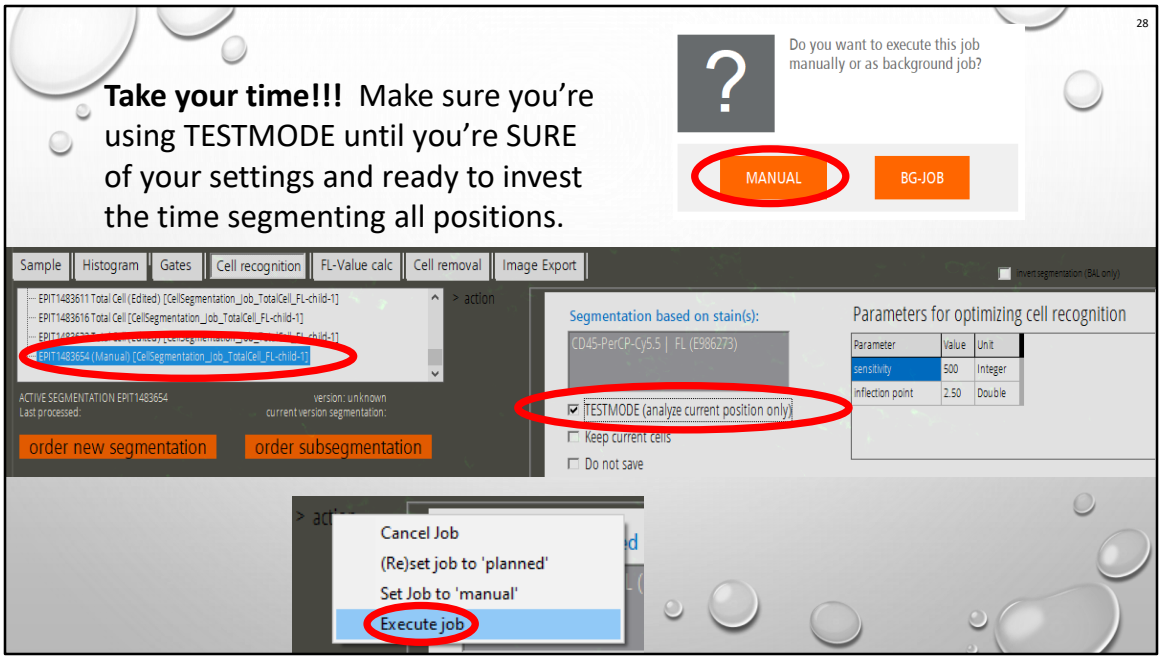
Don’t keep 100 failed segmentation attempts. They can’t be deleted.

- Select markers that are common to the cells you want to compare.
  - Don't try to select all the markers and try to identify all the cells in one go. (You will cry.)

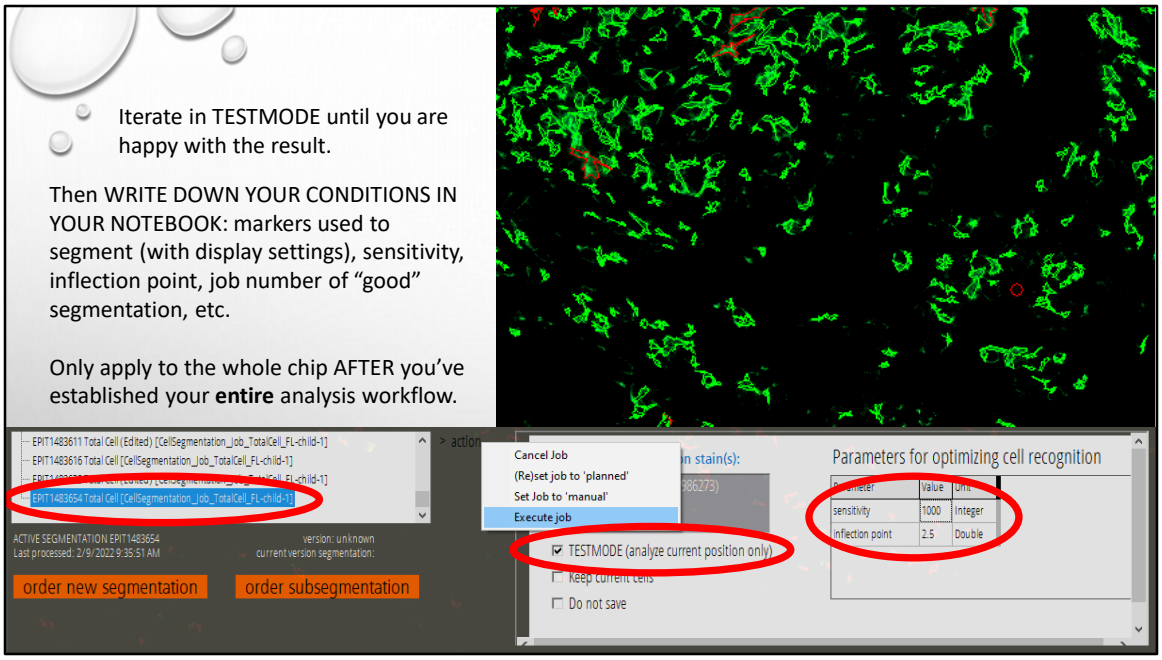
You may want to try an intracellular marker (e.g. DNA) in addition to a surface marker, but be flexible.

Remember, you can concatenate (combine) FCS files in FlowJo, so DIVIDE AND CONQUER your cell populations!



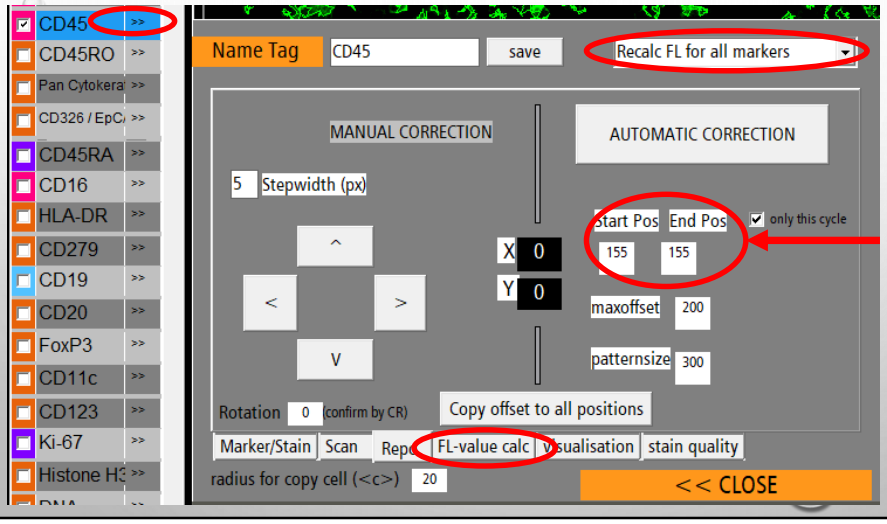


Unfortunately, there's no way to kill a long job, (rebooting the program just restarts the job), so get in the habit of checking your settings before executing!



Sensitivity 100 (low) – 5000 (high) (or 2500?)
Inflection Point 1 (super squiggly) – 5 (more smooth/round)

You can't analyze via dot plots or gates until the software measures the fluorescence in each cell. Recalculate the fluorescence after you're happy with the segmentation.



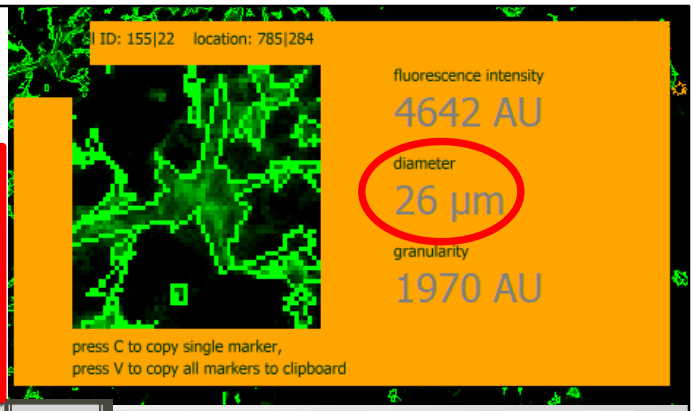
Limit the FL recalculation to the active position until after you establish your analysis workflow.

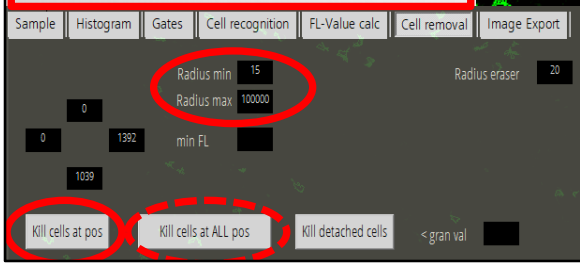
Similarly, check this area if you're trying to segment the whole chip and it won't do more than the current position.

Gate on cell size:

Radius Min/Max defines the cells you want to KILL, so you may want to run it more than once. (e.g. once to kill the doublets and higher, and then again to get rid of debris)

Kill cells at current position, until you are ready to deal with the whole chip.





Other ways to gate:

- Manually sweep through and remove, say, cells outside tumor region using Eraser (hold down shift to see it… cursor will be a yellow circle)
- Brightness (e.g., get rid of bright artifacts of staining)
- Granularity (more for cell samples vs. tissue… "kill detached cells")

Gate on cell size:

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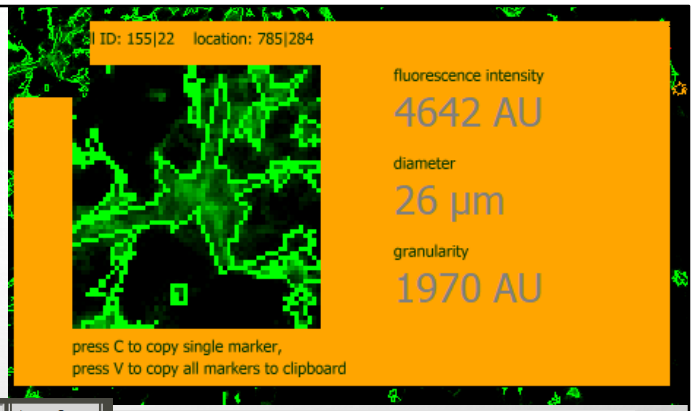

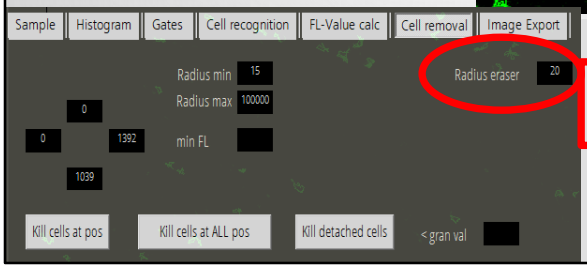

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When using eraser, save first, then after.

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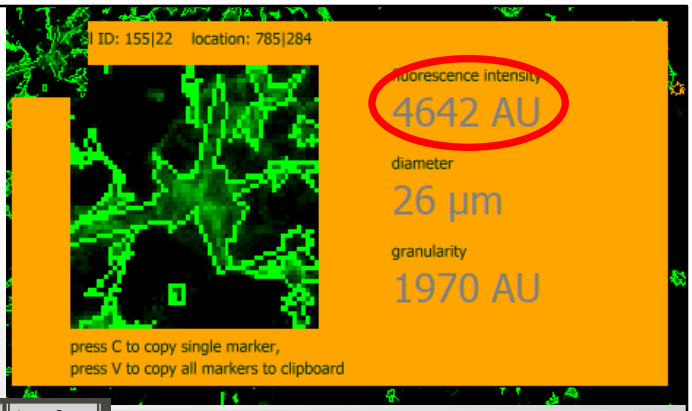
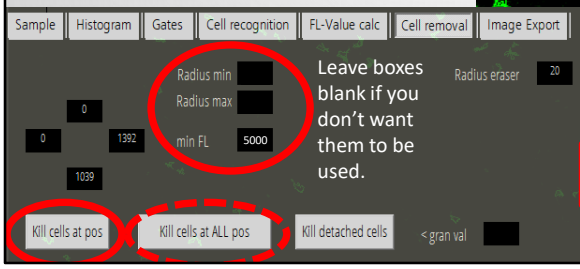
Kill cells at current position, until you are ready to deal with the whole chip.





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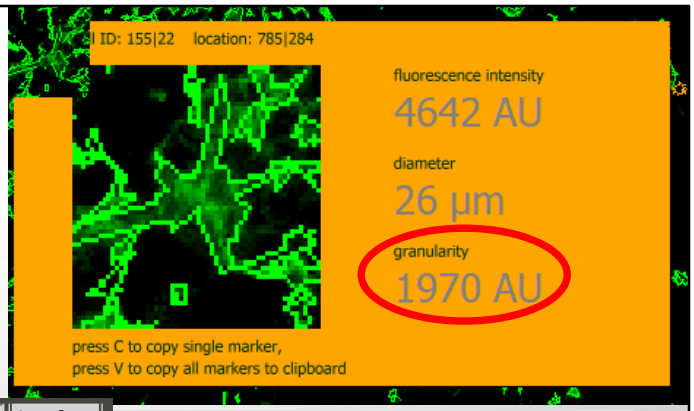

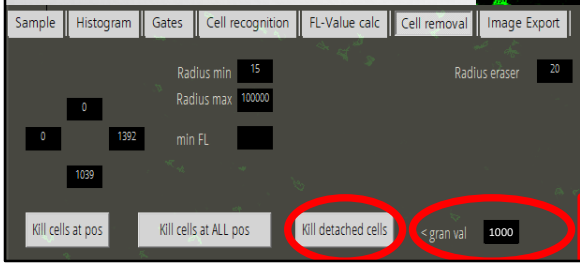

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- Manually sweep through and remove, say, cells outside tumor region using Eraser (hold down shift to see it… cursor will be a yellow circle)
- Brightness (e.g., get rid of bright artifacts of staining)
- Granularity (more for cell samples vs. tissue… "kill detached cells")

Don’t worry about the scary red warning.

If you accidentally delete things you shouldn’t have, just reload that segmentation job without saving changes.

Similarly, if you DO like how things are going, save it by reloading and saying yes to the changes.

“Save as csv” is for exporting data (e.g. to FlowJo, but you can do FCS files directly another way).

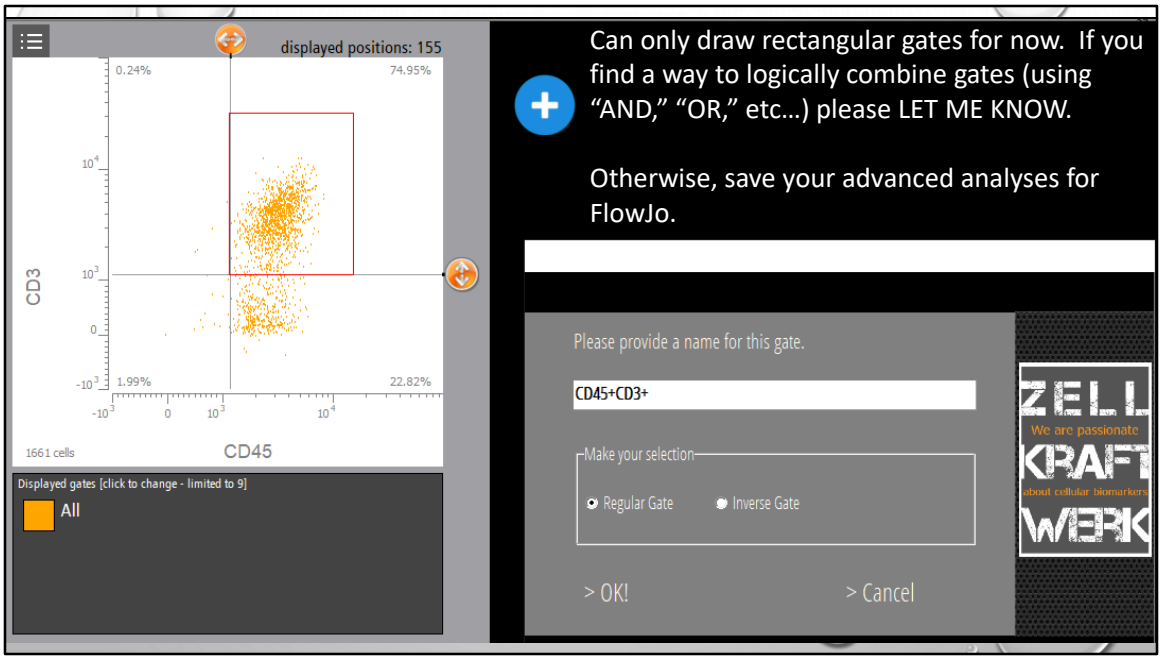
Reload = click another job, then click that one again.

Finally, you can start working with dot plots!

Create dot plot with blue plus
Drag/drop markers onto x and y axes.

Note that there is >1 little menu.

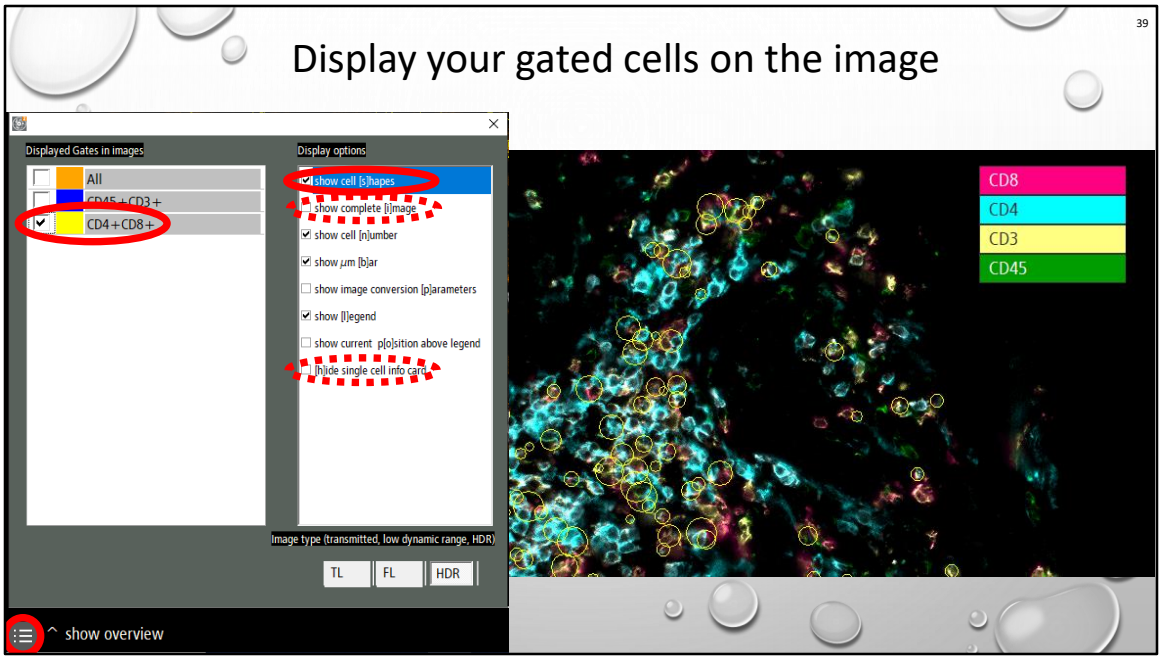
Pay attention to whether you are analyzing (or deleting!!!) cells from all the positions, or specific ones.



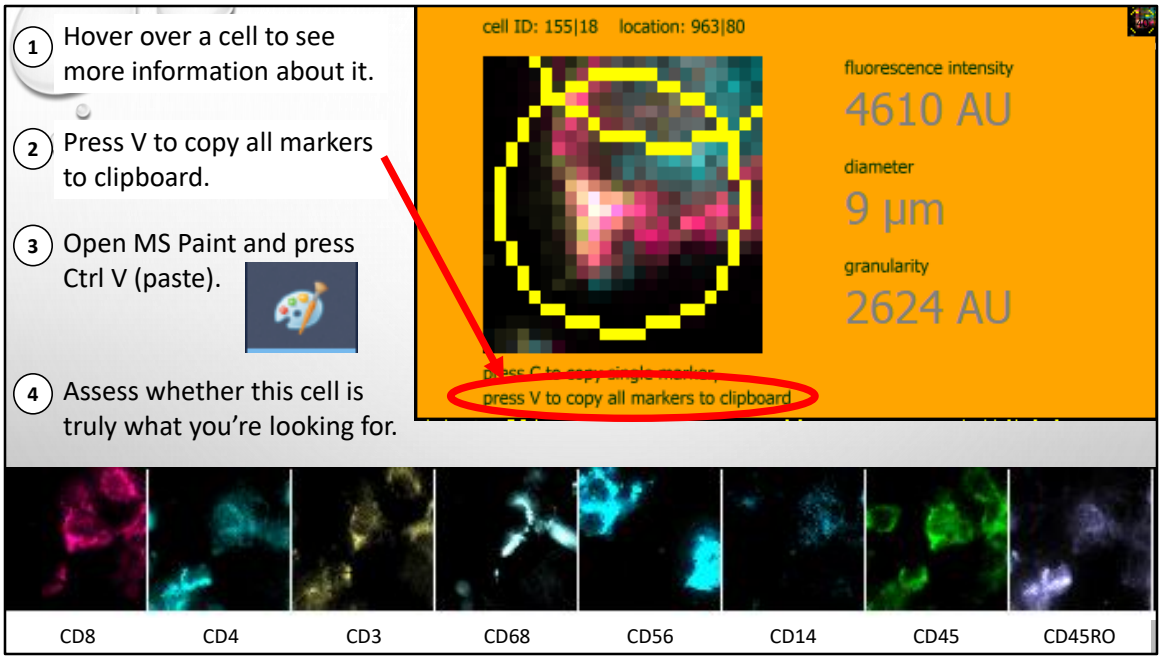
Draw your rectangular gate.

Quick quiz: why are ALL my cells CD45+?
Because that is the marker I used to define the cells. That's my "first" gate.

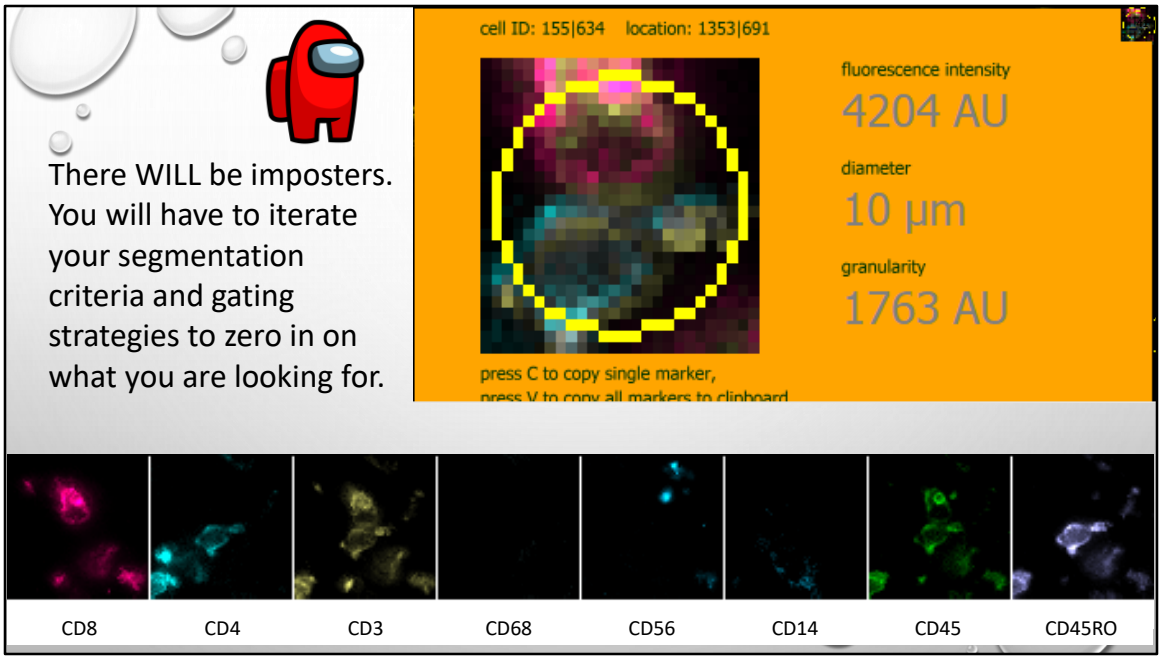

displayed positions: 155
38
28.08%
19.12%
4.00%
48.80%
CD8
CD4
1250 cells
SELECT DISPLAYED GATES
All
CD45+CD3+
Change displayed (parent) gate for next plot BEFORE drawing child gate.
Consider using gates to select the cells you DON'T want, and then deleting them from your segmentation as a strategy. (Watch out as to whether you're applying the gate to one or all positions)
x close
[K] - delete all cells that belong to this gate
Displayed gates [click to change - limited to 9]
CD45+CD3+
CD4+CD8+



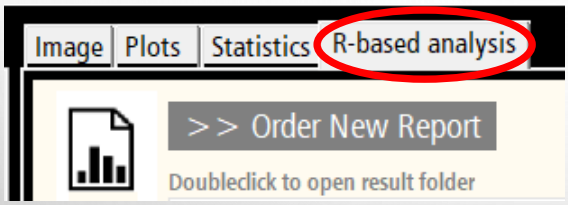
I chose yellow b/c it's easier to see. Notice that gates don't follow cell shapes anymore.



Note: Do not "show complete (i)mage" or the cursor location and cell selection won't match up anymore. Just use the default view.

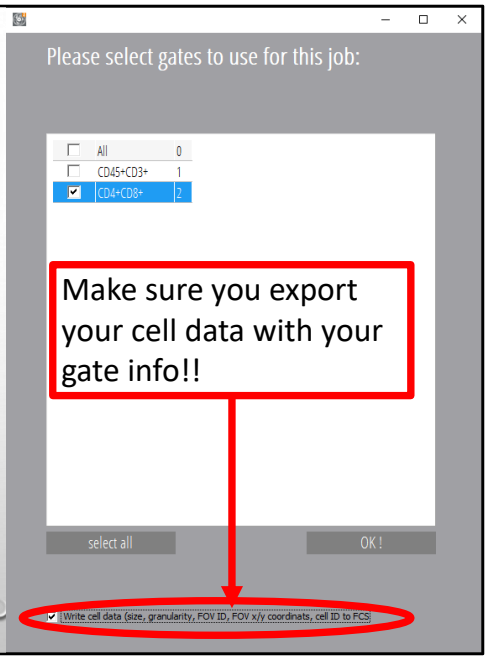

There WILL be imposters. You will have to iterate your segmentation criteria and gating strategies to zero in on what you are looking for.
cell ID: 155|634 location: 1353|691
fluorescence intensity
4204 AU
diameter
10 µm
granularity
1763 AU
press C to copy single marker,
CD8
CD4
CD3
CD68
CD56
CD14
CD45
CD45RO

(Optional) Export Gate(s) as *.fcs for Analysis (e.g. in FlowJo)



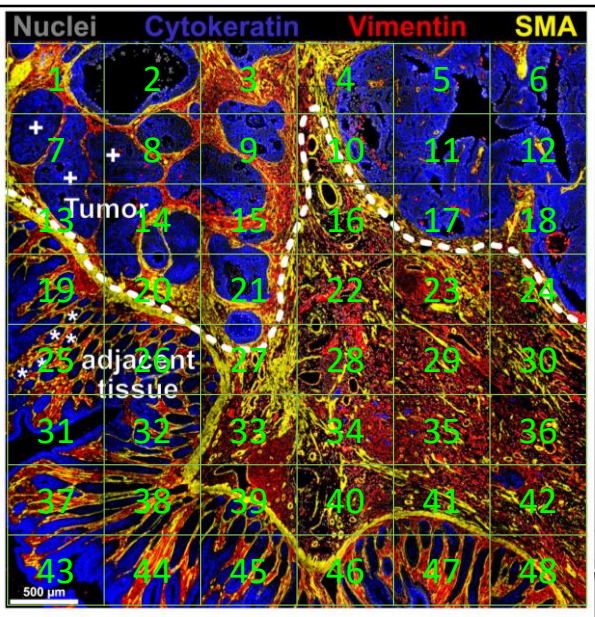
NOTE: You can IMPORT gates to ZKWApp as well. (Maybe export from FlowJo as CSV???)

If you do this, let me know.



An example strategy

- Define segmentation for entire tissue for a given cell type.
- Divide your segmentation into 3 regions based on position.
  - Boundary regions will have to be dealt with manual refinement, but this can be done after you establish whether this is worth pursuing.
- Analyze your 3 regions separately and draw conclusions.



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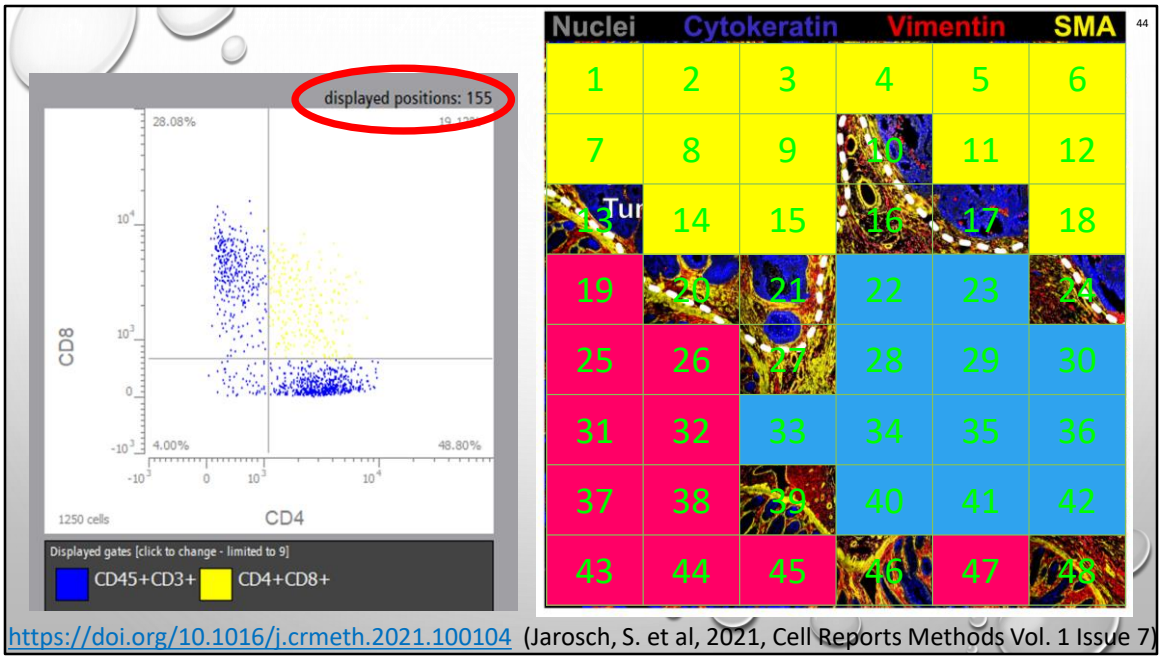
https://doi.org/10.1016/j.crmeth.2021.100104 (Jarosch, S. et al, 2021, Cell Reports Methods Vol. 1 Issue 7)

Part of Fig2 showing published image quality and illustrating approaches to analysis.
Don't reuse colors though. Very confusing.
Don't show all the colors/layers either. Better to use say, drab colors for background/reference and bright for labels?

Figure 2. High-quality multiplexed staining of FFPE colon tissues with ChipCytometry
(A) Representative image of colon tumor with non-tumor adjacent tissue (above and below the dashed line, respectively). Asterisks (∗) and crosses (+) indicate normal and abnormal crypts, respectively.
(B) Representative images of high multiplex staining in an inflamed colon biopsy. Zoom in is indicated by white rectangles and shown in (C).
(C) Representative images of mutually exclusive markers (CD3/CD20/CD14, CD45RA/CD45RO, and CD4/CD8) and co-expressed markers (CD4/Foxp3). Exemplary cells are annotated from left to right as ∗, CD14+; +, Ki-67+; #, CD4+Foxp3+ cells.
(D) Multiplex ChipCytometry overlay of a human pancreatic cancer tissue (number of markers = 13). See also Figure S4.



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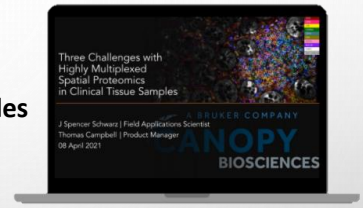
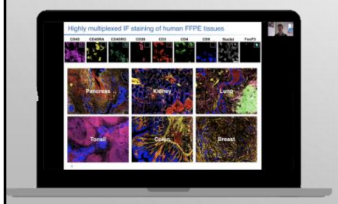
Canopy Webinars

https://info.canopybiosciences.com/webinars

- Cost = sign up for email list (otherwise free)

Three Challenges with Highly Multiplexed Spatial Proteomics in Clinical Tissue Samples
J. Spencer Schwarz





Multiplexed Imaging and Automated Signal Quantification in Formalin-Fixed Paraffin-Embedded Tissues by ChipCytometry™
Sebastian Jarosch

https://doi.org/10.1016/j.crmeth.2021.100104

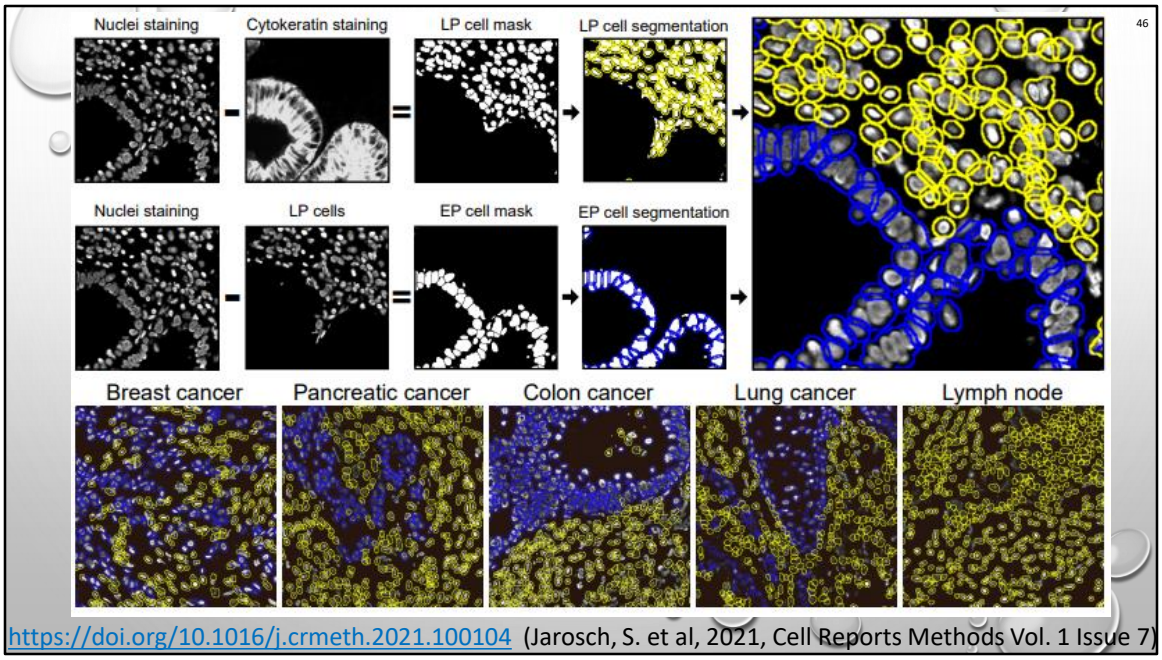
Sebastian's free ImageJ/Fiji plugin for segmentation & stitching artifact removal.

GitHub

https://github.com/SebastianJarosch/ChipCytometry-Image-Processing

I strongly recommend checking out this paper and webinar for recommendations on segmentation strategies.

YES, ZellScanner data can be read by Fiji/ImageJ, but ALWAYS MAKE A COPY BEFORE MESSING AROUND.



https://doi.org/10.1016/j.crmeth.2021.100104 (Jarosch, S. et al, 2021, Cell Reports Methods Vol. 1 Issue 7)

d) Healthy colon tissues were stained with pan-cytokeratin. Either pan-cytokeratin staining (epithelial cells, EP) or nuclear staining of lamina propria (LP) cells was subtracted to the image prior to cell segmentation. e) Exemplary images of separate segmentation in different tissues types. Blue = segmented epithelial cells, yellow = segmented non epithelial cells.

Best Practices

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Best Practices – Validate your Assay!

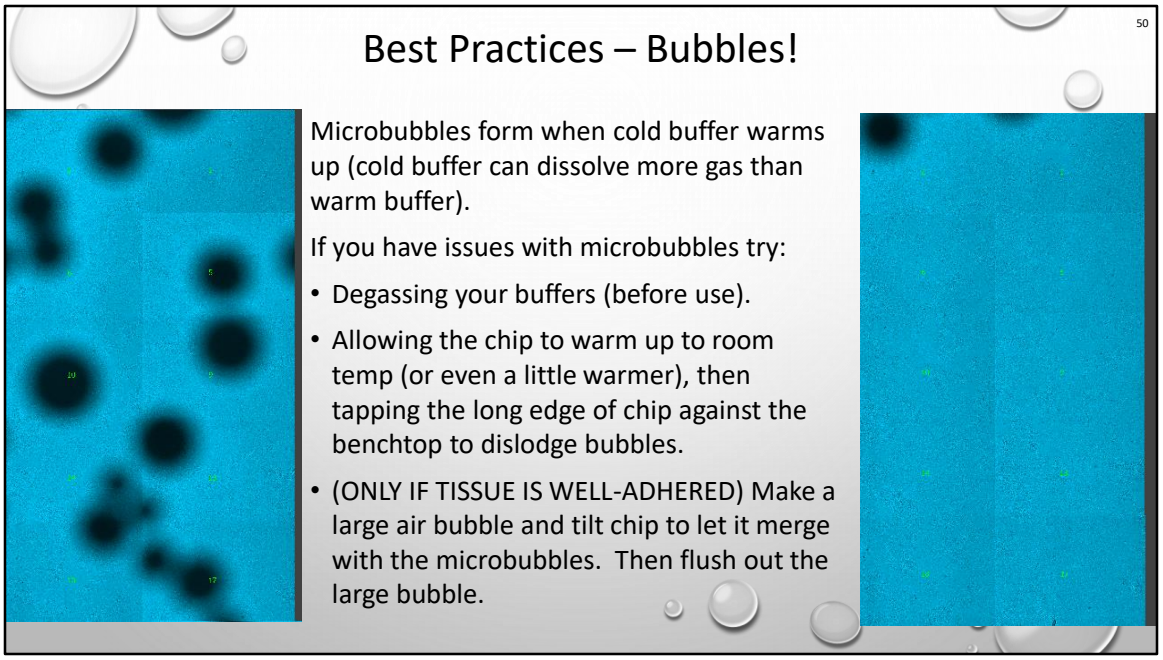
- Do NOT use your precious, one-of-a-kind, expensive, tissue for your first chip. Use your first chip to **VALIDATE YOUR ASSAY** on cheap tissue.
- Make sure you can find your favorite cells in your POSITIVE CONTROL TISSUE using your proposed antibody (Ab) panel.
- **Titrate Ab concentrations** or at least confirm that the recommended dilutions work for you.
  - Note, you can restain the practice tissue many times with different dilutions. Better to go from weak to strong.
  - What a great time to **test your data processing procedure** as well! Can you segment EASILY with what you have or do you need another marker??

Please share any technical gems you discover with us.

Best Practices – Be Strategic about Staining

- You can't reliably unpermeabilize your tissue, so ideally, **do your intracellular stains AFTER finishing up all of your surface stains.** (At least be consistent!)
- The nuclear stains: **DAPI, Hoechst, and Propidium Iodide do NOT photobleach** according to Canopy.
  - Maybe you can chemically bleach them???? Let me know if you can.
    - E.g. PBS + 24 mM NaOH + 4.5% H_2O_2 reference: https://doi.org/10.1002/cpch.14
  - You can run intracellular staining cycles after them on OTHER CHANNELS, though.
    - DAPI & Hoechst = both are visible on FS395 & FS421; Propidium Iodide = FS560
- Consider using your weaker stains on the PE channel (FS560 is the most sensitive filter set according to Canopy).
- Either **TITRATE your stronger stains** to a more moderate level, or run them AFTER the weaker stains on the same channel.

Please share any technical gems you discover with us.



Bubbles are subtle in fluorescence but really screw up position recognition because they move around.

Cold buffer stores more gas than warm buffer.
It's why you see little bubbles when heating water to boil before you actually reach boiling.
It's why warm beer or soda tastes flat.
B/c scope is at RT, buffer will warm up and off-gas/make bubbles.
Warming up the buffer makes bubbles bigger, and easier to dislodge.

Best Practices - General

- Tissue can last 2+ years in storage buffer, **ONLY IF** you don't introduce bacteria, fungi, etc...
  - **KEEP STORAGE BUFFER STERILE.**
- **Be strategic about the number of positions** you cover:
  - Initial bleaching scan costs about 1 min per position.
  - Once you omit a position, the ZellScanner won't scan or analyze it anymore.
- **EXCLUDE scans/stains that didn't work** well by highlighting the marker (in DataWizard) and pressing **e**.
- Don't bounce your leg (or type vigorously at the keyboard) while an image is being acquired **(vibrations can make the image blurry)**.
  - We're working on getting an antivibration platform.
- **Don't leave chip on scope if not actively acquiring or bleaching.**

Please share any technical gems you discover with us.

Storage buffer is basically blocking buffer, so bugs love it.

Storage buffer has antifungal in it, but it can be overwhelmed (it's been done).

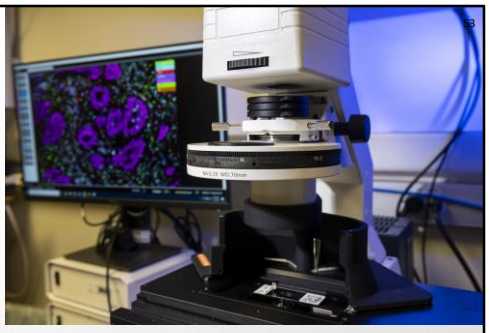
Request for Ideas and Advice

- Tissue adherence tricks?
- Can you pop bubbles with ethanol or will that harm tissue/antibodies?
- Can we use an old chip code as a dummy to test stain quality on traditional slides?
- Tips/tricks on segmentation and gating work flows?
- Do you wish I'd covered something in this talk that isn't here?
  - Or could something I did cover be better?
  - Let me know! (Nicole) **nrb18@pitt.edu**

Pitt Photos: Tom Altany

Please share any technical gems you discover with us.

For More Information:



- Canopy Bioscience ZellScanner Manuals/Protocols
  - https://canopybiosciences.com/zs-support/
- Canopy Bioscience Webinars
  - https://info.canopybiosciences.com/webinars
- Publications
  - https://canopybiosciences.com/resource-center/?_resources_tags=chipcytometry
- Validated Antibodies/Clones (human)
  - Example Assay from Canopy:

    Canopy Zellscanner.zip\04 Example Assay\04 … panel reference….pdf
  - Also See:

    Canopy Zellscanner.zip\03 Assay Development…\04 Current ChipCytometry Clone Information….xlsx
  - Table 1 of Jarosch et al. (https://doi.org/10.1016/j.crmeth.2021.100104)

Thank You!!