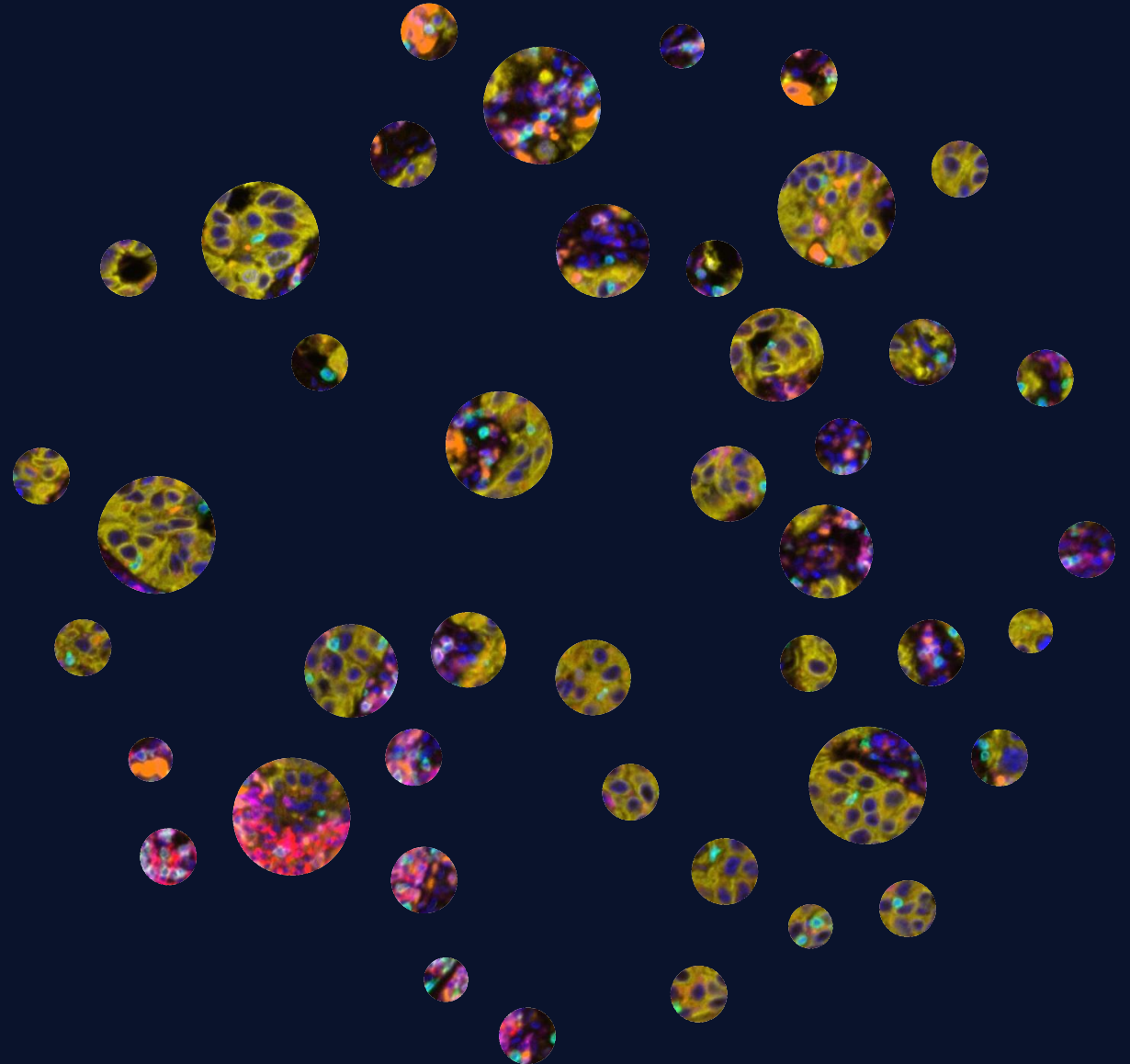


Phenolmager Data Analysis using QuPath and Python

Software Workflow

Aditya Pratapa, Ph.D.

Akoya Biosciences

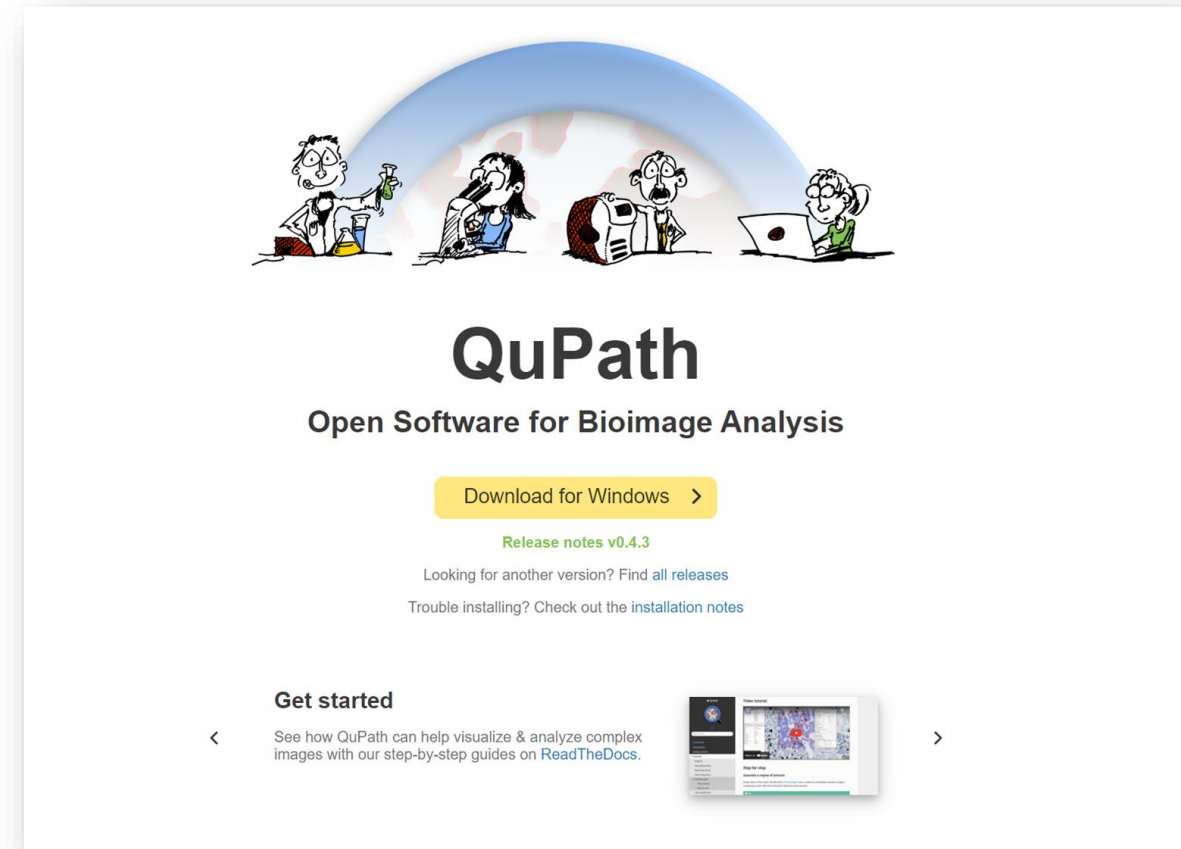


Learning Objectives

- QuPath
 - QuPath project set-up
 - Cell segmentation using StarDist
 - Data export
- Python
 - Anaconda environment set-up
 - Data import
 - Automated phenotyping
 - SpatialScore computation

QuPath set-up

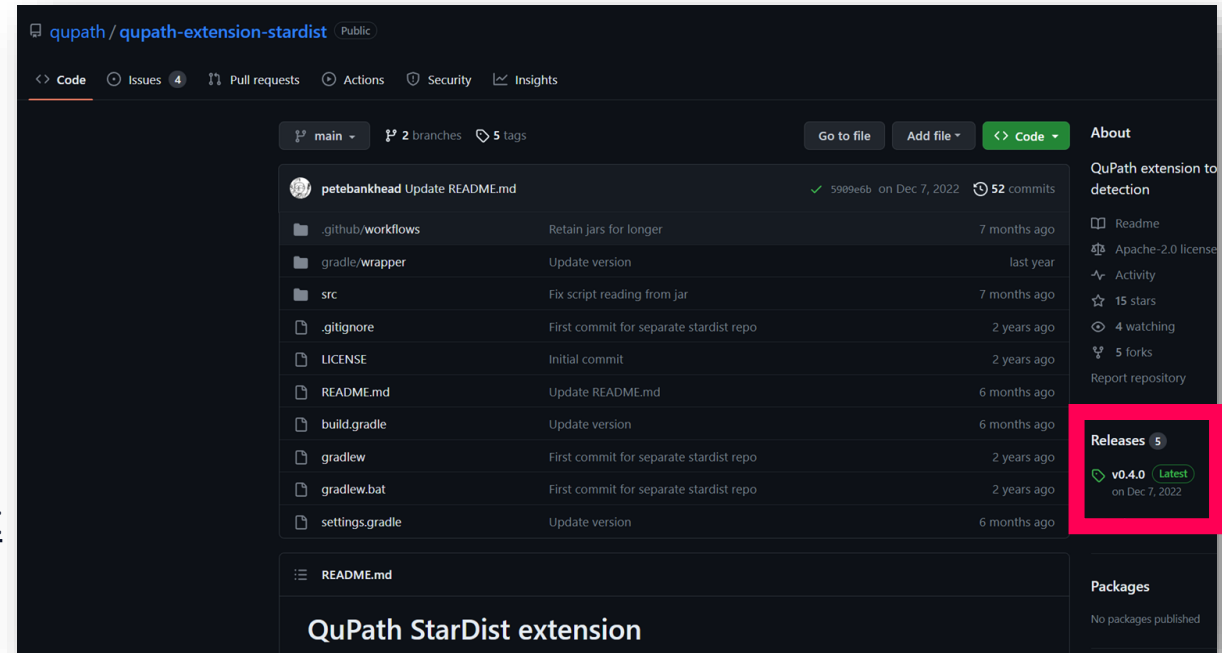
- Basic QuPath:
 - Grab the latest version of QuPath v0.4.3
 - <https://qupath.github.io>



The image shows the QuPath website landing page. At the top, there is a cartoon illustration of four scientists in a lab setting, with a blue and red rainbow arching over them. Below the illustration, the text "QuPath" is written in a large, bold, black font. Underneath that, "Open Software for Bioimage Analysis" is written in a smaller, bold, black font. A yellow button with the text "Download for Windows" and a right-pointing arrow is positioned below the text. Under the button, there is a link for "Release notes v0.4.3" in green text. Below that, there are two links in blue text: "Looking for another version? Find all releases" and "Trouble installing? Check out the installation notes". At the bottom, there is a "Get started" section with a left-pointing arrow, a paragraph of text, and a right-pointing arrow. The text in the "Get started" section says: "See how QuPath can help visualize & analyze complex images with our step-by-step guides on ReadTheDocs." To the right of the text is a small screenshot of the QuPath software interface showing a microscopy image with red and blue annotations.

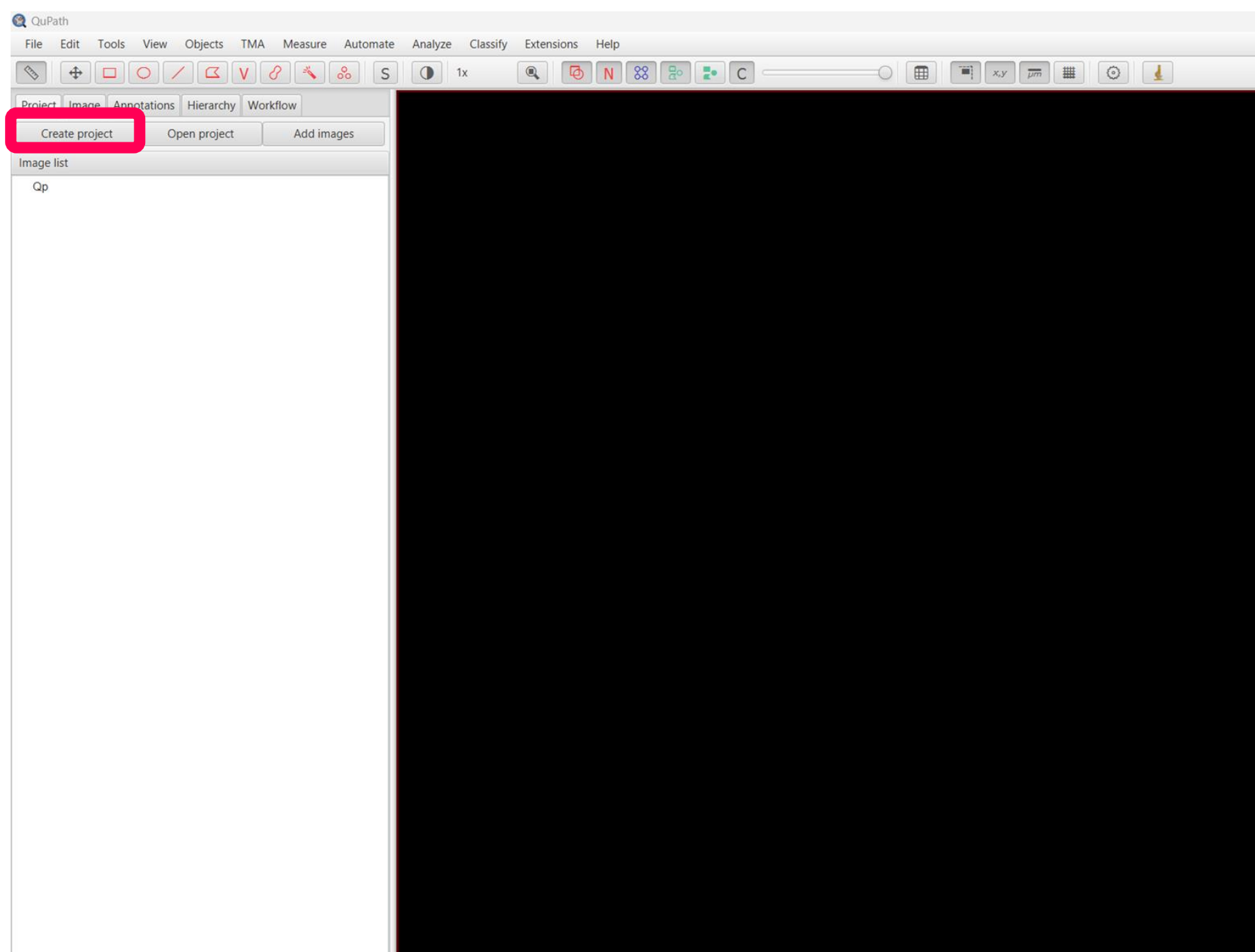
QuPath set-up

- Basic QuPath:
 - Grab the latest version of QuPath v0.4.3
 - <https://qupath.github.io>
- For Cell Segmentation
 - QuPath's StarDist extension
 - <https://github.com/qupath/qupath-extension-stardist>
 - To install the StarDist extension, download the latest qupath-extension-stardist-[version].jar file from releases and drag it onto the main QuPath window.



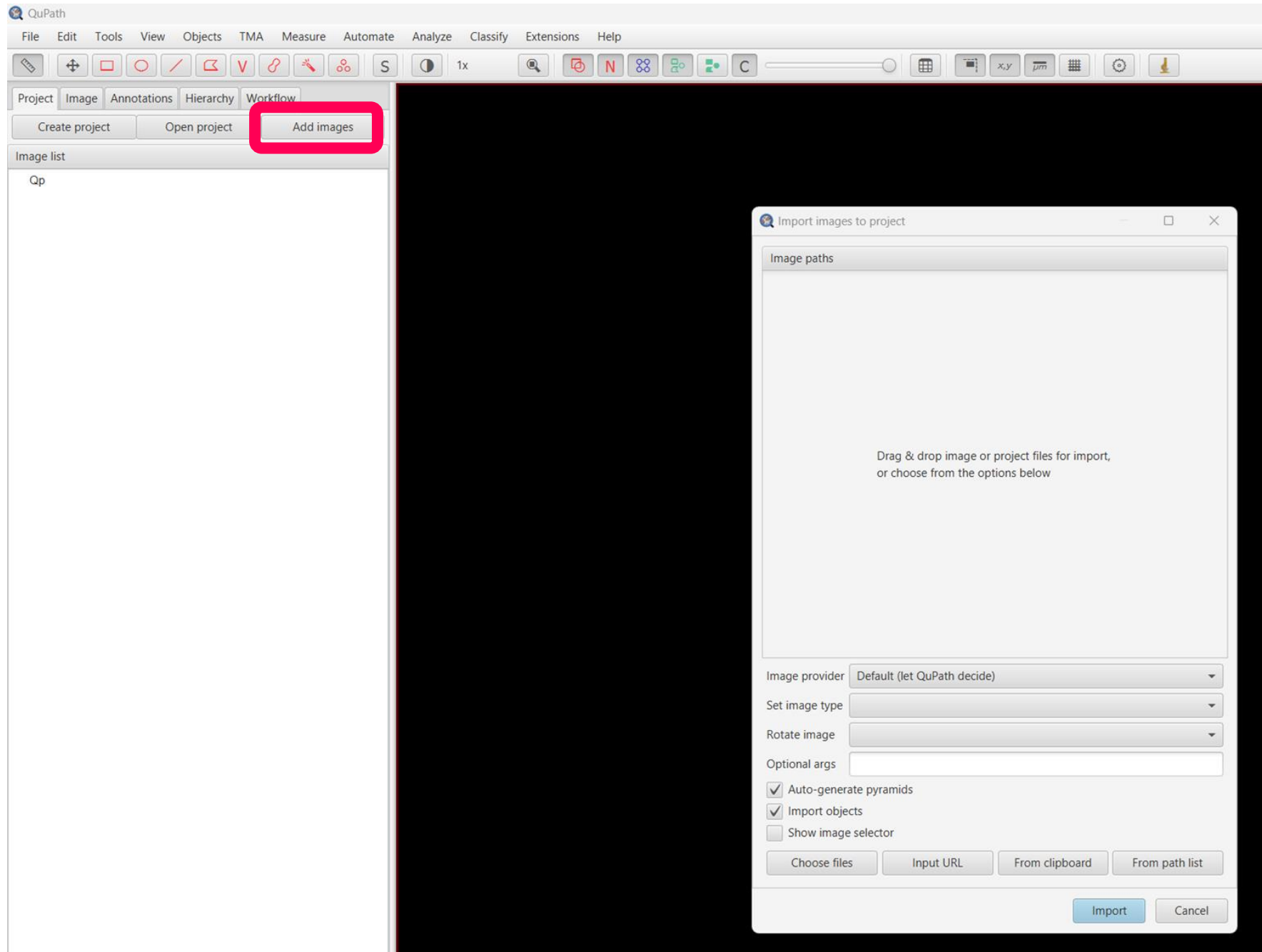
Create Project

- Create a new QuPath project in an empty folder



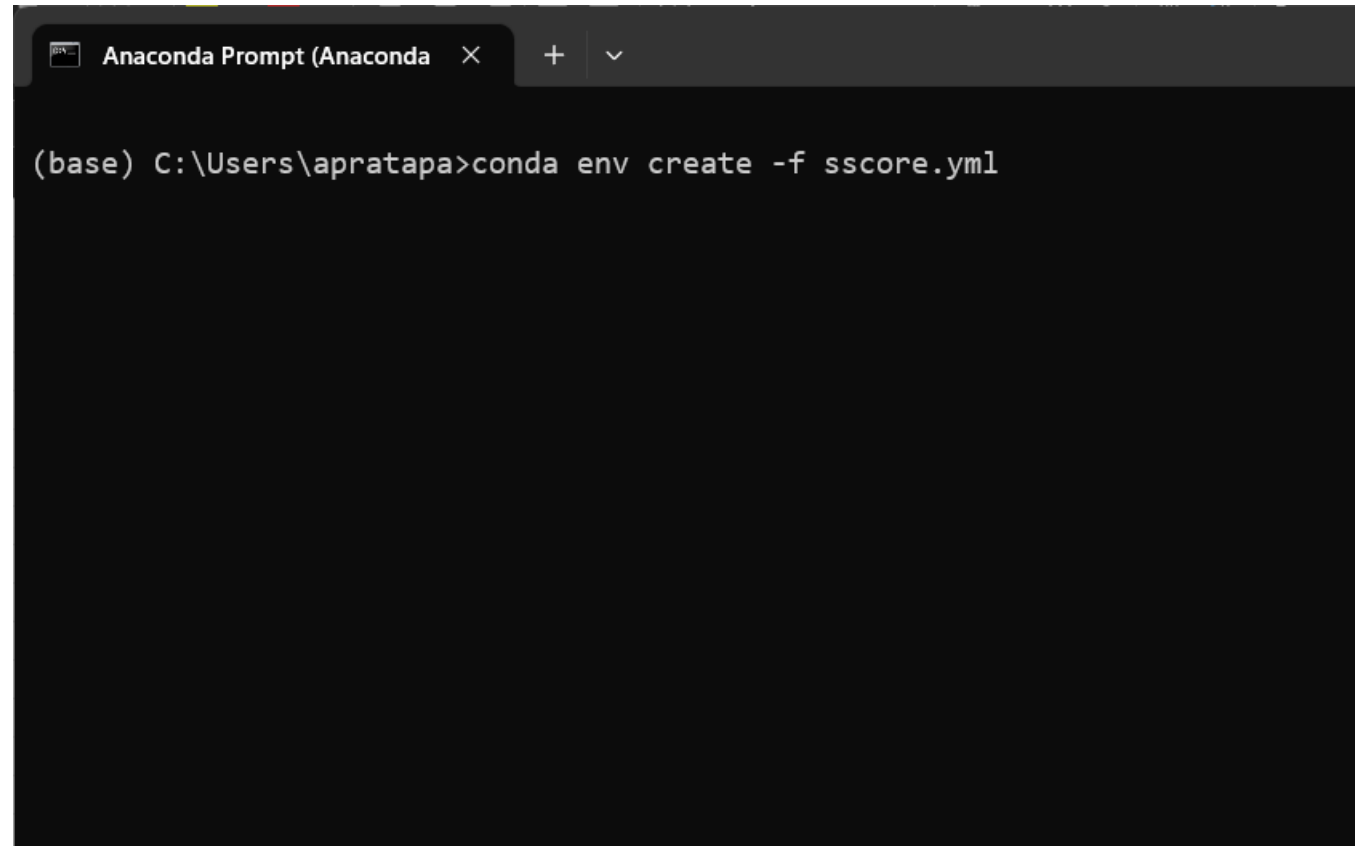
Add images

- Add unmixed ome.tif files



Python Environment Set-up

- Download and install Anaconda
 - <https://anaconda.org/>
- Anaconda prompt
 - Run 'conda env create -f sscore.yml'



```
Anaconda Prompt (Anaconda) x + v  
  
(base) C:\Users\apratapa>conda env create -f sscore.yml
```

Start the notebook server

- Activate newly installed environment
 - `conda activate sscore`
- Start a jupyter notebook server
 - `jupyter-notebook --port 8989`

```
Anaconda Prompt (Anaconda) x + v
(base) C:\Users\apratapa>conda activate sscore
(sscore) C:\Users\apratapa>jupyter-notebook --port 8989

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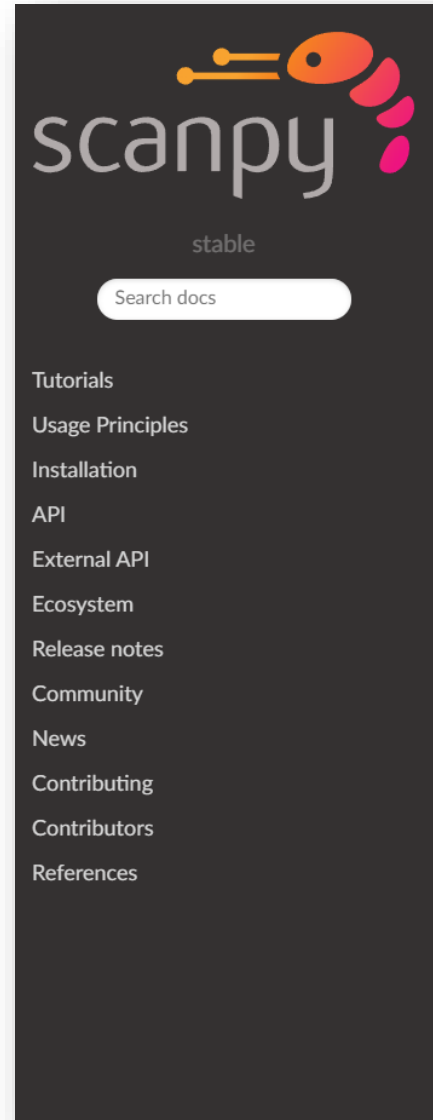
Read the migration plan to Notebook 7 to learn about the new features and the actions to
take: https://jupyter-notebook.readthedocs.io/en/latest/migrate\_to\_notebook7.html

Please note that updating to Notebook 7 might break some of your extensions.

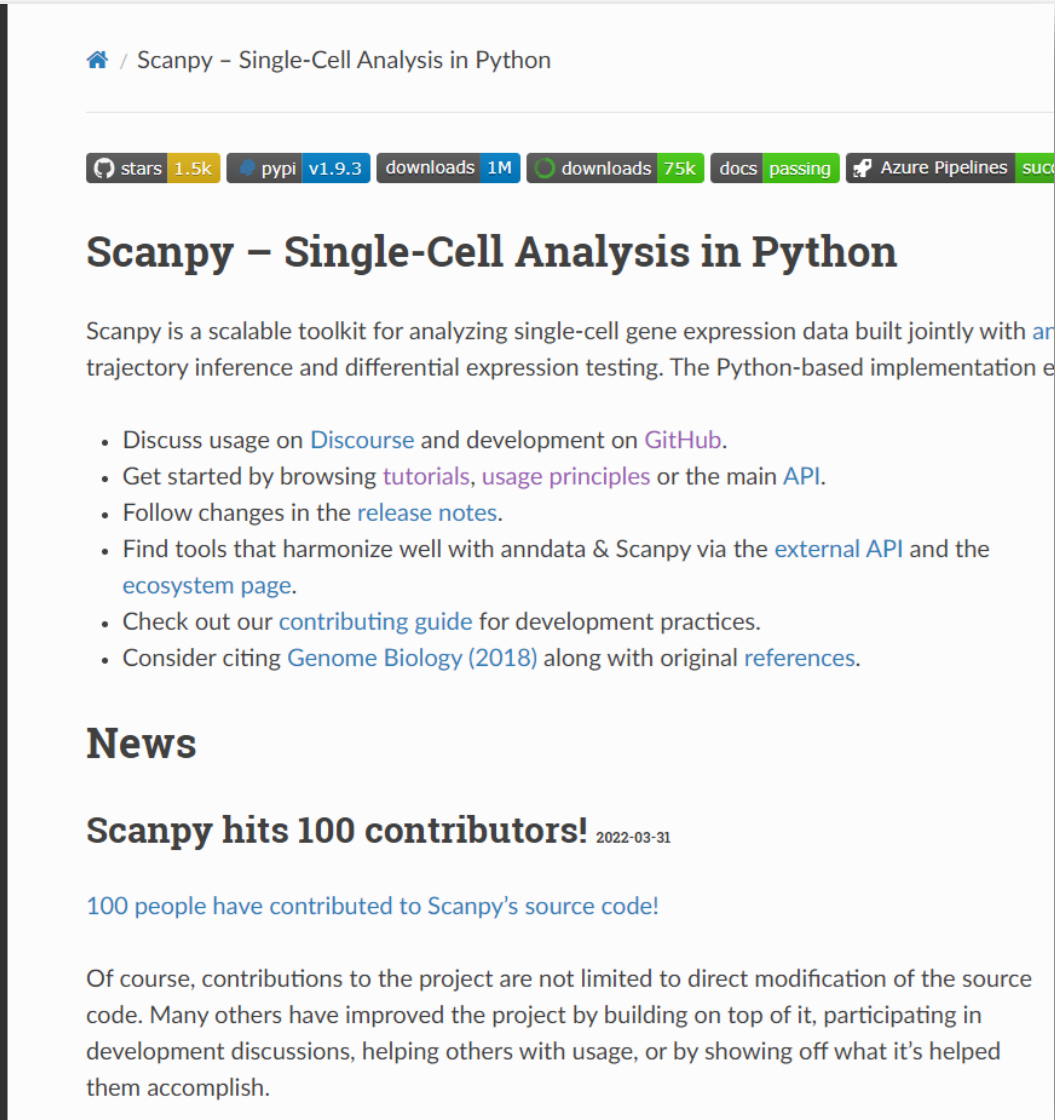
[I 22:10:07.921 NotebookApp] The port 8989 is already in use, trying another port.
[I 22:10:07.925 NotebookApp] Serving notebooks from local directory: C:\Users\apratapa
[I 22:10:07.925 NotebookApp] Jupyter Notebook 6.5.4 is running at:
[I 22:10:07.925 NotebookApp] http://localhost:8990/
```


Step-1: Data Import

- Scanpy library for single-cell analysis
- Read measurements.tsv and create an AnnData object



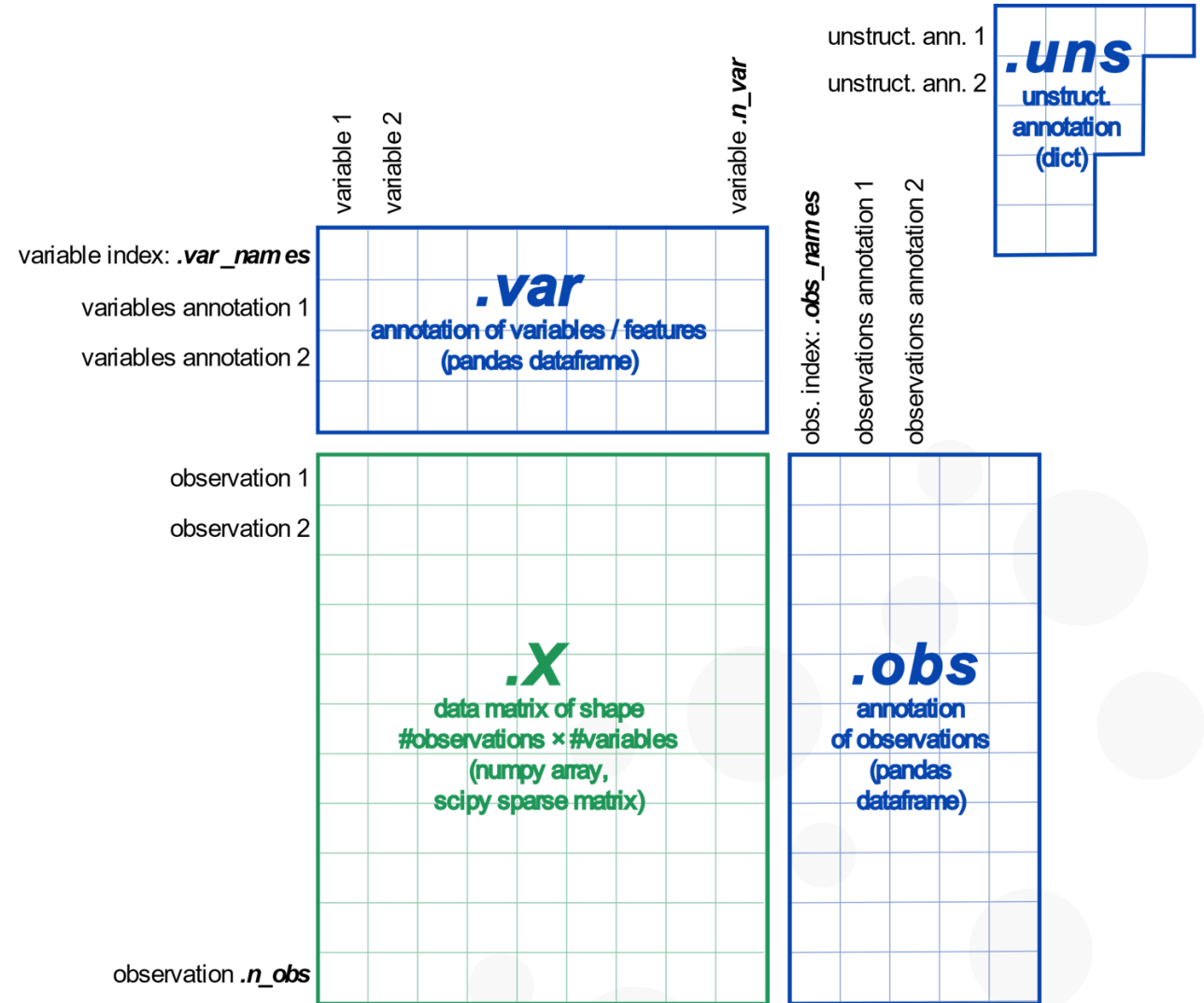
The image shows the Scanpy logo, which consists of the word "scanpy" in a lowercase, sans-serif font. Above the "y" is a stylized orange and pink graphic resembling a cell or a cluster. Below the logo, the word "stable" is written in a smaller font. A search bar with the text "Search docs" is positioned below "stable". A vertical navigation menu on the right side of the logo area lists the following items: Tutorials, Usage Principles, Installation, API, External API, Ecosystem, Release notes, Community, News, Contributing, Contributors, and References.



The image is a screenshot of the Scanpy website. At the top, there is a navigation bar with a home icon and the text "/ Scanpy - Single-Cell Analysis in Python". Below this, there is a row of statistics: stars 1.5k, pypi v1.9.3, downloads 1M, downloads 75k, docs passing, and Azure Pipelines success. The main heading is "Scanpy - Single-Cell Analysis in Python". Below the heading, there is a paragraph describing Scanpy as a scalable toolkit for analyzing single-cell gene expression data. A list of links follows: Discuss usage on Discourse and development on GitHub, Get started by browsing tutorials, usage principles or the main API, Follow changes in the release notes, Find tools that harmonize well with anndata & Scanpy via the external API and the ecosystem page, Check out our contributing guide for development practices, and Consider citing Genome Biology (2018) along with original references. The "News" section is titled "Scanpy hits 100 contributors!" with a date of 2022-03-31. Below the news title, there is a link "100 people have contributed to Scanpy's source code!". A paragraph of text follows, explaining that contributions to the project are not limited to direct modification of the source code.

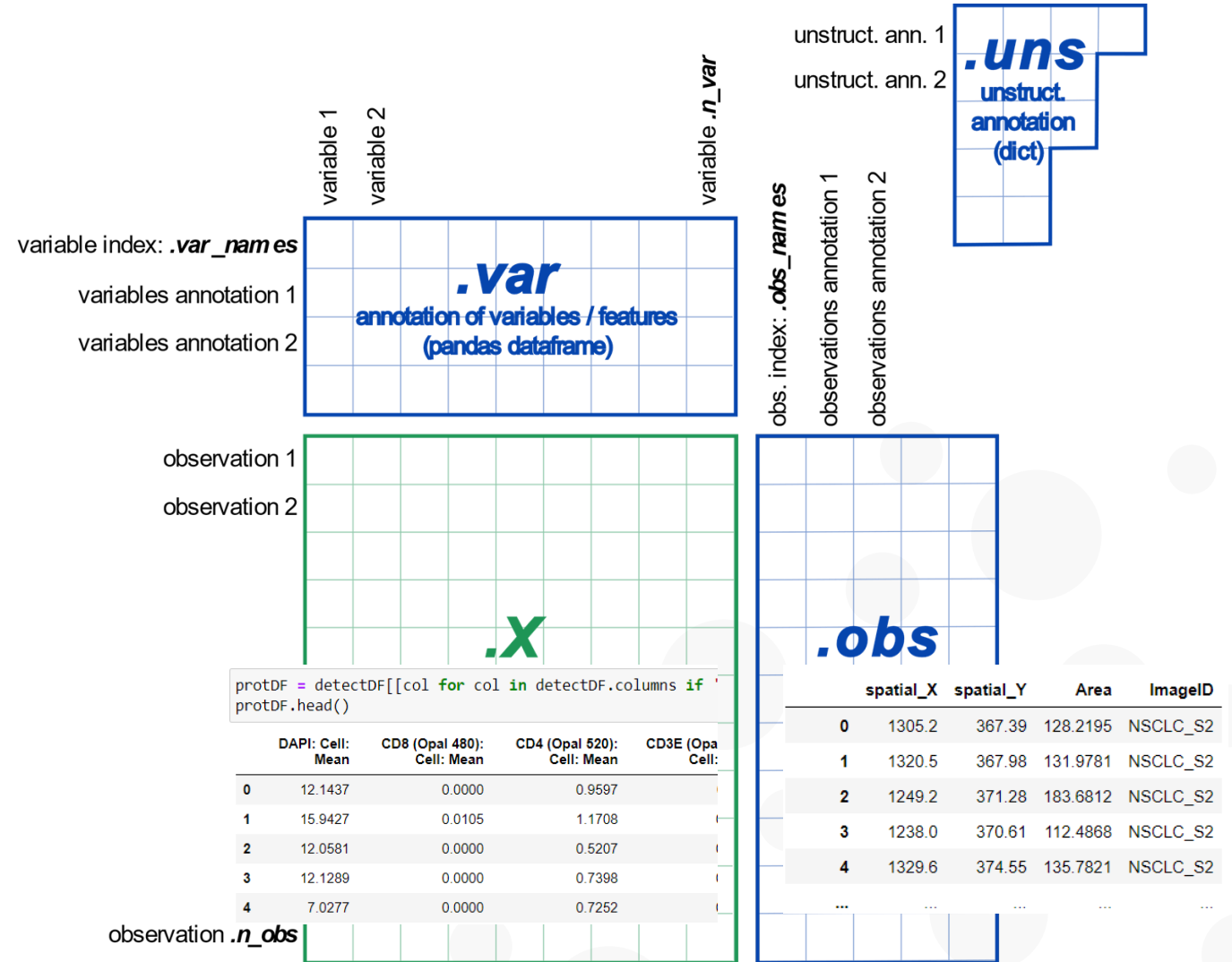
Step-1: Data Import

- AnnData
 - Expression data: `.X`
 - Spatial data: `.obs`
 - Cell metadata: `.obs`



Step-1: Data Import

- AnnData
 - Expression data: .X
 - Spatial data: .obs
 - Cell metadata: .obs



Step-2: Evaluate Staining Quality

- Compute Mean of top 20 cells and divide it by the mean of the bottom 10% of the cells of each marker
- Use ratio of top20:bottom10 as the signal-to-background(noise) ratio
- Ideally, greater than 10
 - Typically > 100



Multiplex Immunofluorescence and Multispectral Imaging: Forming the Basis of a Clinical Test Platform for Immuno-Oncology

Clifford C. Hoyt^{1*}

¹Akoya Biosciences Inc., Malborough, MA, United States

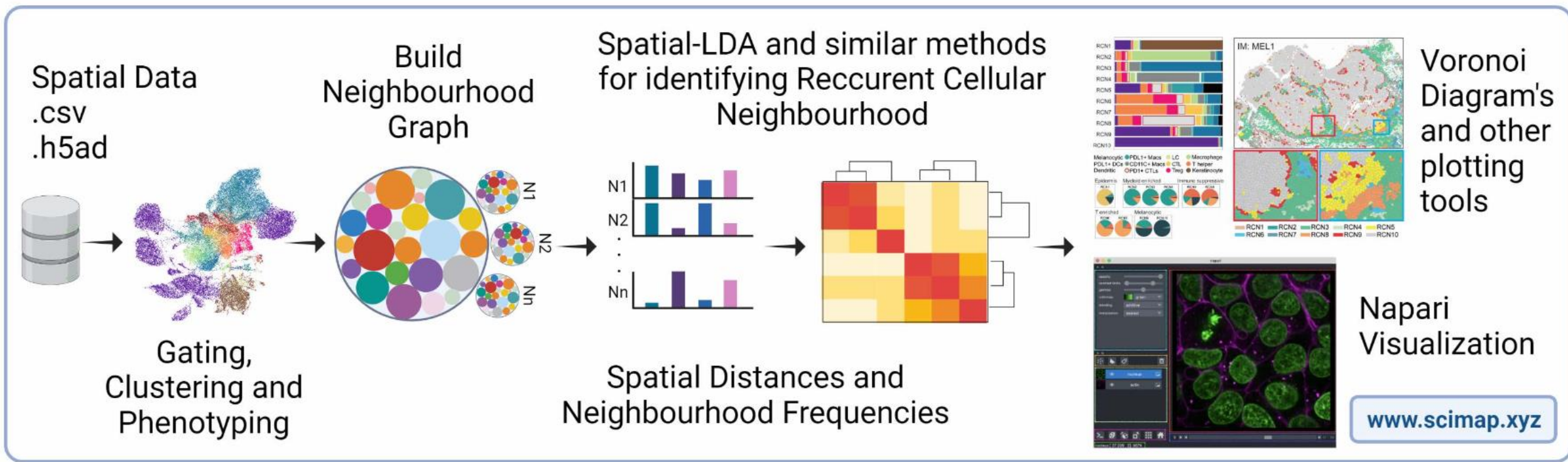
Dynamic Range Our standard approach assessing dynamic range is to calculate a signal-to-background (SNR) ratio by dividing the average of the top 20 brightest cells by the average intensity of the weakest 10% of cells. An SNR of 10 or more supports reliable image analysis, including accurate counting of positive cells and quantifying expression levels. While we recommend an SNR of 10 or greater, typical ratios are well in the 100s with high-performing antibodies, or as low as 3-to-1 that still provide analytical value.

Step-2: Evaluate Staining Quality Control

- Compute Mean of top 20 cells and divide it by the mean of the bottom 10 percentile of each marker
 - `computeTop20Btm10(AnnData)`
- Use ratio of top20:bottom10 as the signal-to-background(noise) ratio
- Ideally, greater than 20
 - Inf indicates zero background

ImageID	Protein	Top20/btm10
NSCLC_S1	CD8	inf
NSCLC_S1	CD4	inf
NSCLC_S1	CD3E	3306.3506
NSCLC_S1	CD20	771.2482
NSCLC_S1	PanCK	436.5684
NSCLC_S1	CD68	959.6335
NSCLC_S2	CD8	inf
NSCLC_S2	CD4	73434.0859
NSCLC_S2	CD3E	1826.7528
NSCLC_S2	CD20	64.1688
NSCLC_S2	PanCK	1425.5354
NSCLC_S2	CD68	276.1166

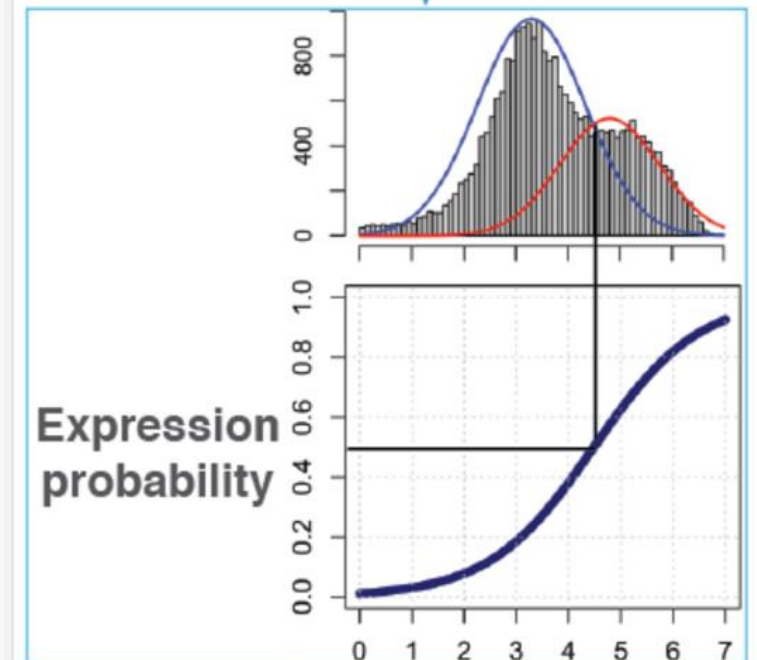
Step-3: Phenotyping



Step-3: Phenotyping

- Step 3a: Rescale data using GMM
 - Fit a 2-class Gaussian Mixture Model
 - Fully automated
 - Converts raw intensities to probabilities, i.e., values between 0-1
 - Value ≥ 0.5 indicated 'positive' for a given marker
 - `sm.pp.rescale(method='by_image')`

CD9	CK	CD7	FAP	CD3	CD4	CD8	...
2.5	4.3	2.7	4.3	6.7	1.3	3.5	...
8.9	2.8	8.5	2.8	7.5	4.8	2.3	...
6.1	0.2	5.1	0.2	2.1	1.2	0.8	...
							...

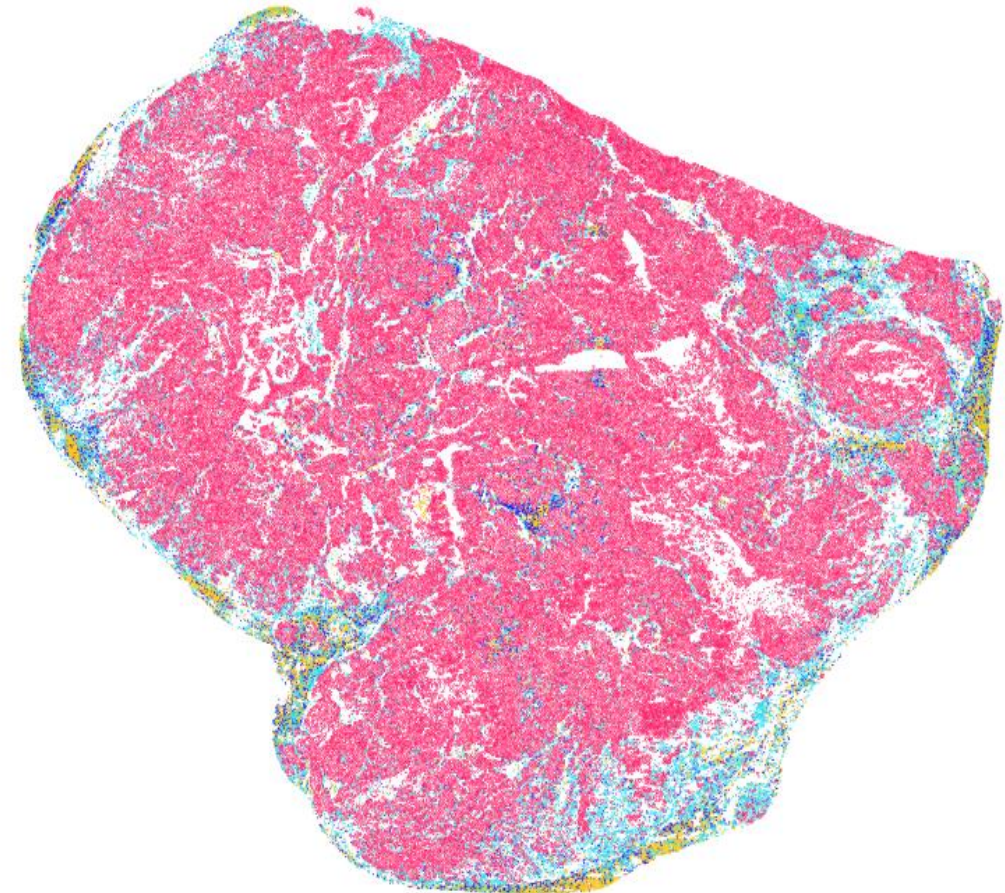
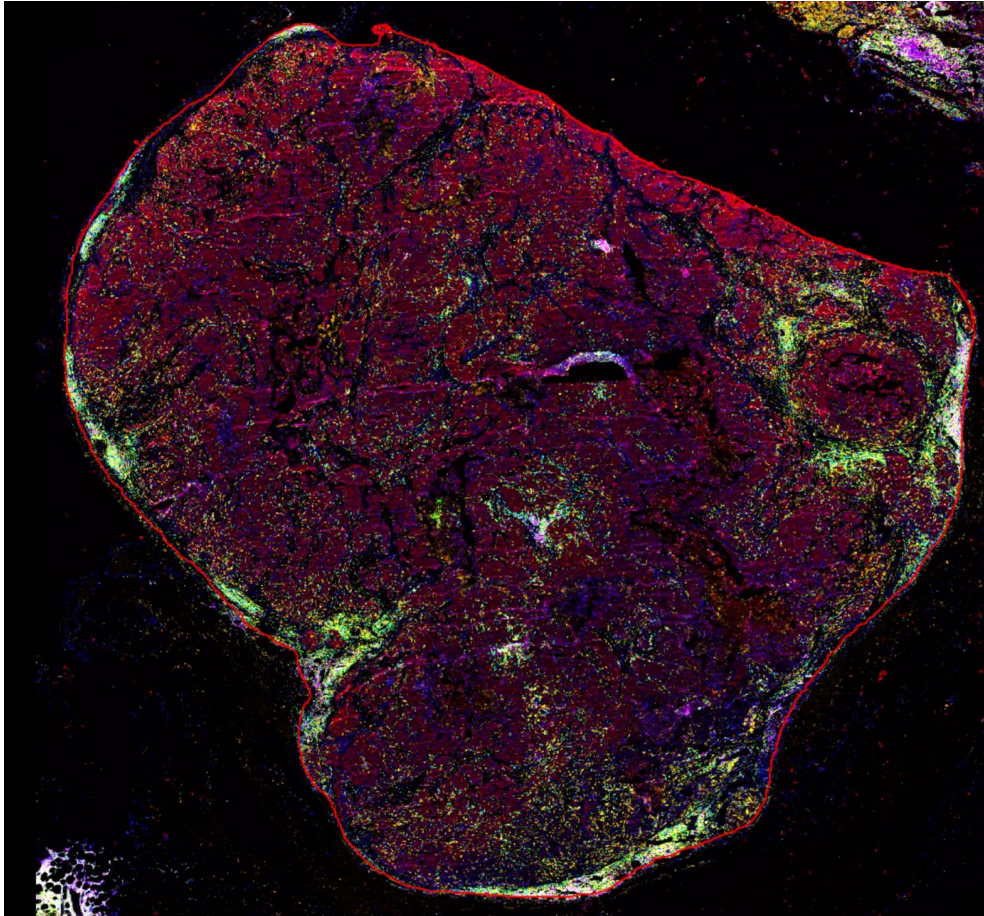


Step-3: Phenotyping

- Step 3b: Rule-based phenotyping
 - Value ≥ 0.5 indicated 'positive' for a given marker
 - If 'pos' -> assign a cell type
 - **CD20+** : B cells
 - **PanCK+**: Tumor
 - ...
 - **CD3E+ CD8+**: Cytotoxic T cells
 - `sm.tl.phenotype_cells()`

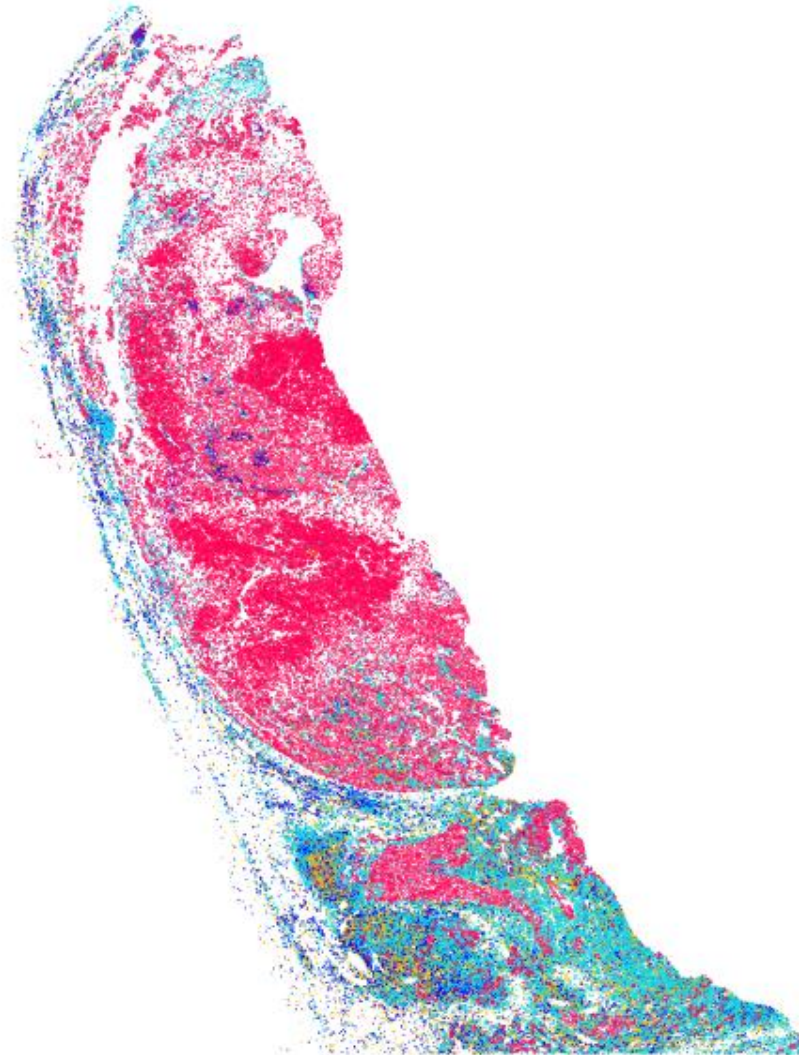
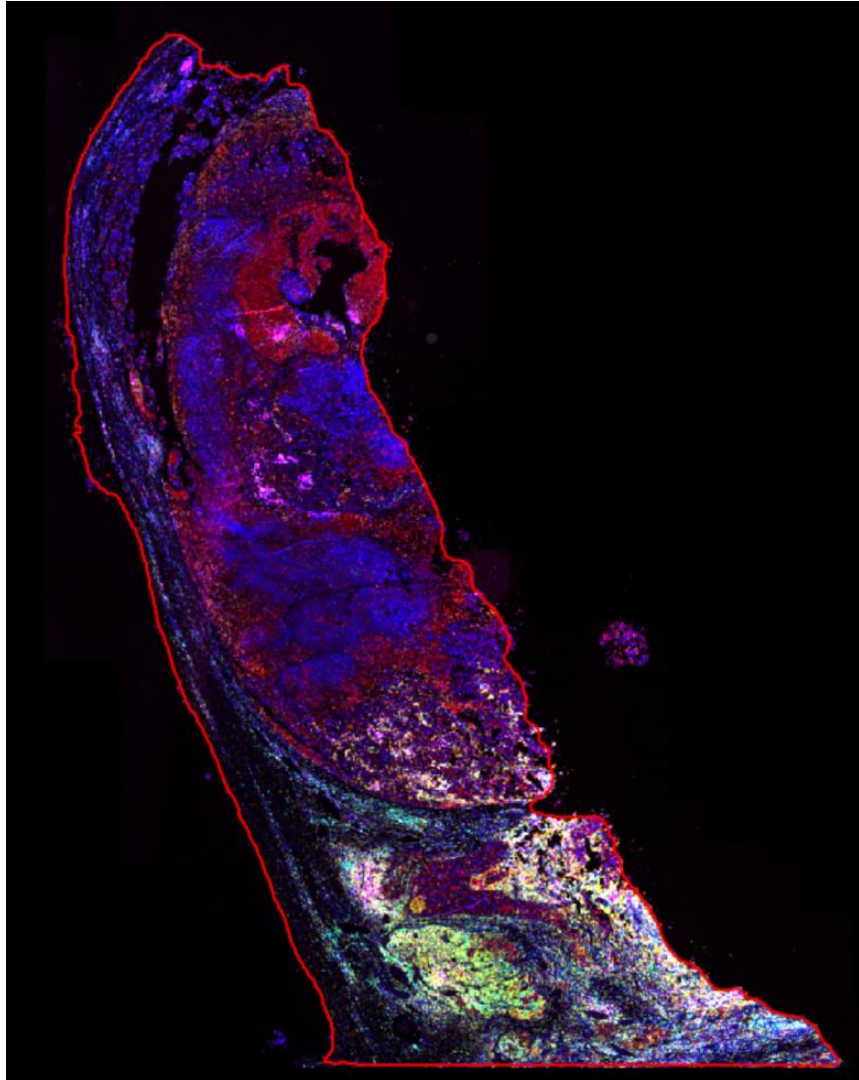
parent	child	CD3E	CD20	PanCK	CD68	CD8	CD4
all	B cells		pos				
all	T cells	pos					
all	Tumor			pos			
all	Macrophages				pos		
T cells	CD8+ T cells					pos	
T cells	CD4+ T cells						pos

Step-3: Phenotyping



- B cells
- CD4+ T cells
- CD8+ T cells
- Macrophages
- T cells
- Tumor

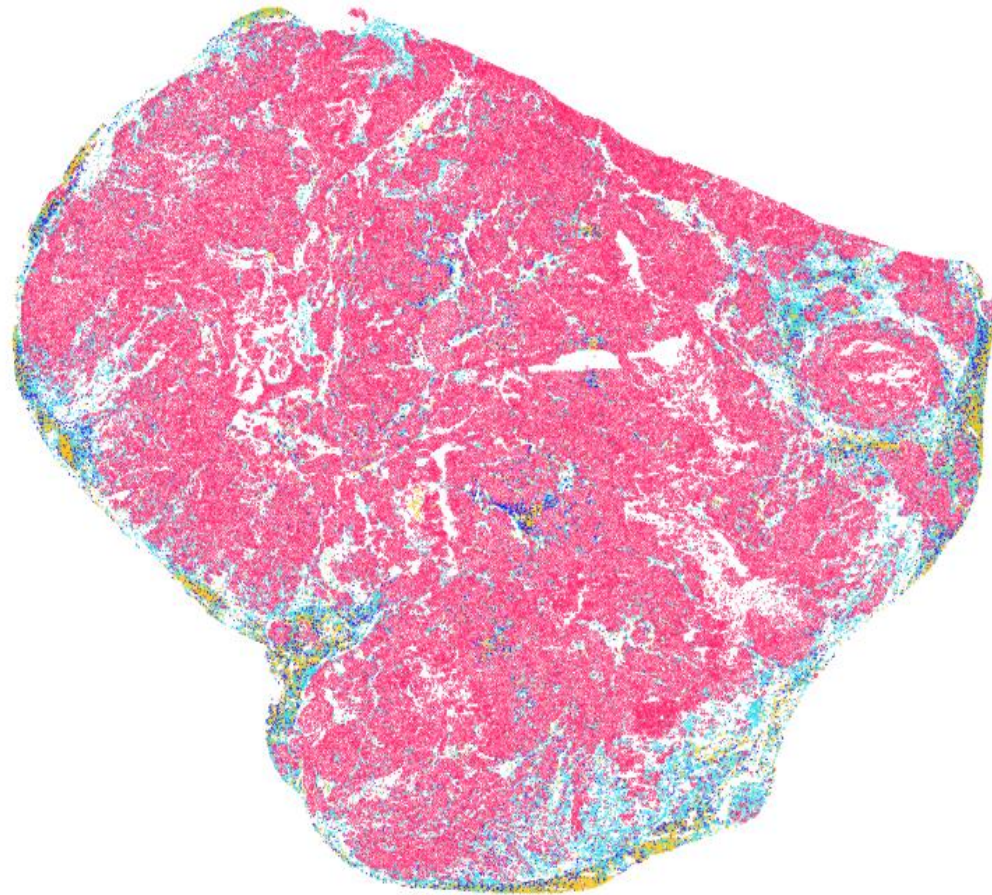
Step-3: Phenotyping



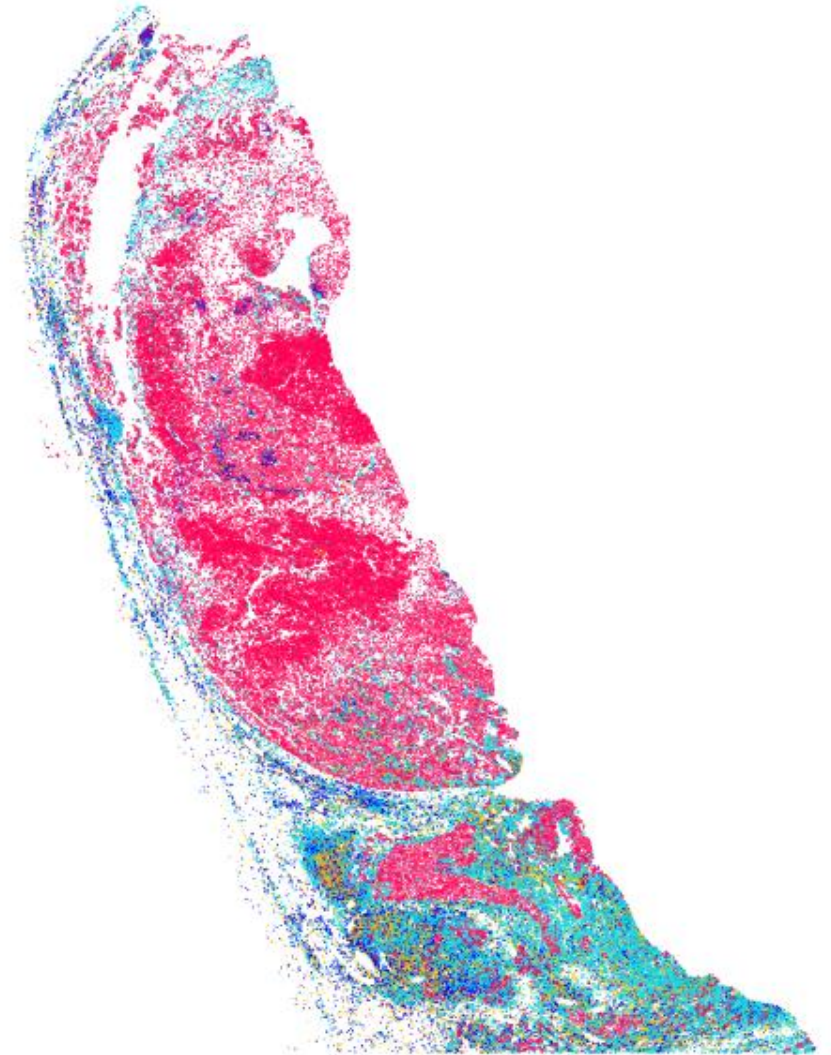
- B cells
- CD4+ T cells
- CD8+ T cells
- Macrophages
- T cells
- Tumor

Step-3: Phenotyping

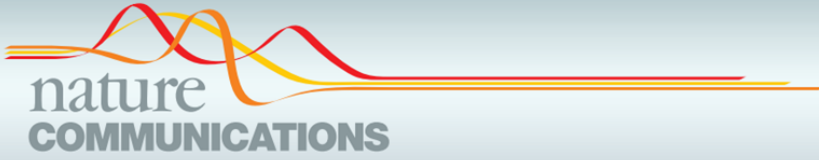
Responder vs. Non-responder



- B cells
- CD4+ T cells
- CD8+ T cells
- Macrophages
- T cells
- Tumor



Step-4: SpatialScore













nature
COMMUNICATIONS

ARTICLE Check for updates

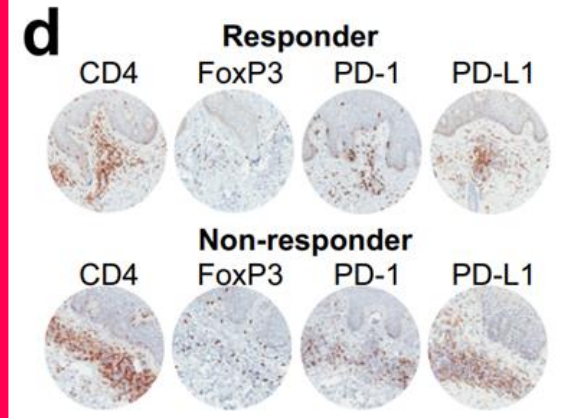
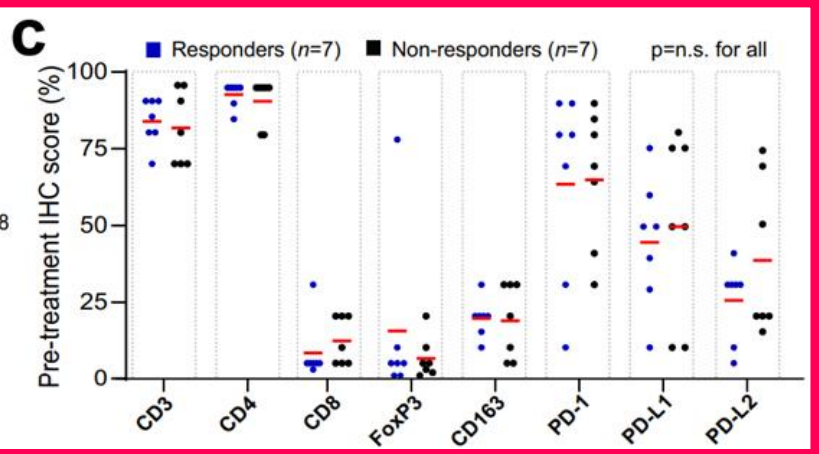
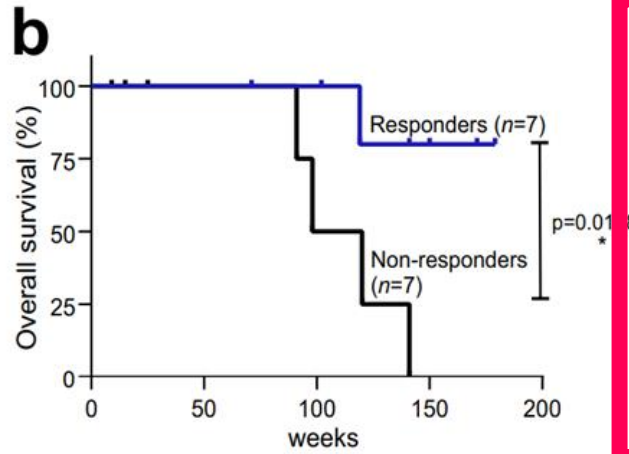
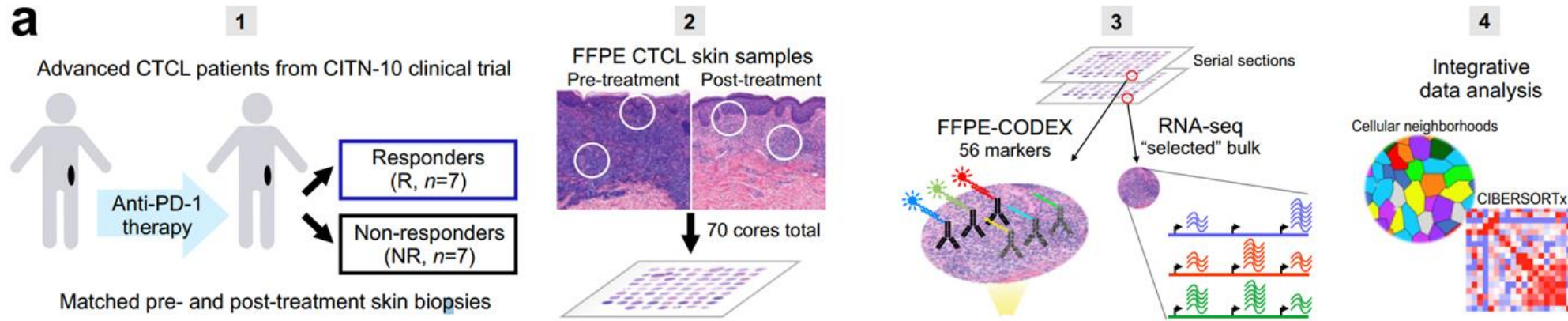
<https://doi.org/10.1038/s41467-021-26974-6> OPEN

Immune cell topography predicts response to PD-1 blockade in cutaneous T cell lymphoma

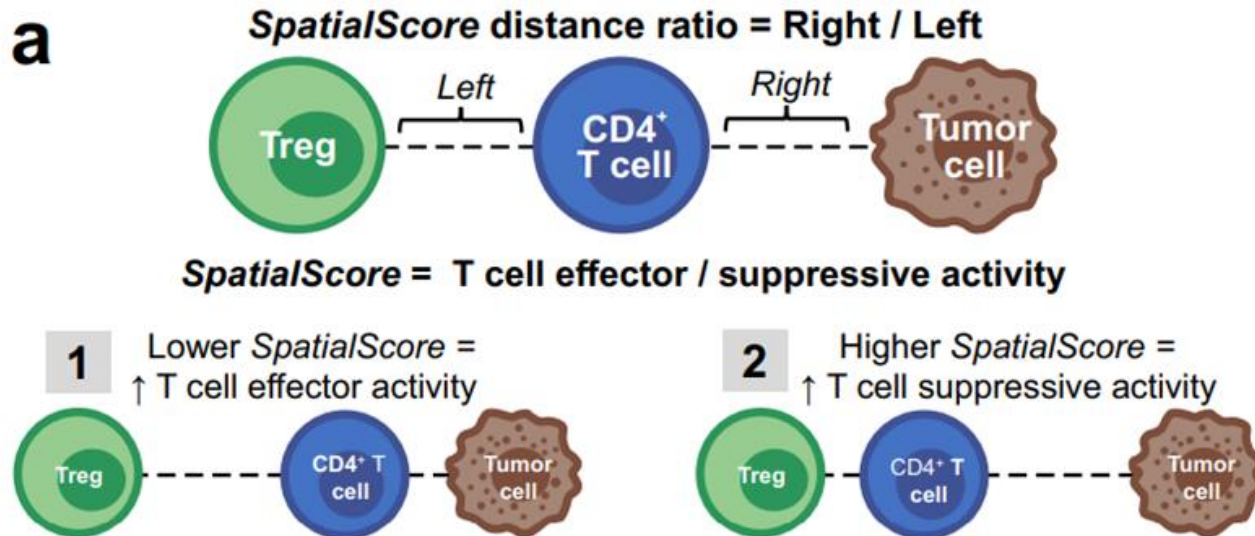
Darci Phillips ^{1,2,3,9}, Magdalena Matusiak^{3,9}, Belén Rivero Gutierrez³, Salil S. Bhat^{1,3,4}, Graham L. Barlow ^{1,3}, Sizon Jiang ^{1,3,5}, Janos Demeter ¹, Kimberly S. Smythe ⁶, Robert H. Pierce⁶, Steven P. Fling ⁶, Nirasha Ramchurren ⁶, Martin A. Cheever⁶, Yury Goltsev ^{1,3}, Robert B. West³, Michael S. Khodadoust^{7,10}, Youn H. Kim^{2,7,10}, Christian M. Schürch ^{1,3,8,10} ✉ & Garry P. Nolan ^{1,3,10} ✉

Cutaneous T cell lymphomas (CTCL) are rare but aggressive cancers without effective treatments. While a subset of patients derive benefit from PD-1 blockade, there is a critically unmet need for predictive biomarkers of response. Herein, we perform CODEX multiplexed

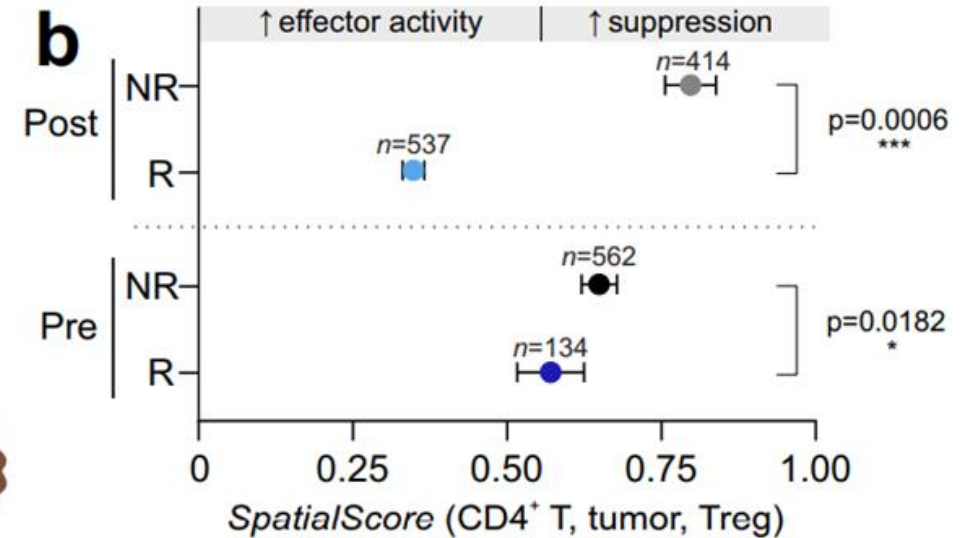
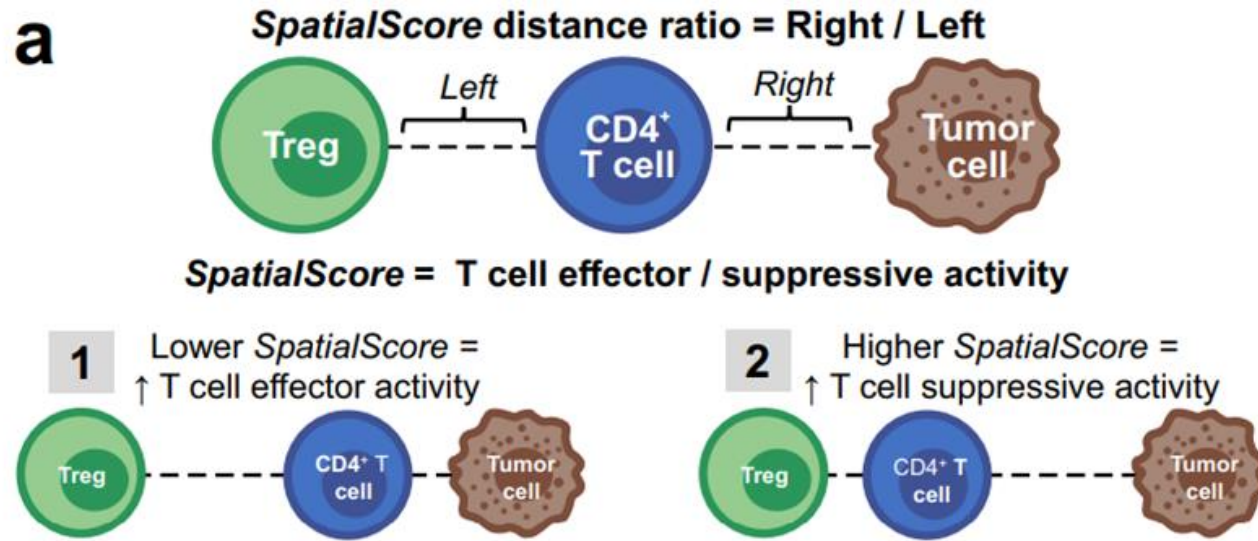
Step-4: SpatialScore



Step-4: SpatialScore

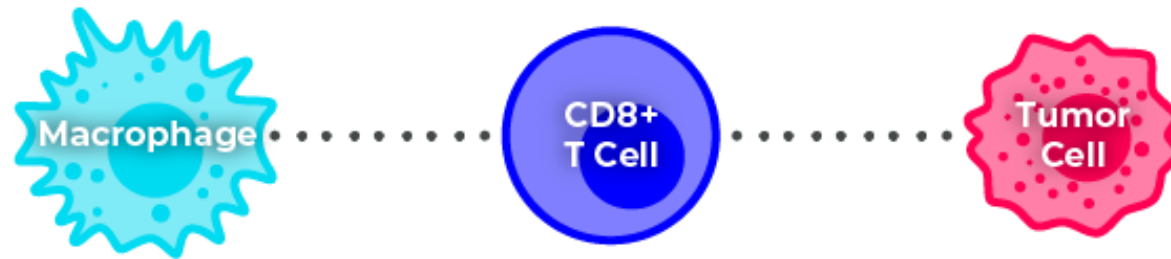


Step-4: SpatialScore



Step-4: SpatialScore

SpatialScore distance ratio = CD8+ T Cell to Tumor Cells / Macrophage to CD8+ T Cell

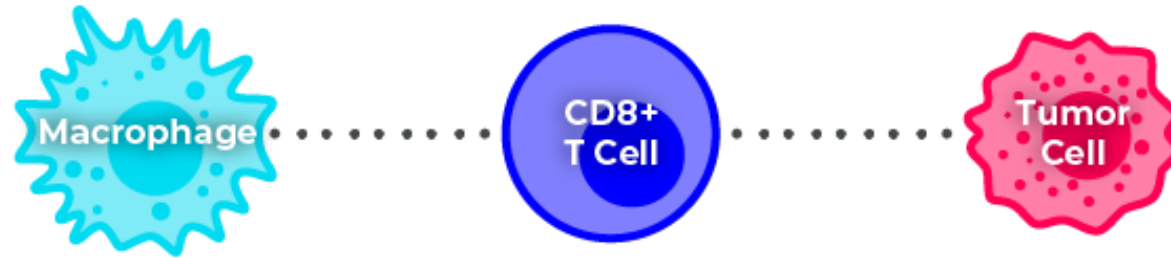


Low spatial score: High T cell *effector* activity; better outcome

High spatial score: High T cell *suppressive* activity; poor outcome

Step-4: SpatialScore

SpatialScore distance ratio = CD8+ T Cell to Tumor Cells / Macrophage to CD8+ T Cell

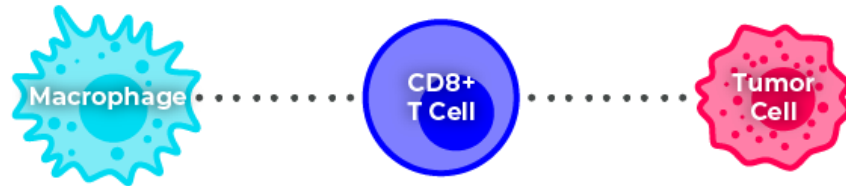


Low spatial score: High T cell *effector* activity; better outcome

High spatial score: High T cell *suppressive* activity; poor outcome

Step-4: SpatialScore

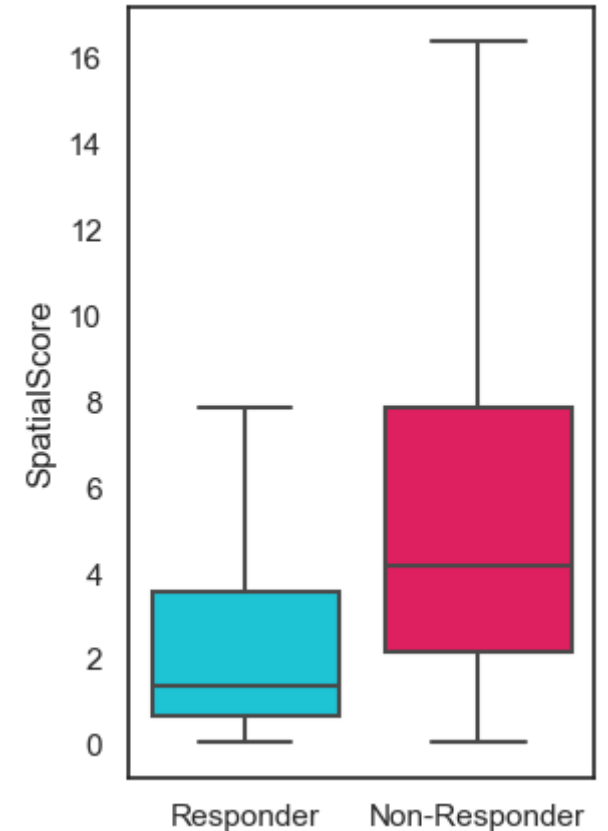
SpatialScore distance ratio = CD8+ T Cell to Tumor Cells / Macrophage to CD8+ T Cell



Low spatial score: High T cell *effector* activity; better outcome

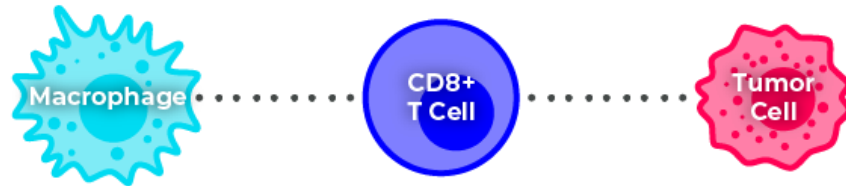
High spatial score: High T cell *suppressive* activity; poor outcome

- `computeSpatialScore(left='Macs',middle='CD8',right='Tumor')`
- Compute and plot value for each CD8+ T cell



Step-4: SpatialScore

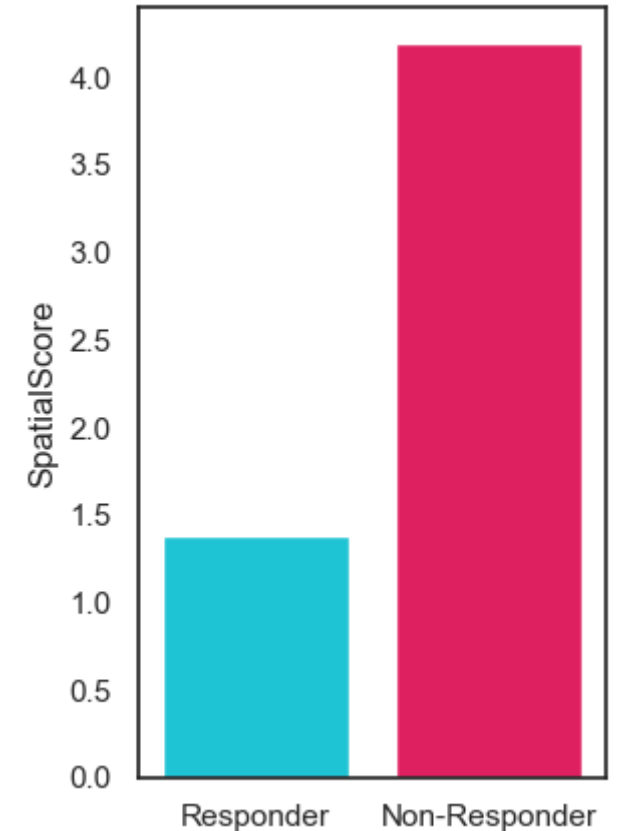
SpatialScore distance ratio = CD8+ T Cell to Tumor Cells / Macrophage to CD8+ T Cell



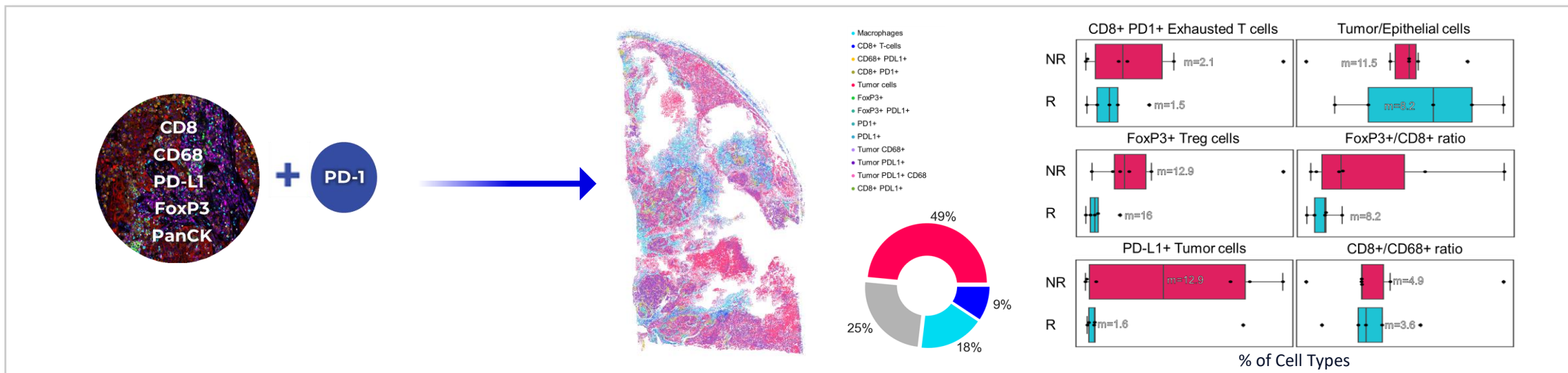
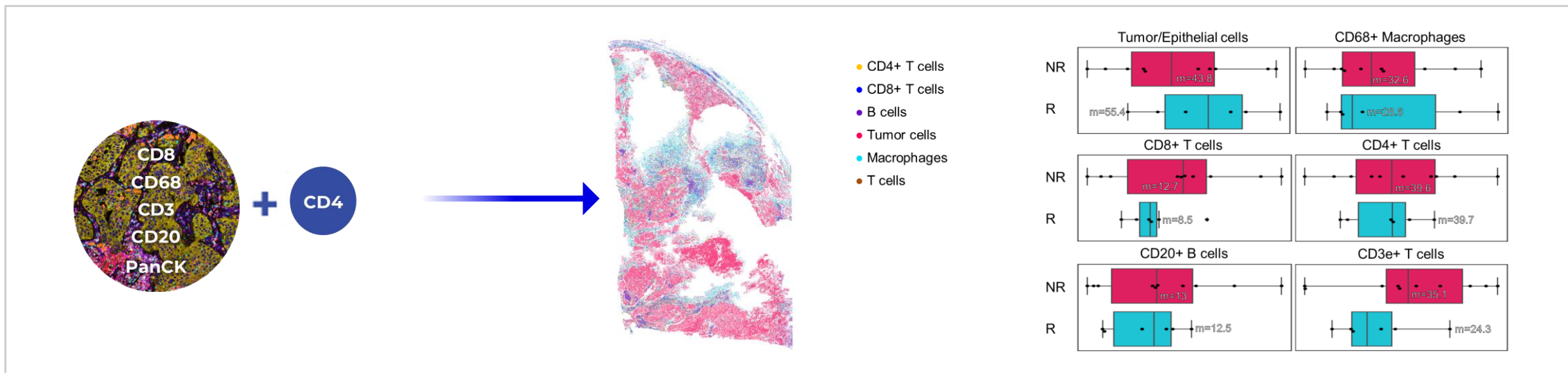
Low spatial score: High T cell *effector* activity; better outcome

High spatial score: High T cell *suppressive* activity; poor outcome

- `computeSpatialScore(left='Macs',middle='CD8',right='Tumor')`
- Compute and plot median value per sample



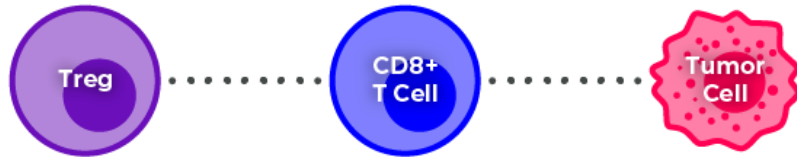
6-plex Spatial Phenotyping with PSP Validates Immune Landscape but Rules out Differences in Immune Cell Composition



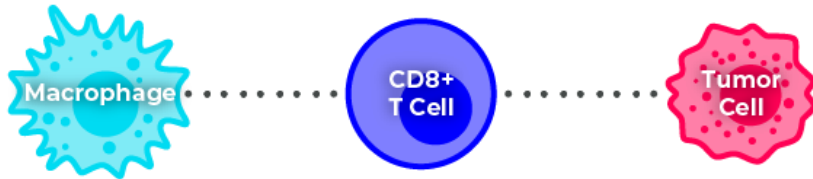
A Predictive Spatial Signature for Treatment Outcomes

SpatialScore

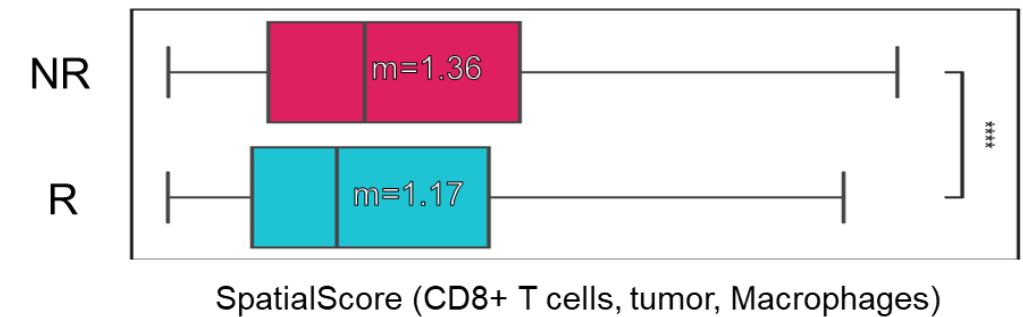
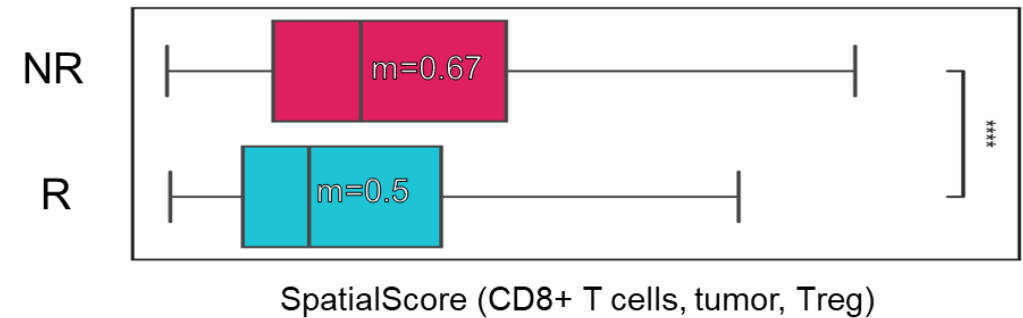
SpatialScore distance ratio = CD8+ T Cell to Tumor Cells / Treg to CD8+ T Cell



SpatialScore distance ratio = CD8+ T Cell to Tumor Cells / Macrophage to CD8+ T Cell



Prediction of ICI Response



Conclusion

- QuPath and Python-based workflow for Phenolmager analysis
 - QuPath: Visualization, segmentation
 - Python: Data processing, phenotyping, spatial metrics
- SpatialScore helps identify spatial insights into your Phenolmager data
- **Data and code available at: https://bit.ly/akoya_0622**

Questions from Zoom Webinar

Question	Answer
Hello! Is this webinar recorded? Can I follow it again in my own time?	Yes, this webinar will be available in a few days on the Akoya webpage under Resources and Akoya Academy.
Please also provide a programming note for the software used.	The code and data can be found here: https://bit.ly/akoya_0622
QuPath is not compatible with the latest Mac system Ventura. Any solution?	The latest version of QuPath is compatible with Ventura (see: https://twitter.com/petebankhead/status/1681554982464544768). The Python scripts are compatible with data from the older version of QuPath (<0.4) as well, so the general workflow remains the same.
The `modeltoPath` step is unclear.	QuPath provides helpful documentation on getting the segmentation model, please refer to QuPath's StarDist extension
Are there any plugins/scripts/software available to create a membrane mask based on membrane markers?	You can try CellPose: https://github.com/MouseLand/cellpose/
If I have to analyze the whole image, sometimes there are some artifacts in the tissue, can we excluded some areas?	Yes, you can include/exclude ROIs using QuPath's Annotation tool.
Can the whole workflow be replicated in R instead of Python?	Yes, instead of Scanpy/Scimap, you can use Seurat/CELESTA for automated phenotyping. Additional resources are linked on the Github repo.
Is the distance measured in microns?	Yes, QuPath stores cell coordinates in microns, so all distance calculations are in microns as well.
Is it possible to also say negative while tagging. In order to eliminate mistakes during scanning. especially when one dye bleed into another.	Yes, refer to Scimap's documentation for all the combination of rules you can set.
How do you convert the QuPath data formatting into AnnData - What if you already phenotyped in QuPath?	QuPath's export measurements will store the Classification information under the Class variable. You can include that column while creating the AnnData object.
Is there a difference in phenotyping by using QuPath's sequential classifiers compared to scimap?	QuPath's classifier does not include the normalization step so the results may vary.
How do you generate a fully unmixed WSI that you are using - are you doing any stitching?	Yes, stitching can be done with the QuPath's script here: https://gist.github.com/petebankhead/b5a86caa333de1fdcff6bdee72a20abe
Is the gejson file same as the .tsv format export from your inference?	No, gejson stores additional information like the cell's outline as a polygon, which isn't included in the tsv/csv.
What would be the clear advantage of using QuPath versus ScanPy or SquidPy?	Scanpy does not contain cell segmentation module. However, if one is interested in running cell segmentation programmatically, they can use Stardist's Python implementation directly.