Scout 2.1 Software

Training Presentation

Welcome!

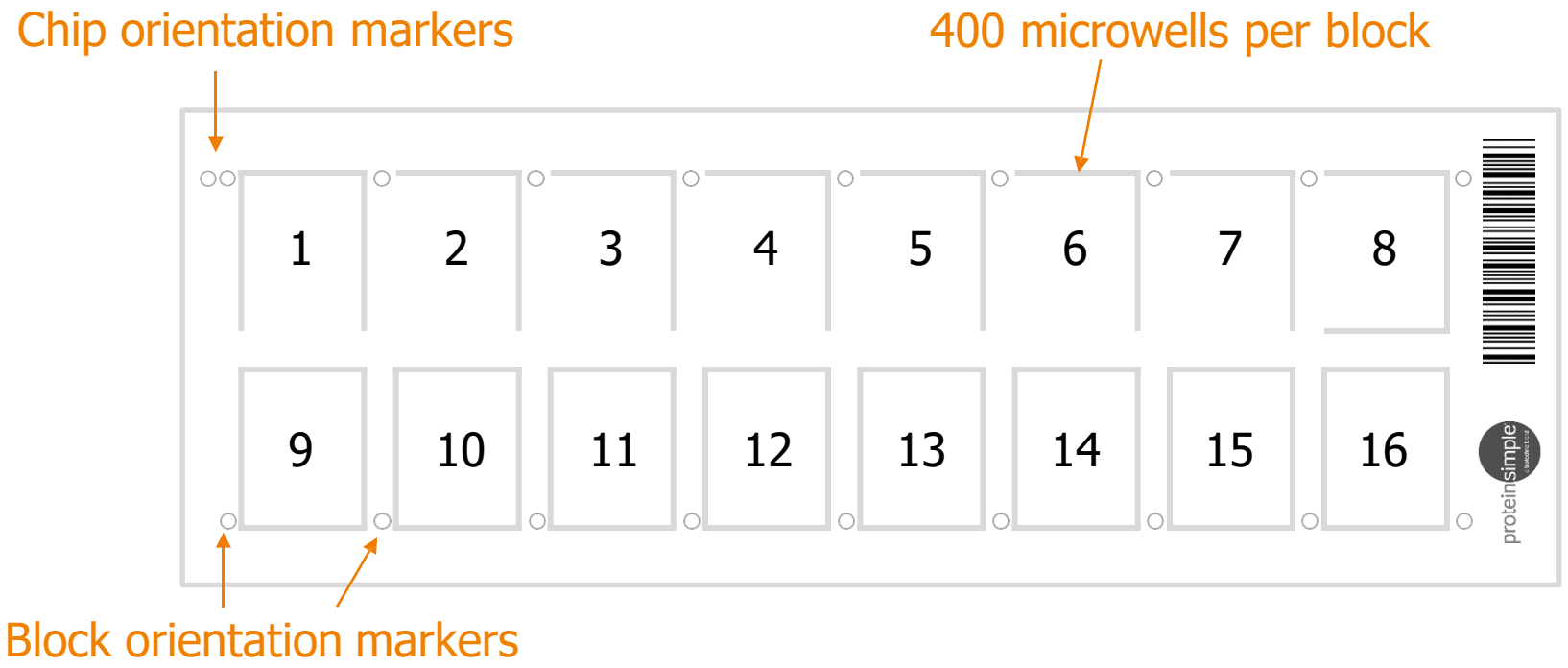
- In this training we will cover:
  - How to analyze scWest chip images in Scout
    - Opening images
    - Detecting peaks
    - Eliminating noise peaks
    - Labeling your peaks of interest
    - Visualizing your data
    - Exporting data for further analysis
  - Advanced features including:
    - Stripping & reprobing
    - Three-Plex Probing Chamber data
    - Molecular weight sizing
    - Normalizing data

System Requirements

- Scout software requires 64-bit versions of Windows 7 and 10 or

  Mac OS-X OS-X 10.11 (El Capitan), 10.12 (Sierra), 10.13 (High Sierra)
- Minimum of 16GB of RAM recommended

A reminder about chip layout



A simple, automated workflow for high quality images

1. Read all images in using auto registration. Peaks will be detected using default settings
2. Generate peak table for each scan
3. Run Auto Tag function for each peak table
4. Label peaks for protein targets of interest
5. Visualize data

Key Steps to Analyzing Your Images

1. Open images
    - Auto registration
    - Manual registration
2. Automatically detect peaks
3. Reject unwanted sections of chip
4. Optimize peak detection settings
5. Remove noise peaks & label peaks for proteins of interest
    - Generate peak table
    - Auto Tag for automated peak curation
    - Manual exclusion of remaining noise peaks
    - Manual peak labeling
    - Inspect function
6. Visualize data
7. (optional) Export data for further analysis

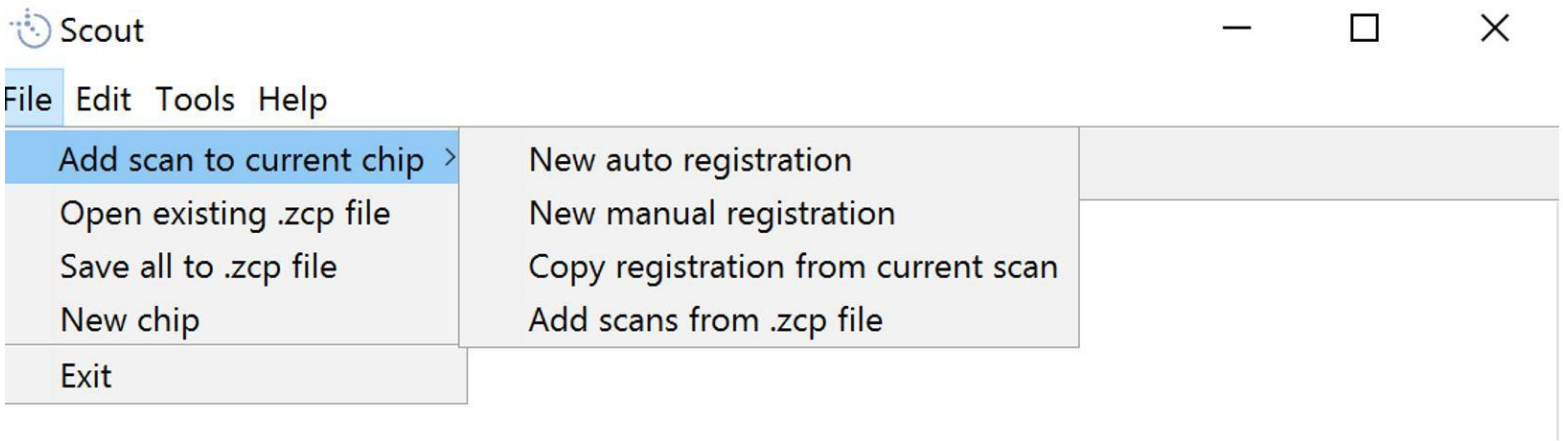
Advanced features:

- Stripping & reprobing
- Images from Three-Plex Probing Chamber
- Molecular weight sizing
- Normalizing peak areas
- Detecting overrun peaks

Opening Images

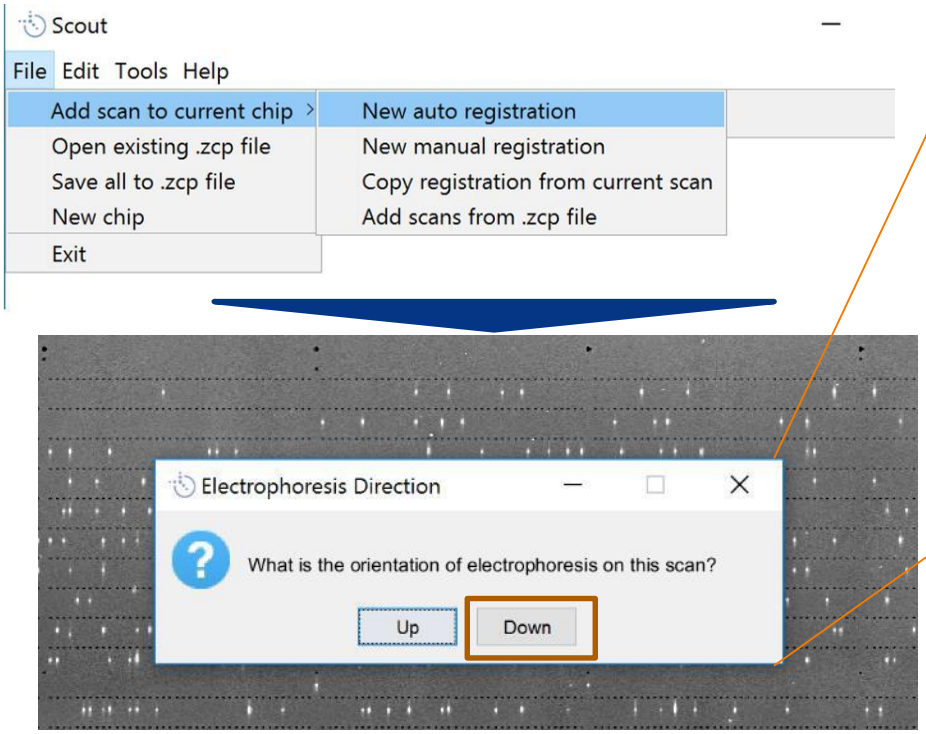
Opening images

- Open new chip window and add scan containing loading control protein as a tab
- After analyzing first image (detailed in following slides), add and analyze additional scans as separate tabs

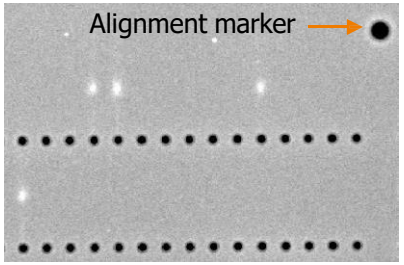


Auto registration

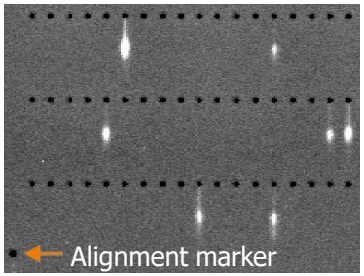
- Automatically aligns your chip image, finds all 6,400 lanes on your chip, and detects peaks in each lane with default peak detection settings
- Can be used in most cases



Select which direction the separation is occurring in the image



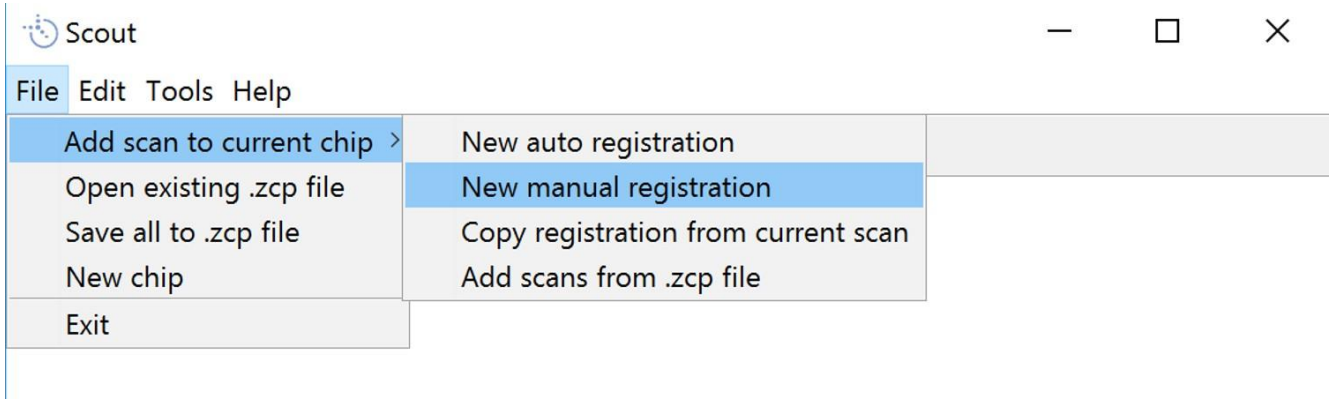
Up



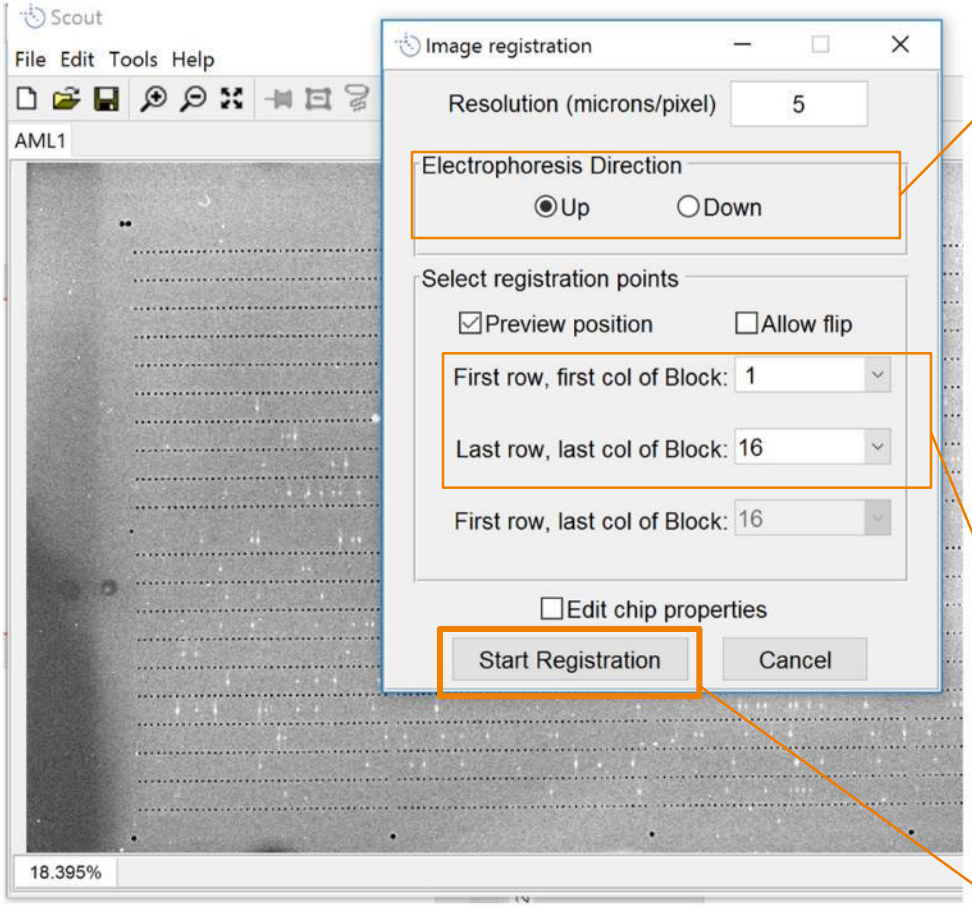
Down

Manual registration

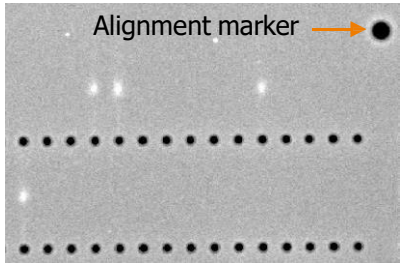
- If the auto registration fails (can occur because of poor scan quality), use manual registration to align chip image



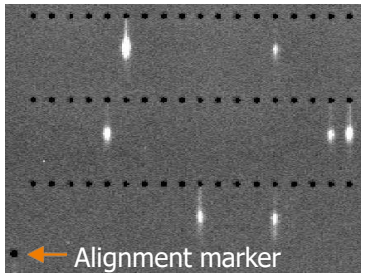
Manual registration



1. Note whether your separations are occurring up or down in the image



Up



Down

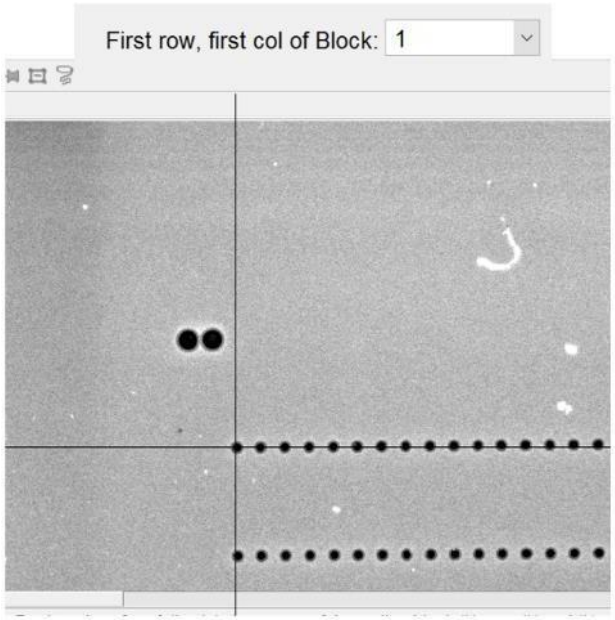
2. Choose 2 of the 16 microwell blocks to register your image

3. Start Registration

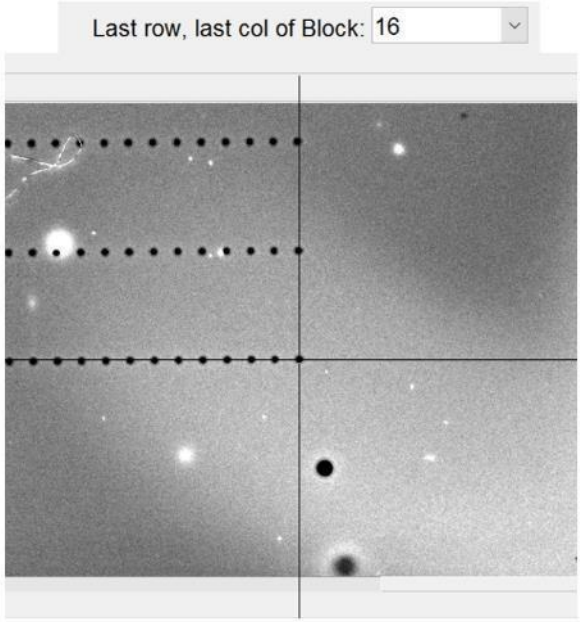
Manual registration

- Click on the center of the 1st well in first specified block and last well of second specified block

First Specified Block (Block 1)



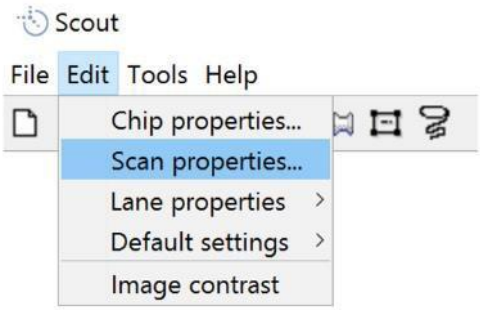
Second Specified Block (Block 16)

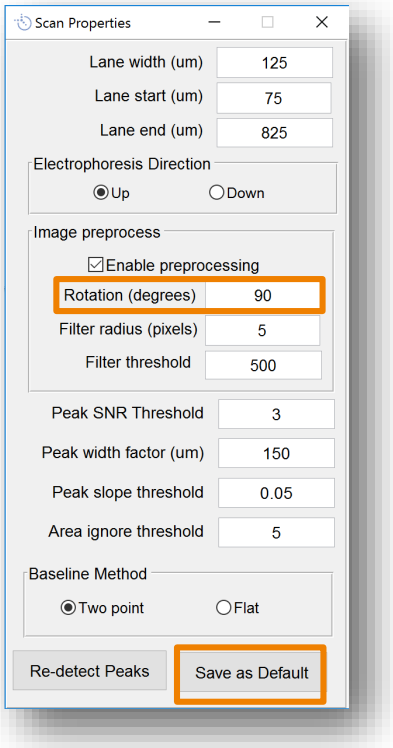


- Software will then automatically align the images, find all 6400 lanes, and detect peaks in each lane with default peak detection settings

(optional) Rotating images for manual registration

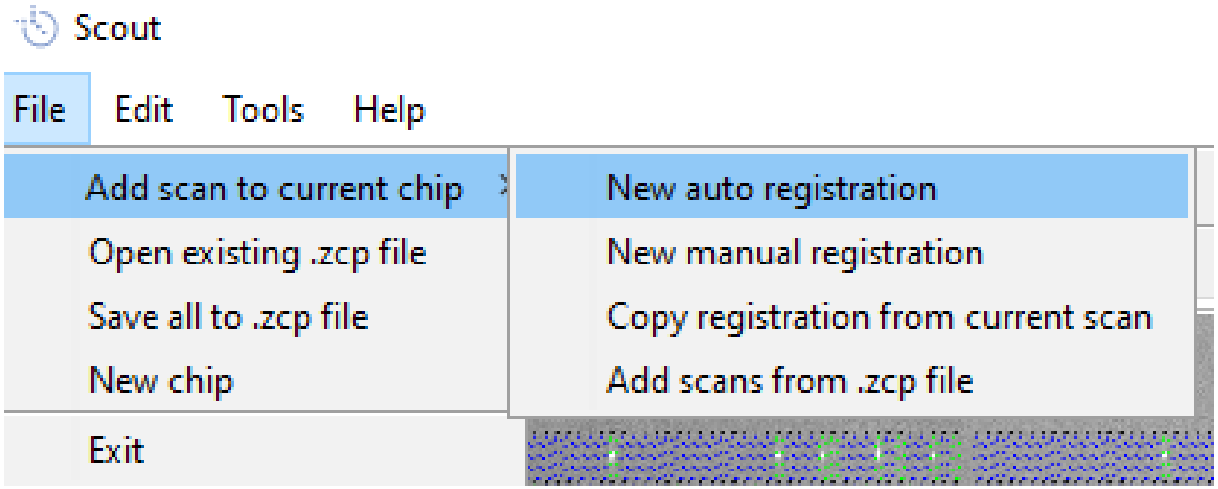
- To input images with default orientation settings, chip image should be vertical with double dot feature in upper right corner
- If microarray scanner image is saved in the horizontal orientation, open Scan Properties window and change image preprocessing rotation to 0 or 180 degrees
- Save as default
- Then open images using manual registration



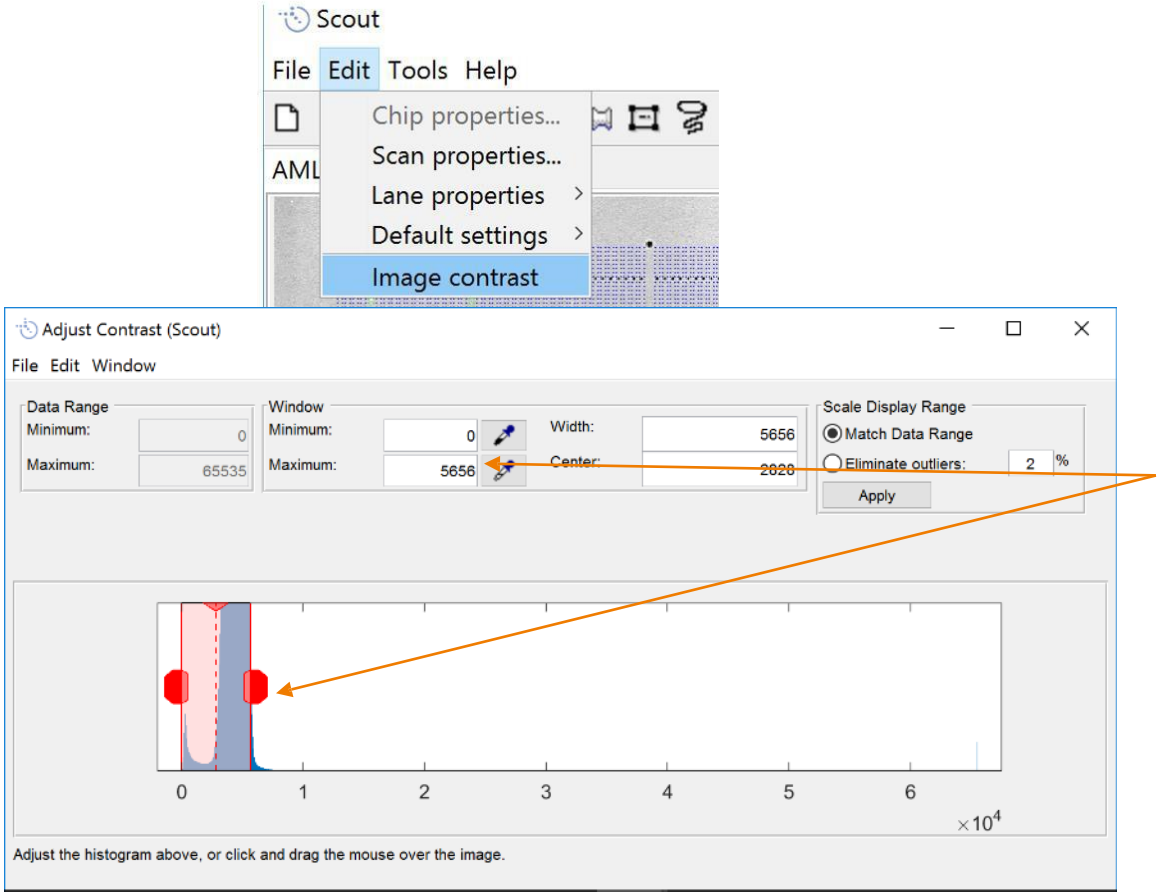


Adding additional scans to chip

- Add additional images to the chip by repeating the New auto registration or New manual registration steps (as shown previously)



(optional) Adjusting image contrast



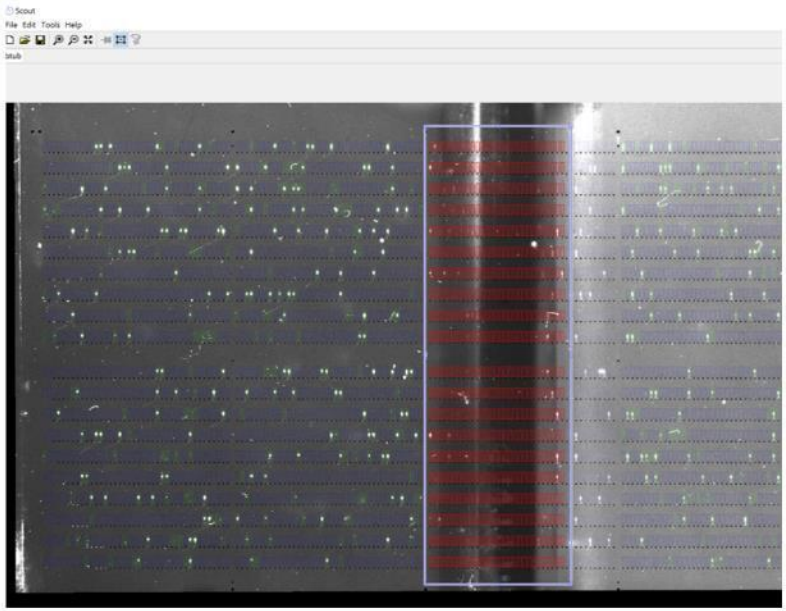
Drag red handles left and right or change minimum/maximum window values to adjust contrast in image

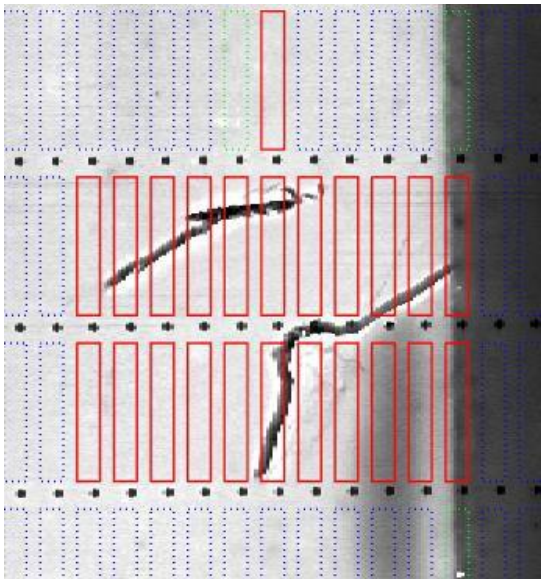
Note: changing the contrast does not change the data

- Adjusting image contrast may be needed for manual registration when the alignment wells are not visible with default settings.

(optional) Reject unwanted regions

- Reject regions of the chip with:
  - Major gel fouling or ripping due to handling errors
  - Areas between chambers that were not probed when using a 3-Plex Probing Chamber

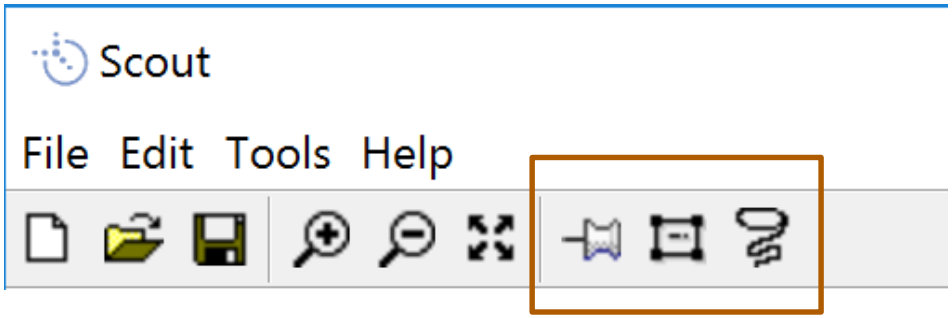




(optional) Reject unwanted regions

- To reject chip regions:
  - Select lanes in any sections that you want to remove and mark them "**Rejected**" (right click, "Mark as Rejected" or [r])
  - Apply selected lanes across all scans (right click menu shown below) and mark them "**Rejected**" across all scans

Selecting multiple lanes in an image



- **Pin**: Click on multiple lanes to select them all (can also be done by holding down Shift key)
- **Rubber band box**: drag to select lanes within rectangular region (can also be done by holding down Ctrl key)
- **Lasso tool**: drag to select lanes within user-defined region

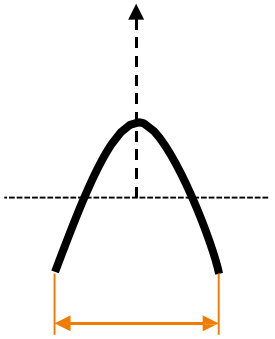
How Does Scout Detect Peaks?

Peak detection process in Scout

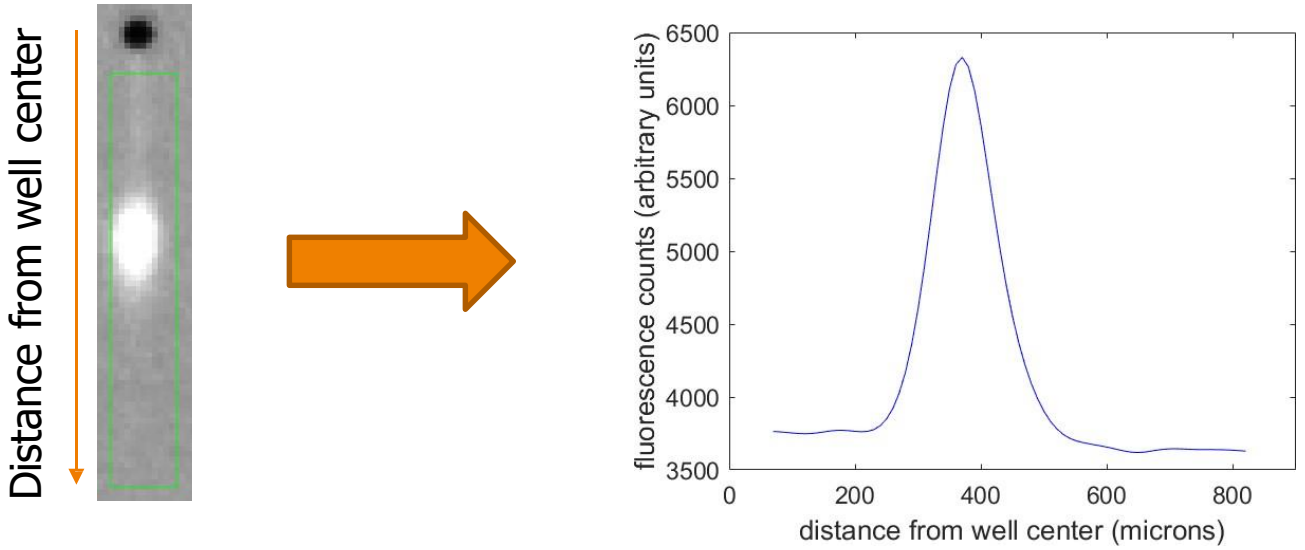
- Scout detects every possible peak (no threshold)
- Estimates which peaks are noise peaks
- Looks for all peaks that have a Signal to Noise Ratio (SNR) ≥ 3
- SNR threshold can be adjusted by user as needed. Decreasing SNR threshold will decrease stringency in peak detection or lead to more peaks being detected.

Peak detection: creation of correlation SNR plot

1. Scout defines a canonical peak shape

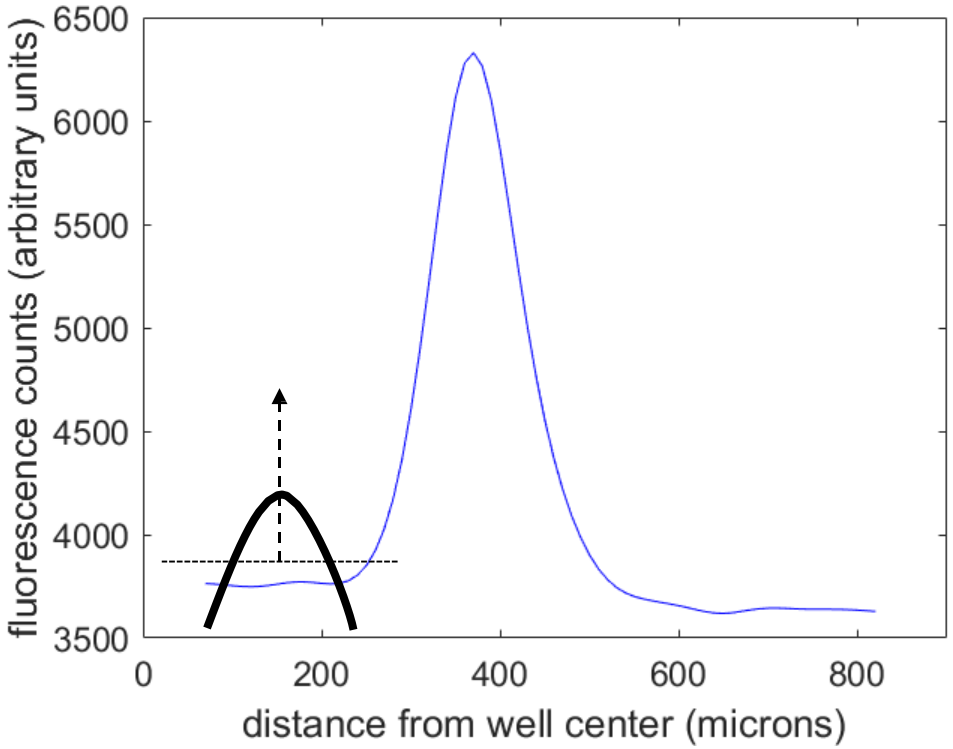


2. Converts 2-D gel images to 1-D intensity plots



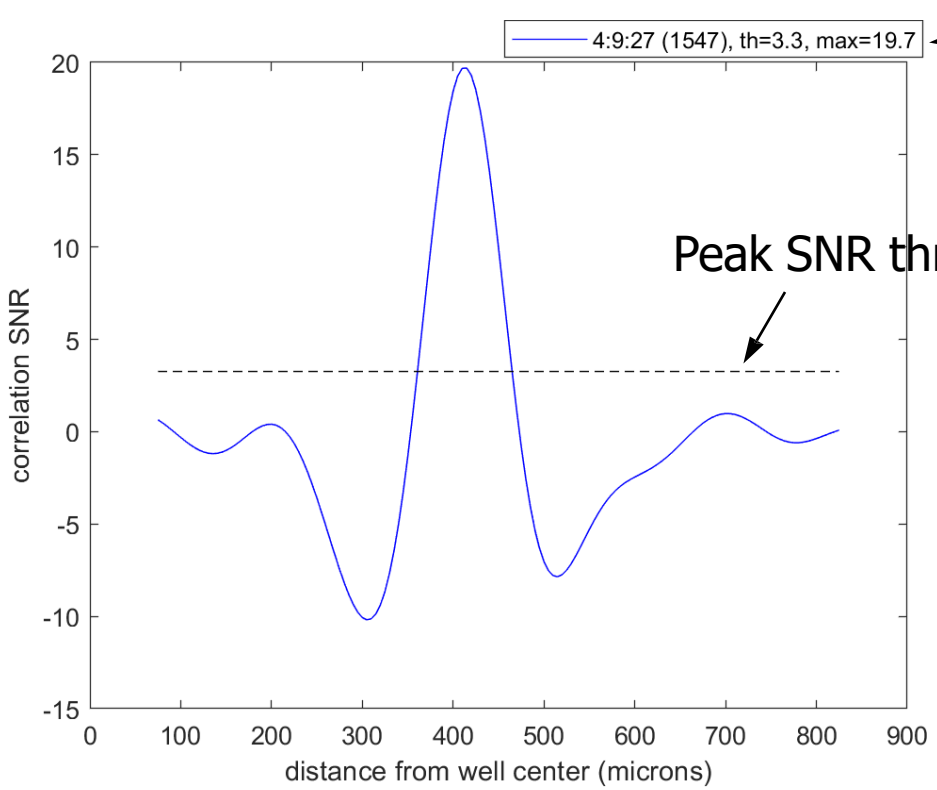
Peak detection: creation of correlation SNR plot

3. Convolves shape with intensity plot



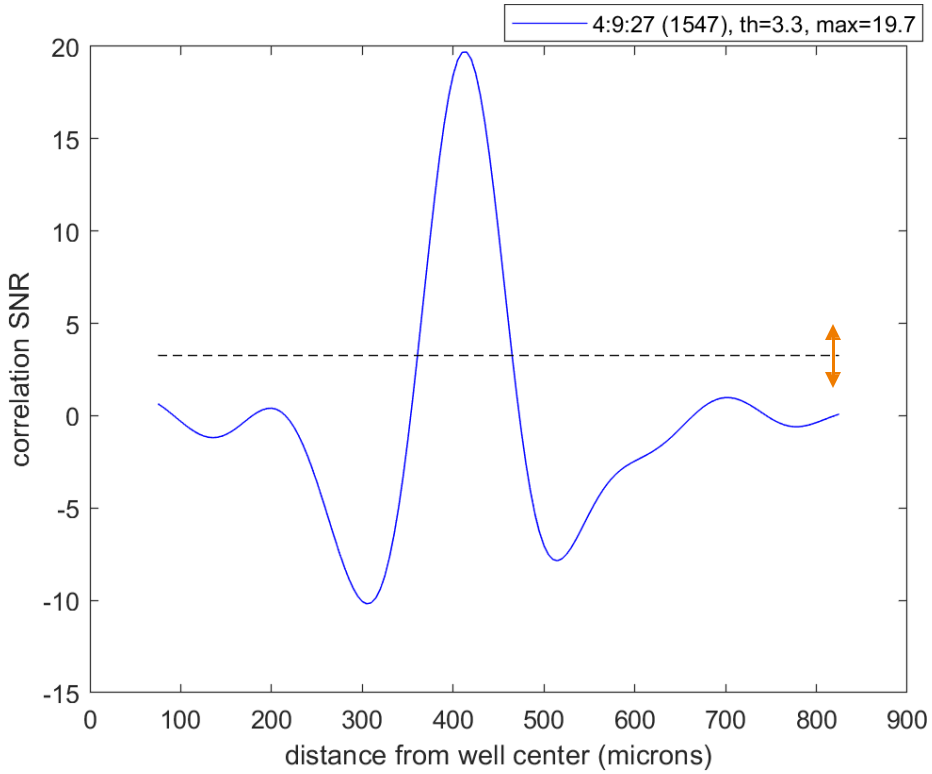
Peak detection: creation of correlation SNR plot

4. Creates correlation plot



Peak detection: creation of correlation SNR plot

5. SNR threshold can be adjusted to detect all peaks of interest (if necessary)



Optimizing Peak Detection Settings

Checking default peak detection

- Once peaks are detected, scan through the image to see if lanes with visible peaks of interest are highlighted in green
- In most cases, default settings will be sufficient to detect all peaks
- However, if some peaks are not detected, proceed to optimize peak detection settings
- It is better to set peak detection settings to capture all protein peaks *and* some noise peaks since noise peaks can be easily removed in the peak curation step

Scan Properties Window

- Changes peak detection settings across the full image

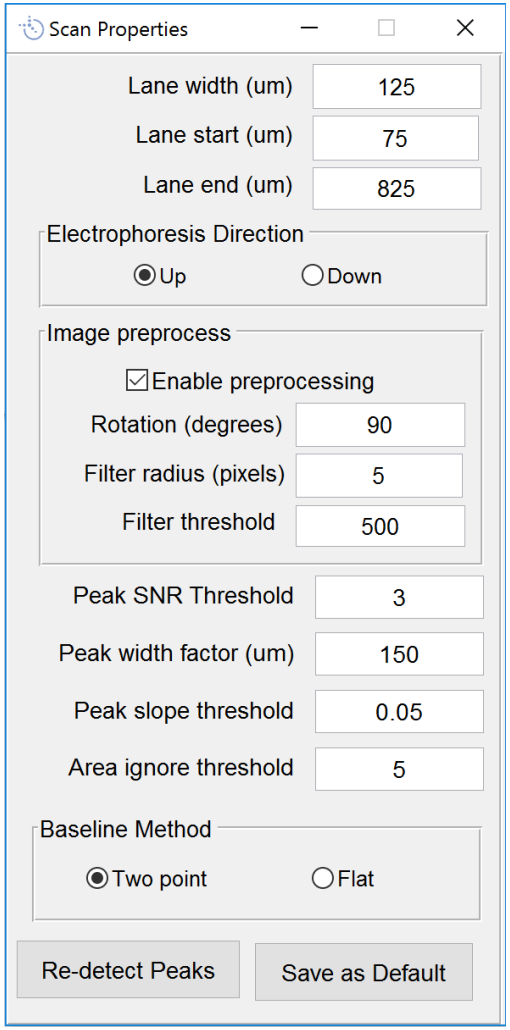
Changes dimensions of lanes used for detection

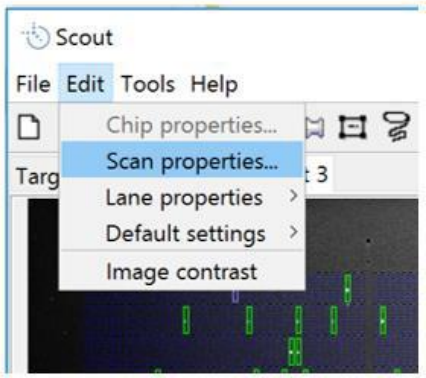
Sets migration direction in image

Sets image preprocessing (typically leave as default)

Parameters used in peak detection algorithm

Different methods of setting peak baseline

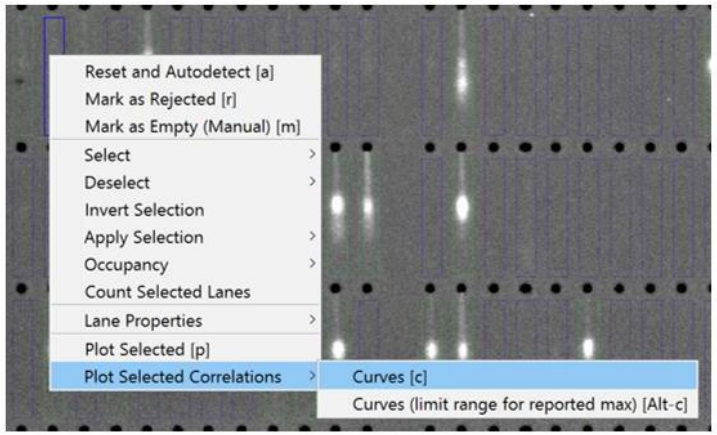




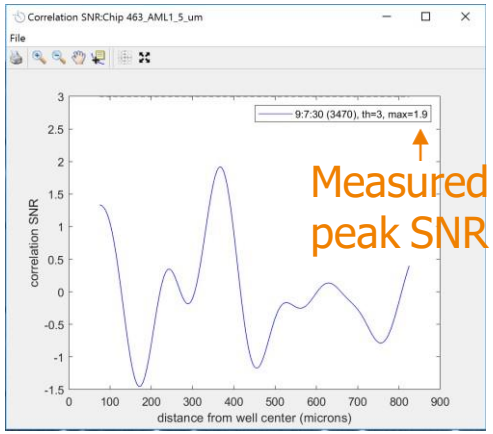
Next slides provide more detail on major parameters to adjust

Adjusting Peak SNR Threshold

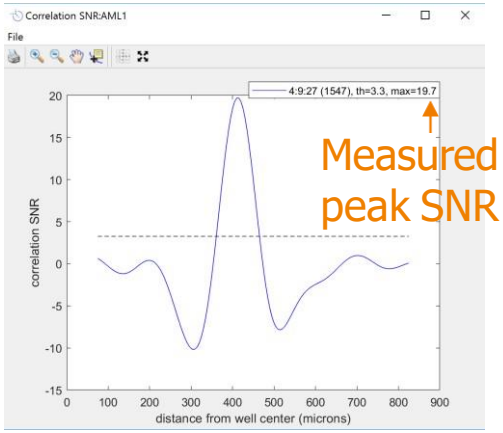
1. Select several lanes in image that have visible peaks but that remain undetected (lane outline still blue)
2. Plot peak correlation SNR for those peaks [c]
3. Set peak SNR threshold for the full scan below lowest peak SNR



SNR threshold too low

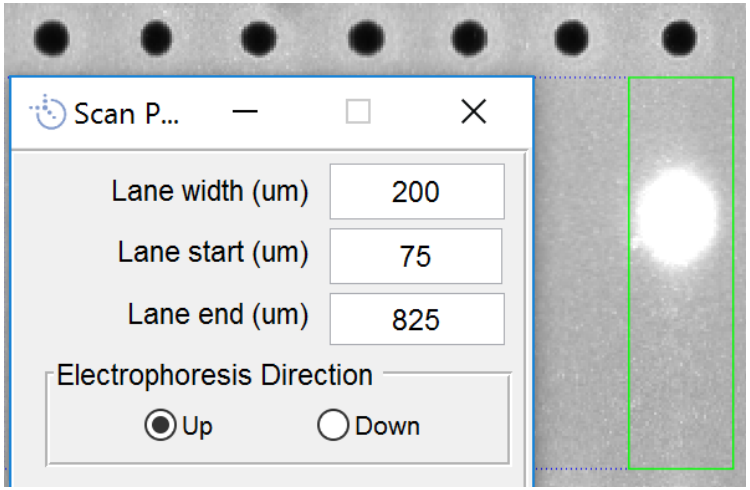


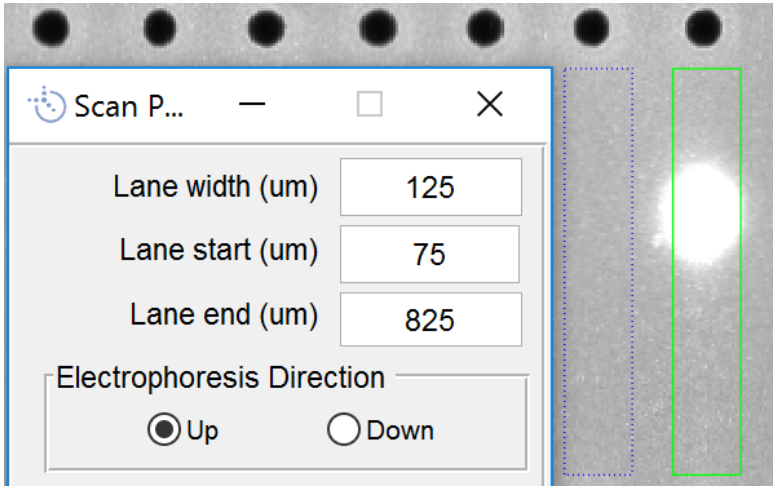
SNR threshold is good



Adjusting Lane Width

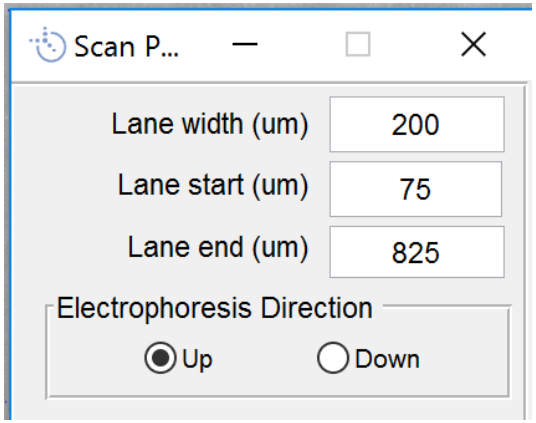
- If protein band is wider than default lane width, adjust lane width to include all band fluorescence (up to 200 microns)





Adjusting Lane Start and Lane End

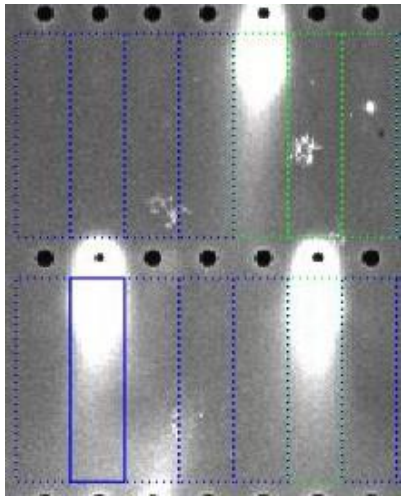
- Lane Start and Lane End can be adjusted to increase or decrease the length of the lane in which Scout detects peaks
- Can also be done on an individual lane level by adjusting local lane properties (see later slide)



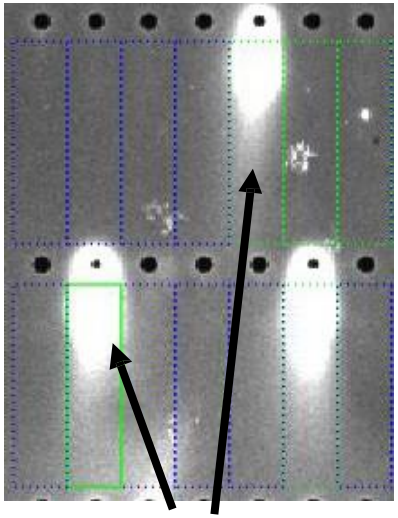
Adjusting Baseline Method

- Two point baseline draws baseline between peak start and peak end
- Flat baseline projects baseline from lower of peak start or peak end points
- If peak is up against well, change to flat baseline and lower lane start value for better peak detection and more consistent peak area measurements

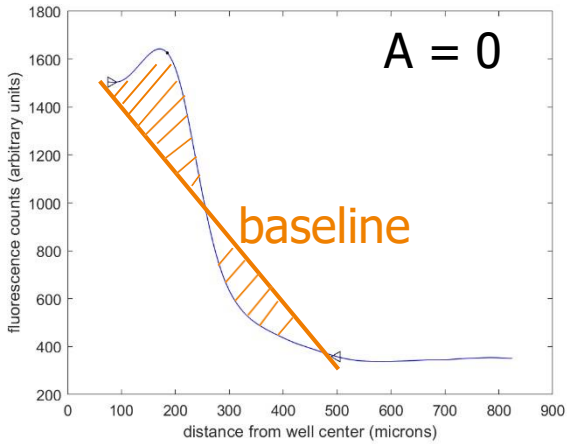
Two-point baseline

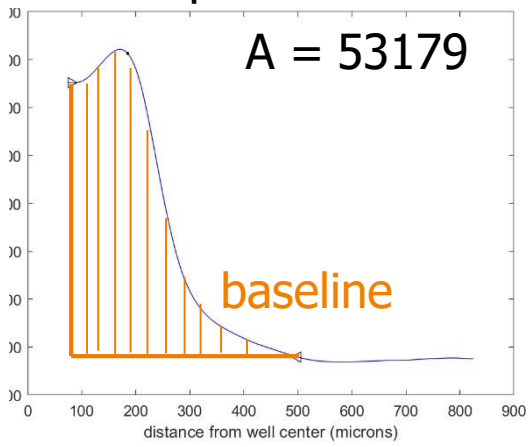


Flat baseline



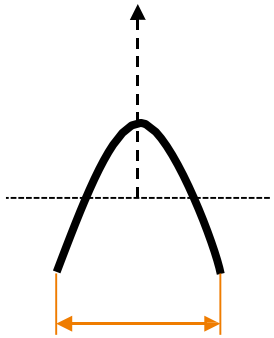
Additional peaks detected





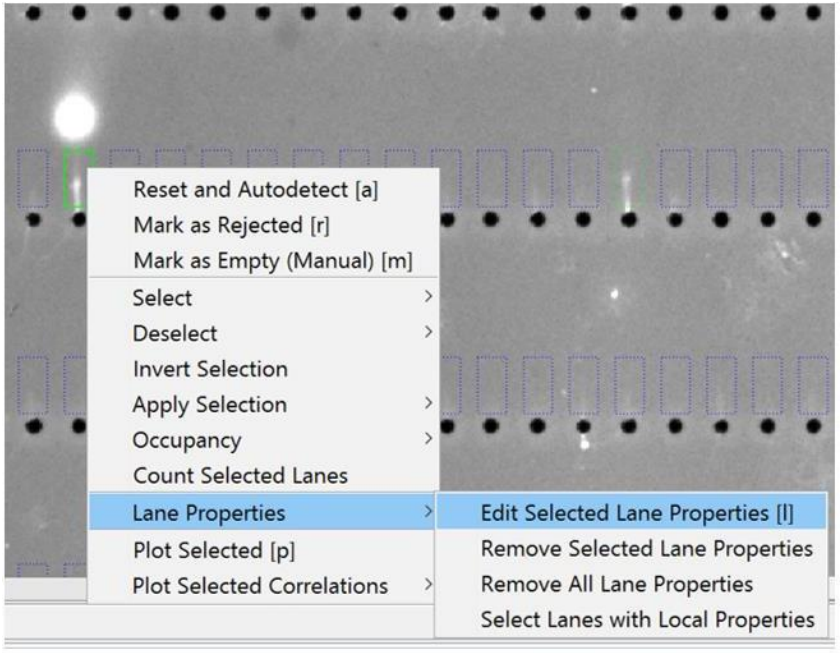
Adjusting Peak Width Factor

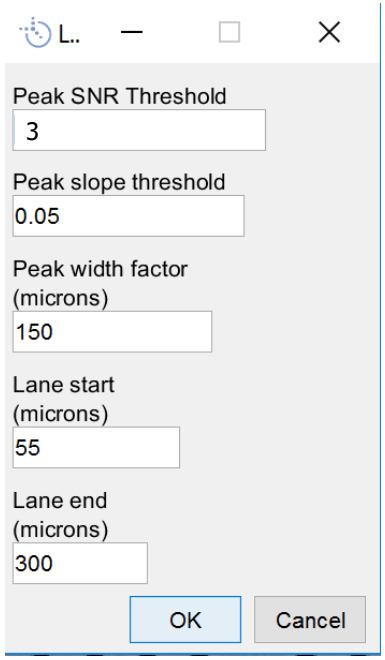
- Changes width of canonical peak shape used in creation of correlation plot
- Increasing the value will improve detection of wider peaks
- Decreasing the value will allow detection of narrower, adjacent peaks



Modifying Local Lane Properties

- Can use to find optimal peak detection settings for a small number of individual lanes and then apply settings to full chip using Scan Properties window
- Can use to detect peaks in a small number of lanes after full chip settings are adjusted using Scan Properties





1. Select lane of interest
2. Right click and select Edit Selected Lane Properties or type [l]

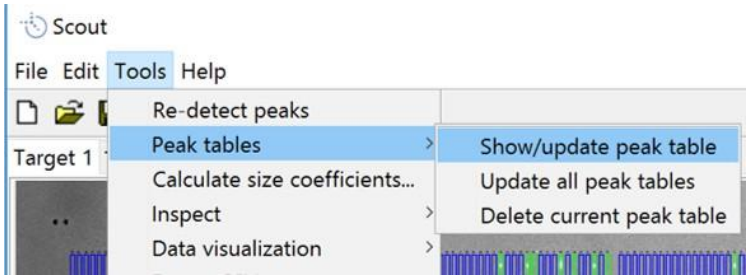
Peak Curation

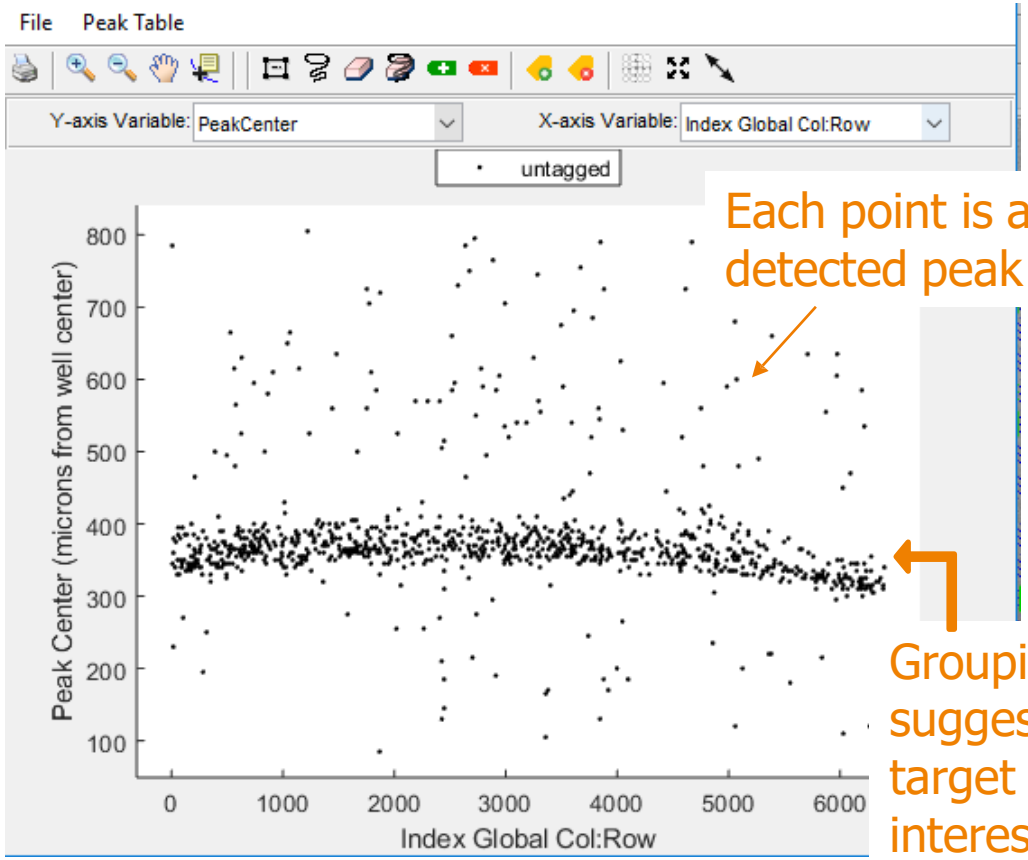
Peak Curation

- Once peak settings have been optimized to detect all peaks of interest, use peak curation tools to tag (label) peaks
- Goals:
  1. Tag & remove noise peaks
  2. Tag peaks of interest
- "Auto Tag" function and advanced tagging workflows available (both detailed in this section)

Peak Table

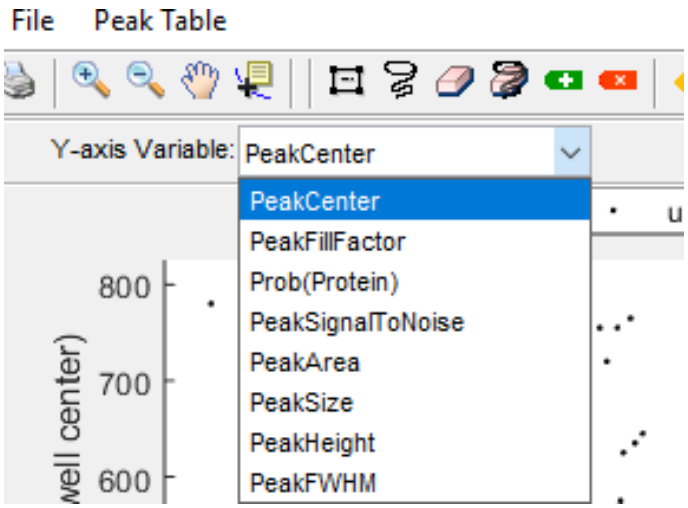
- Primary tool to rapidly identify and tag noise and real peaks
- Displays each peak detected in each single-cell separation
- 1 peak table is generated per image
- Default x-axis shows all 6400 lanes on a chip
- Multiple parameters can be plotted on the x- & y-axes (detail in next slides)





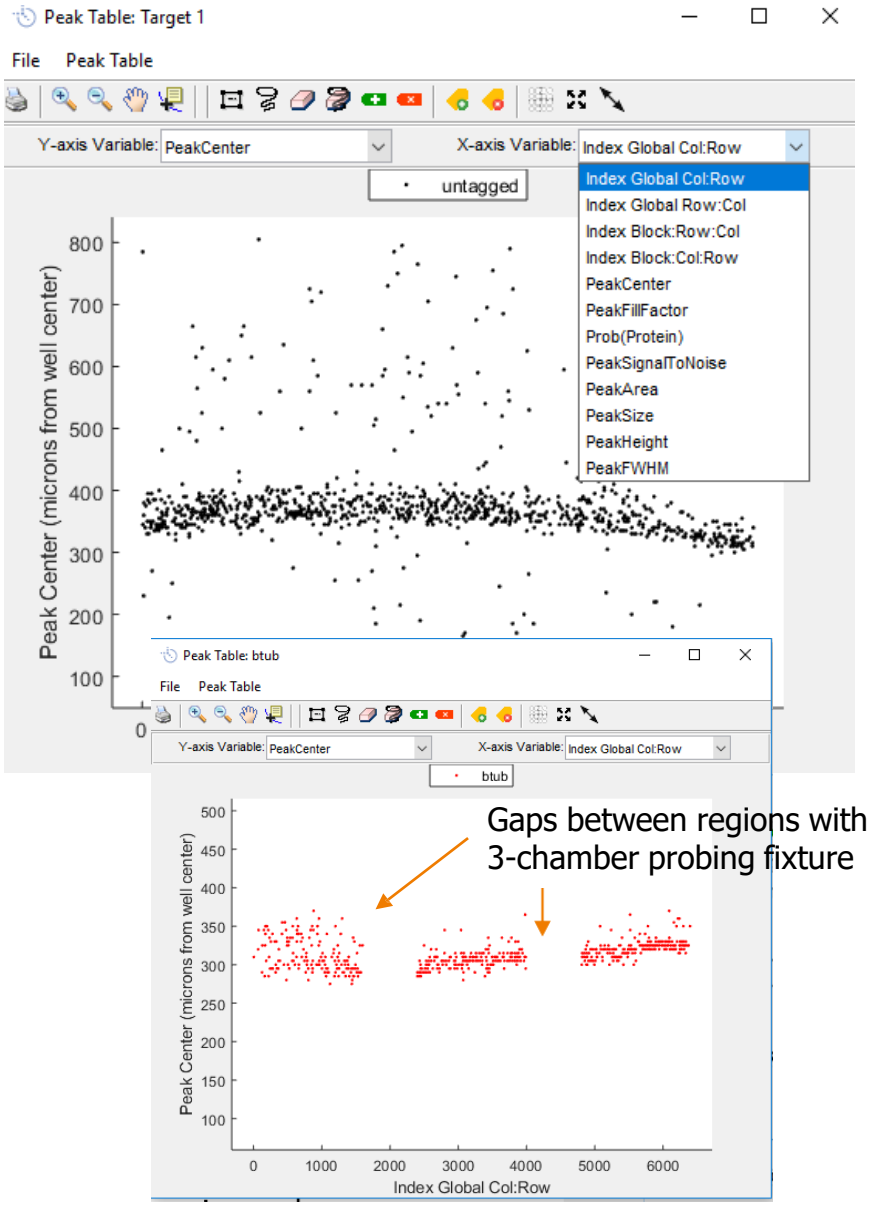
Peak Table: y-axis

- Different peak parameters can be plotted on the y-axis to look for outliers, including:
  - Peak Center: migration distance or how far the peak has traveled into the gel
  - Peak Fill Factor: proportion of lane that is filled by the peak
  - Prob(Protein): probability assigned by Scout that a peak is a real protein peak (1 = 100%). Used by the AutoTag function.
  - Peak Signal to Noise Ratio
  - Peak Area
  - Peak Size (i.e., peak molecular weight if molecular weight sizing assay has been designed and run)
  - Peak Width: Full width half max value (FWHM)
  - Peak Height



Peak Table: x-axis

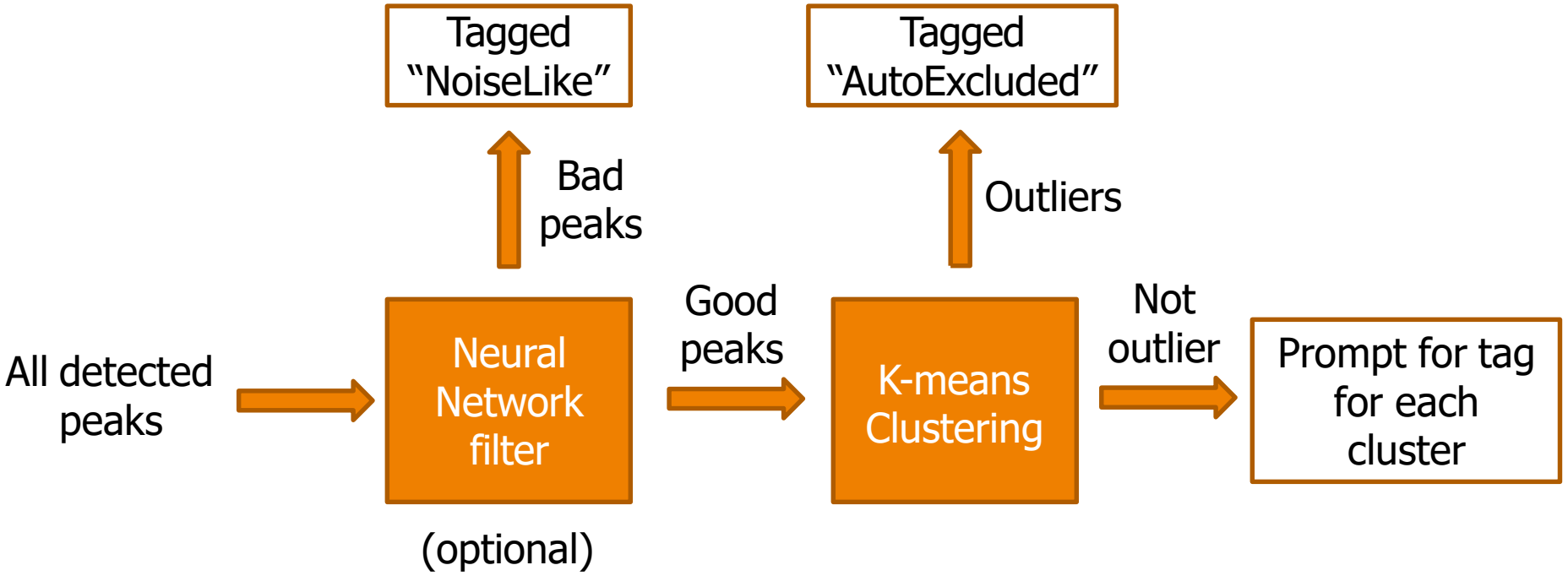
- Scout allows you to choose how to order the 6,400 lanes on the x-axis display
- Default is Global Col:Row which plots lanes one column at a time and clearly separates the adjacent chambers if 3-chamber antibody probing fixture is used
- Plotting PeakFillFactor and other peak metrics on the x-axis can be helpful for manual peak tagging



Automated Peak Curation: Auto Tag

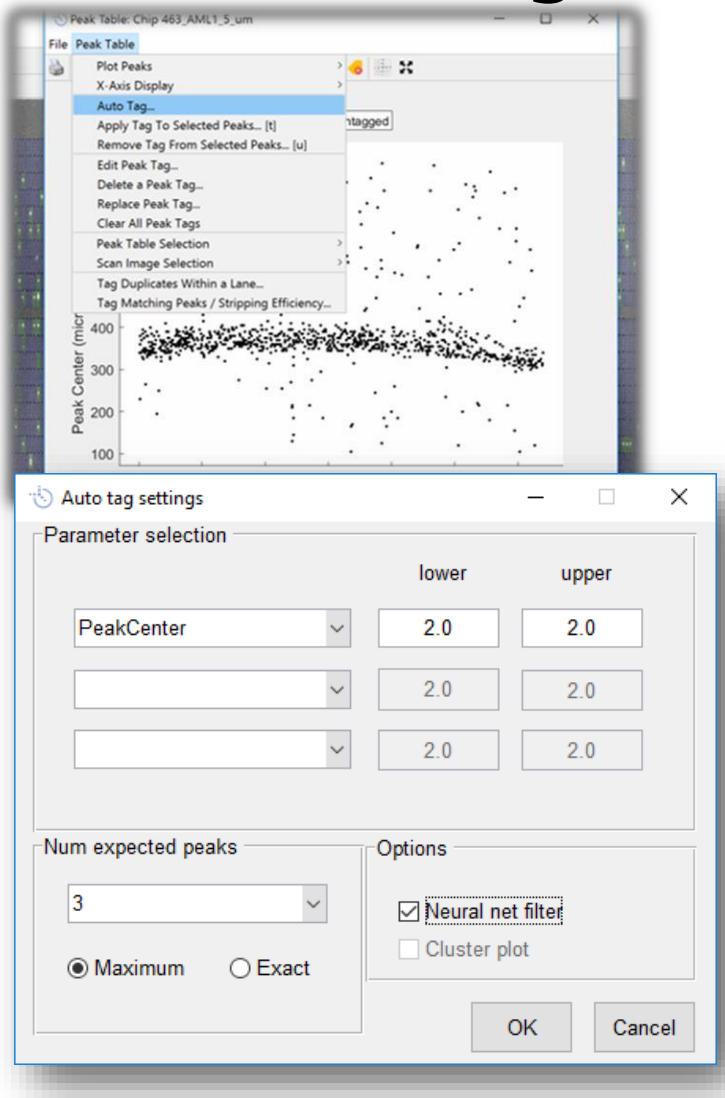
- Simple Wizard that uses Machine Learning to remove noise and find your protein targets of interest
- How does it work?
  - Neural network (machine learning filter) removes noise peaks due to dust, lint, etc.
  - K-means clustering with outlier detection identifies groups of likely protein peaks based on up to 3 specified parameters (e.g., Peak Center, PeakFillFactor)

Auto Tag Function



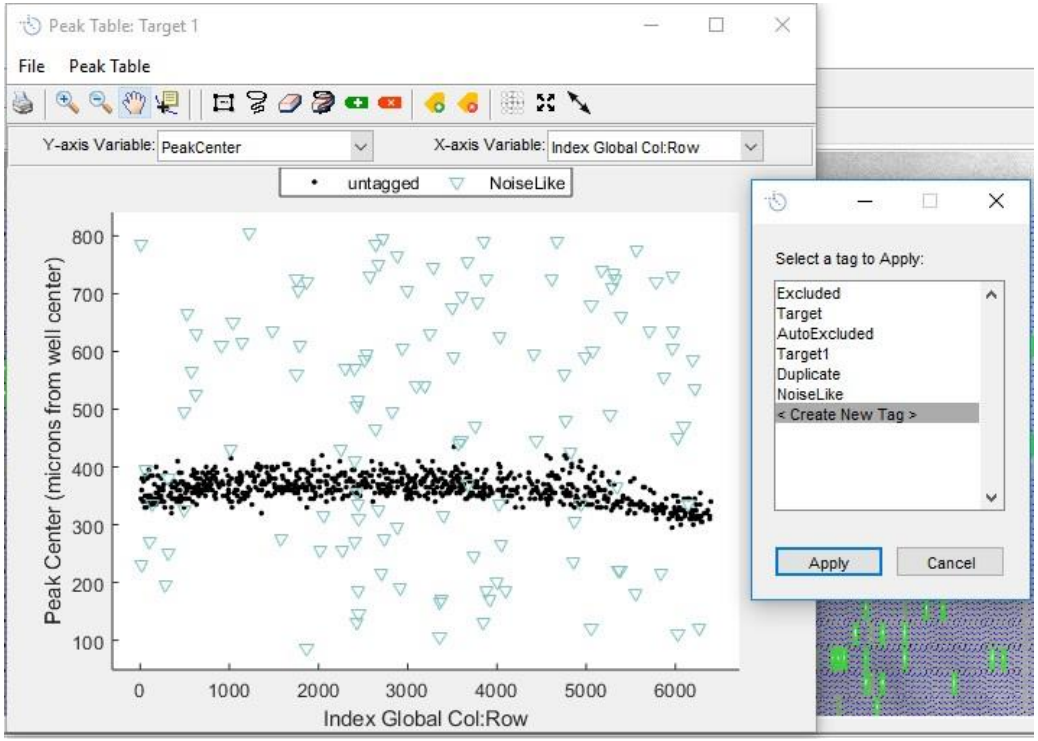
Automated Peak Curation: Auto Tag

- Open Peak Table
- Peak Table > Auto Tag
- Select up to 3 parameters (e.g, PeakCenter & PeakFillFactor) to use in K-means clustering algorithm to find peak clusters
- Set lower and upper limits which determine which peaks are outliers. Limits represent multiples of the interquartile range. Increasing values for lower and upper numbers will accept more peaks
- Enter number of expected peaks per lane in that scan.
  - "Maximum" means Scout will look for at most this many clusters but will return the best fit for the data. Usually best results but slower.
  - "Exact" means Scout will force the number of clusters. Faster but often poorer predictor of real peaks.
- Select whether you want to use neural net pre-filter. Most of the time this is useful. (See Slide 52 for example of situation where neural net is not useful)
- Check cluster plot if you want to display the cluster graph to show peak clusters and outliers (2 or more parameters must be selected)
- Clustering is done on untagged peaks only



Automated Peak Curation: Auto Tag

- Scout labels noise peaks from Neural Network as "NoiseLike".
- Scout labels peaks that are outliers after K-means clustering as "AutoExcluded"
- Select or create a tag for "untagged", selected peaks (should be peaks from your target(s) of interest)
- Any questionable peaks can be visually confirmed in the image later using Inspect function



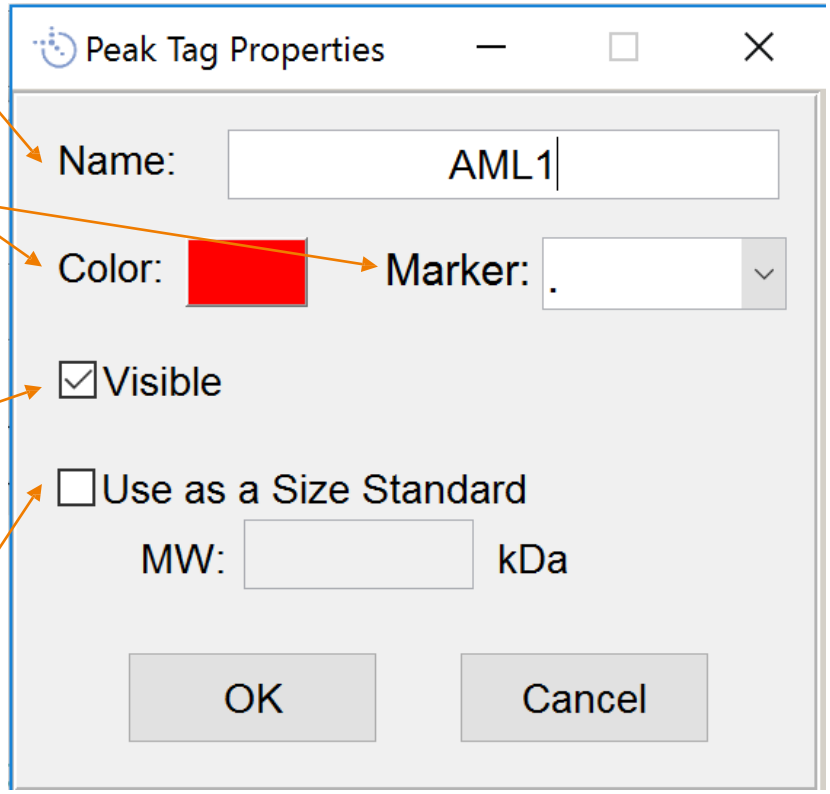
Creating a new peak tag

Name of new peak tag

Select preferred color & marker for display in peak table

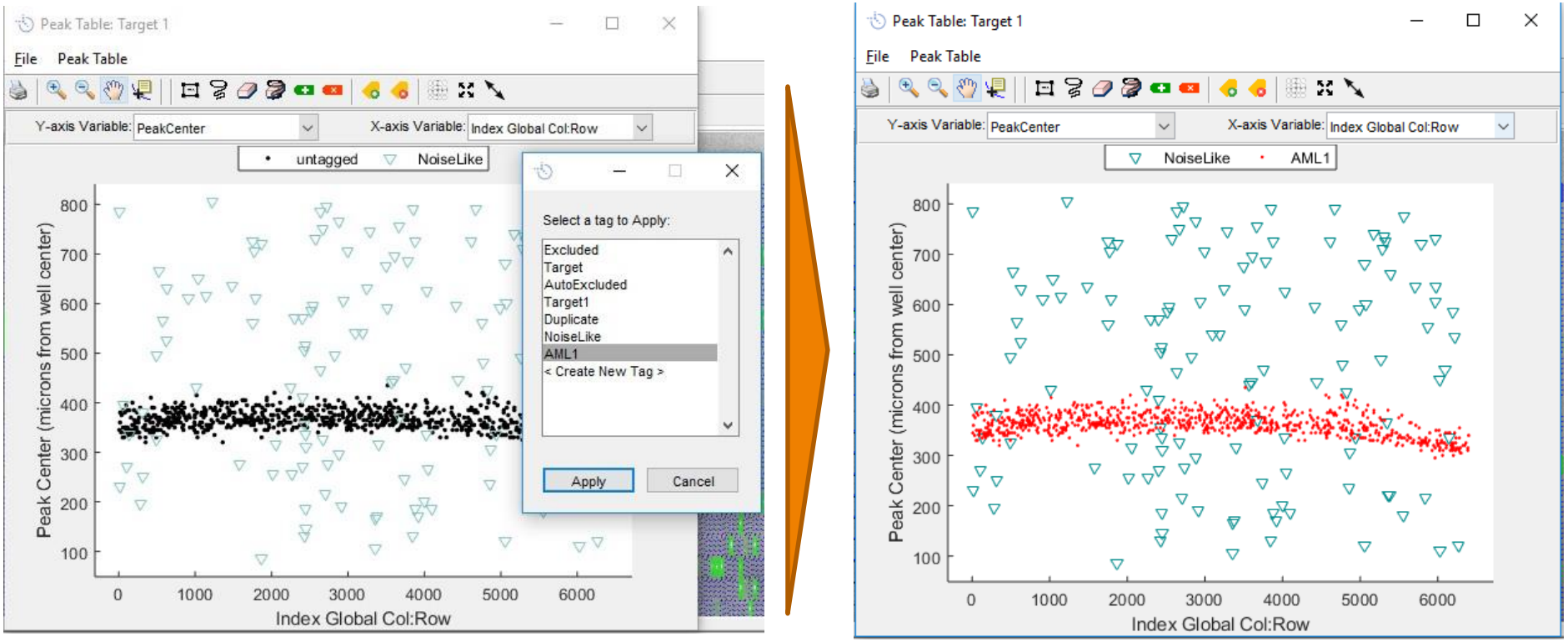
Check if want peaks to be visible in the peak table (e.g, you may not want to visualize excluded peaks)

Leave unchecked unless doing a molecular weight sizing assay (refer to advanced feature section for more information)



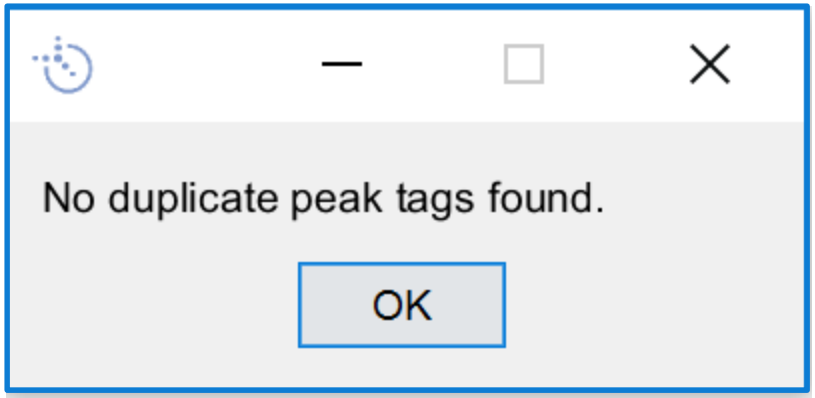
Select tag to apply to “untagged” peaks

- Label untagged peaks as AML1 peaks



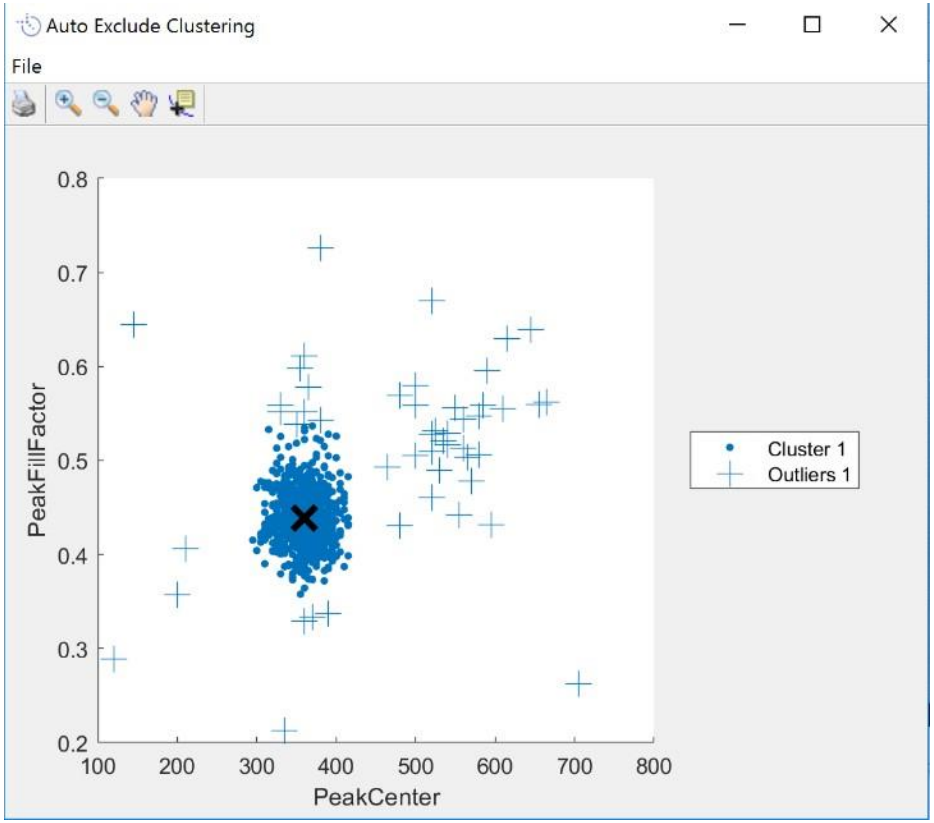
Duplicate peak tags

- If two peaks in one lane are labeled as the same peak, Scout will give a warning and label the duplicate peaks for inspection
- Duplicate peaks can be examined using the Inspect function and curated as needed
- Most data visualization is not possible while duplicate peaks remain (Lane plot is still possible)



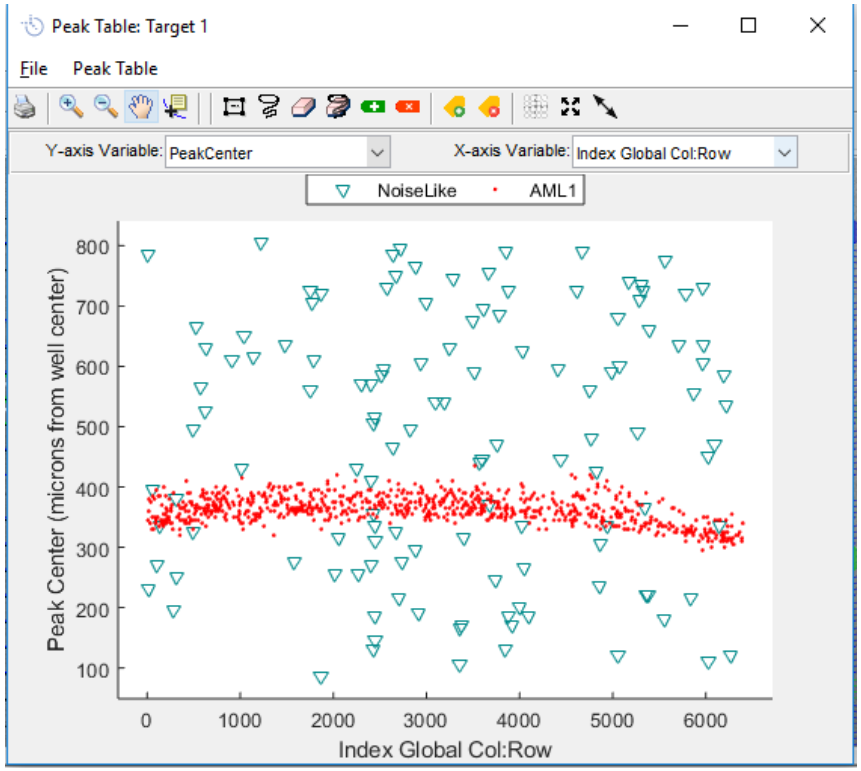
Automated Peak Curation: Auto Tag

- If cluster plot was checked in Auto Tag settings and more than one clustering parameter was selected, a cluster plot will be displayed showing clusters defined in K-means clusters and which peaks are outliers vs "real" peaks in the cluster
- Can visually confirm that clustering is accurate
- To change outlier definition, delete new tags and change upper and lower parameters in AutoTag Settings window

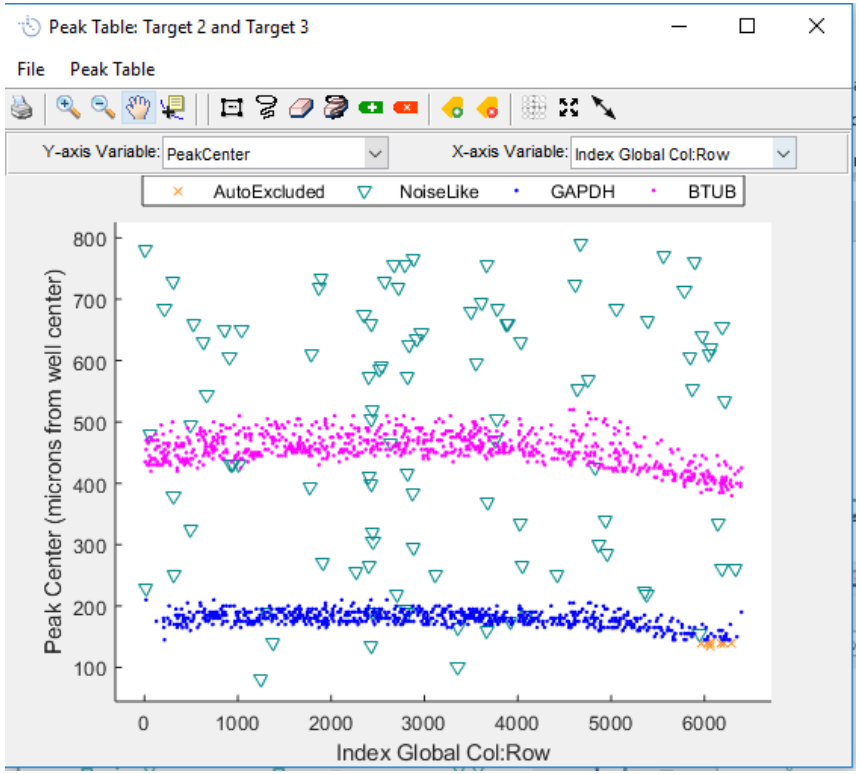


Repeat Auto Tag feature for other scans of chip

555 scan



647 scan



- Once peaks for all targets that you probed for have been labeled in all scans, you can start to visualize your data (detailed in next section)

Inspecting Peaks

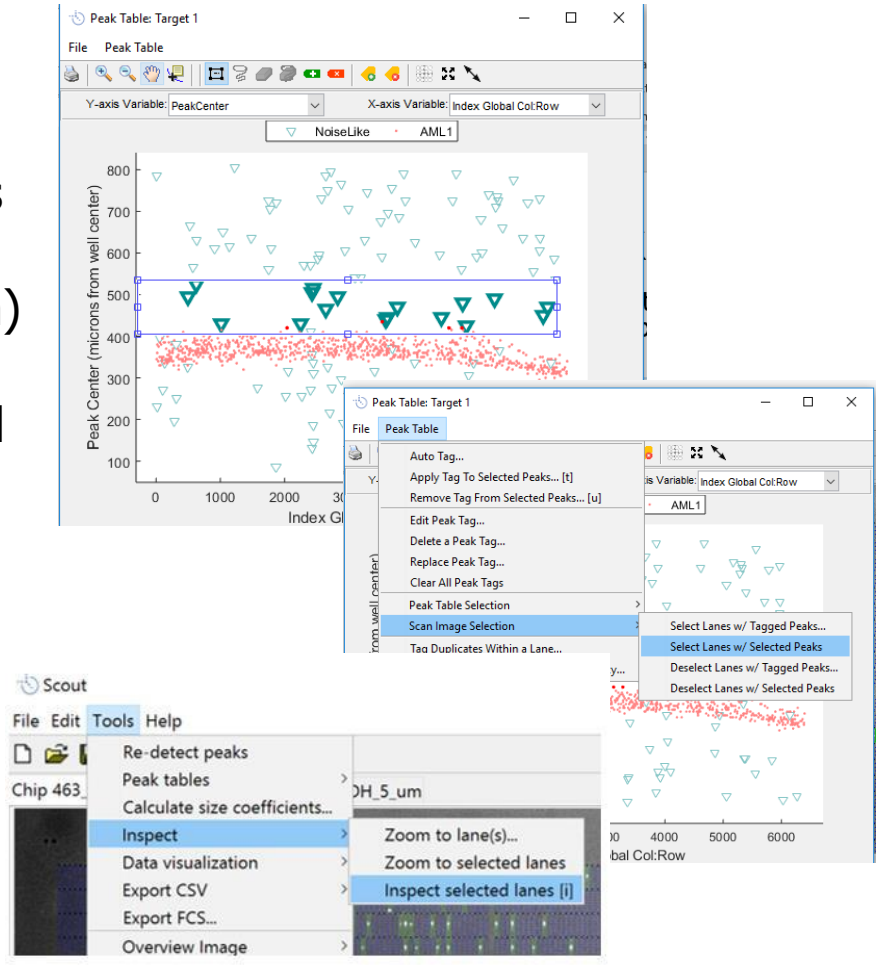
The **Inspect** feature can be used to visually inspect the lanes in the scan image for any peaks that are selected in the Peak Table. This is helpful if you are unsure if a subset of peaks are real or noise.

1. Select peaks you want to inspect in the Peak Table using box or lasso select tools and by selecting or deselecting labeled peaks (Peak Table > Peak Table Selection)

2. Select lanes in image containing selected peaks (Peak Table > Scan Image Selection)

3. Deselect all peaks on Peak Table

4. On scan image, navigate to Tools > Inspect > Inspect selected lanes or [i]



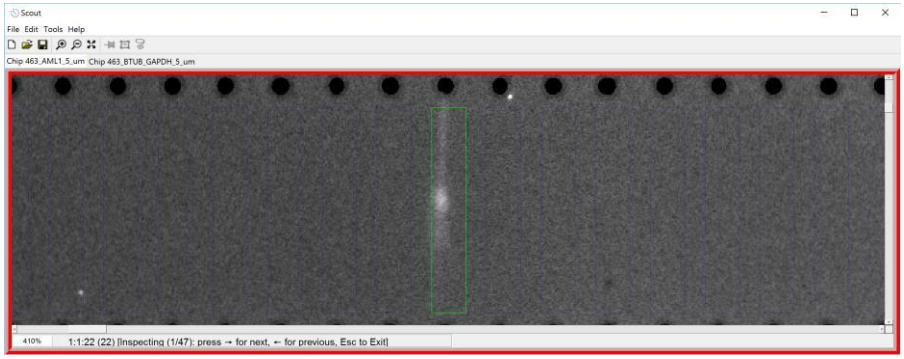
Inspecting Peaks

5. Lanes containing selected peaks can be toggled through with left/right arrows

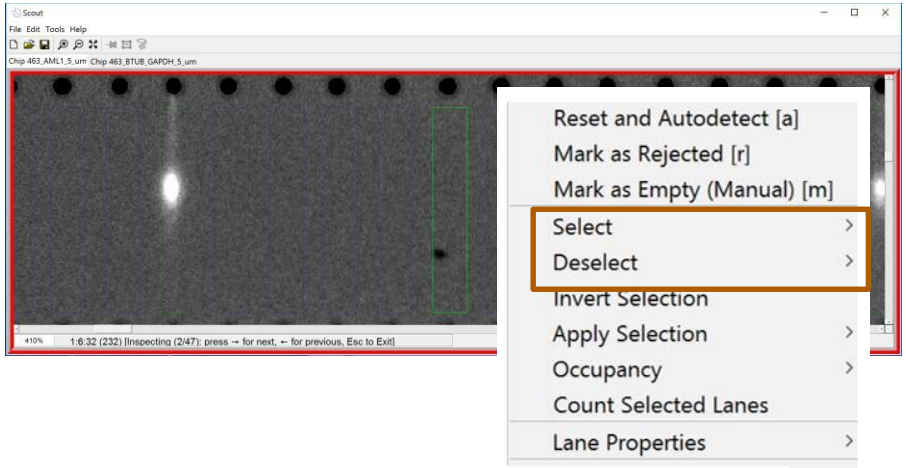
6. To change peak tags for any mis-tagged peaks:
   - Select all peaks in lane on Peak Table (right click > Select menu)
   - Tag peak(s) with appropriate tag on peak table

Alternatively, leave peaks selected on peak table before inspecting, deselect any mis-labeled peaks on peak table as you inspect, and tag all peaks remaining on selection in peak table with desired tag

Selected lane with real peak



Selected lane with noise peak

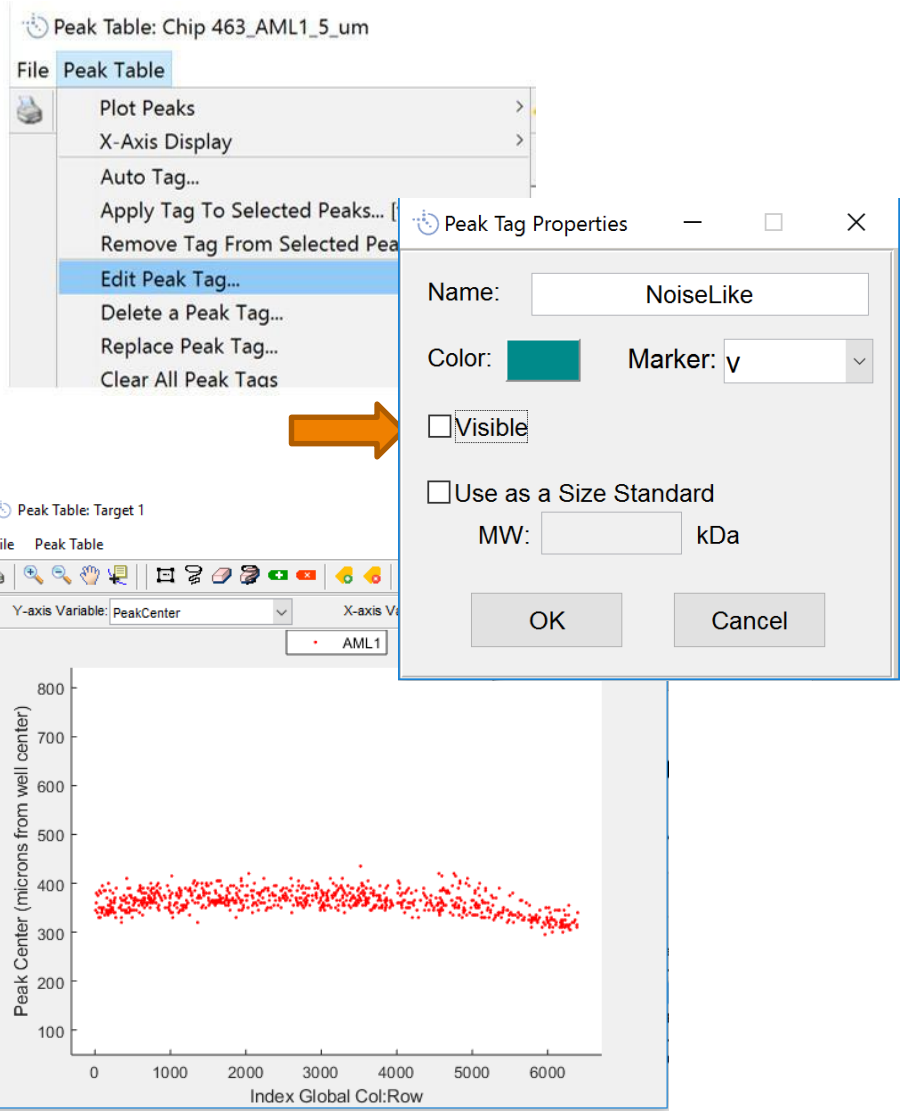


A note about labeling peaks

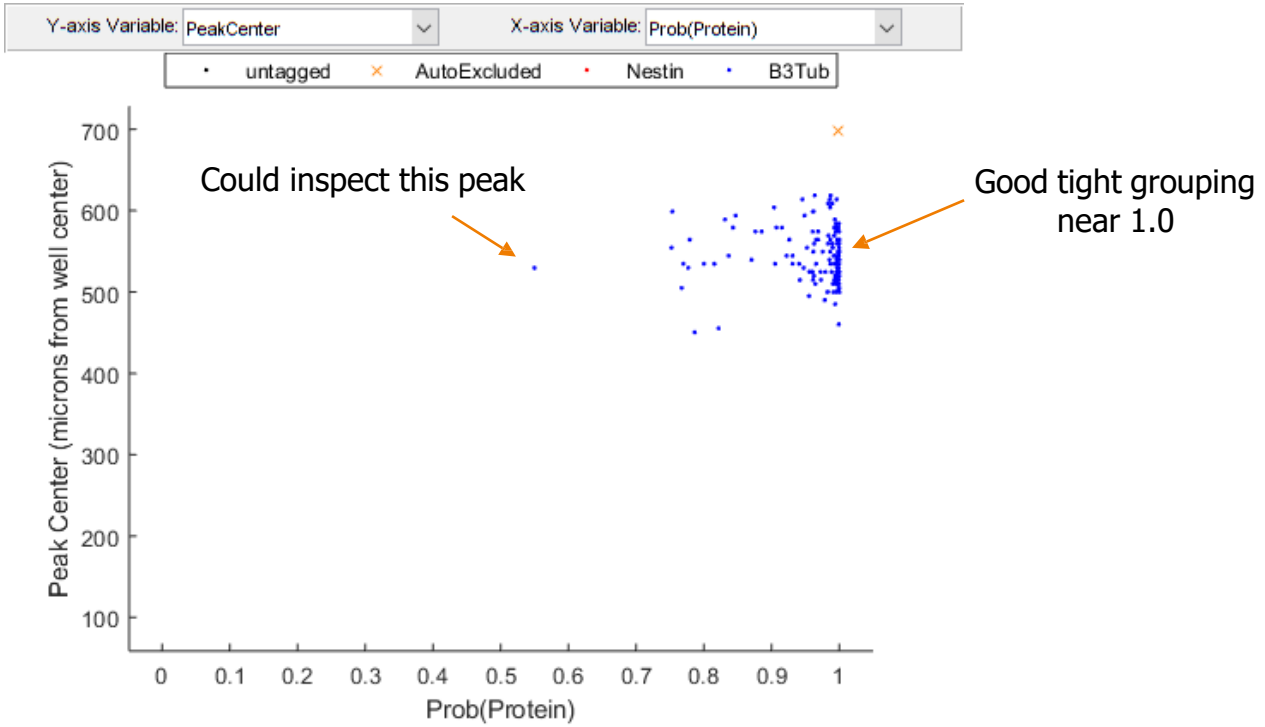
- Peaks can only be labeled in the Peak Table, not the image
- Selection tools should be used to identify which peaks to label in the Peak Table when looking at the image
- When selecting peaks from a lane in the image, remember that *all* peaks in that lane will be selected (if there is more than one peak you may need to deselect some of the peaks from the Peak Table)

Hiding AutoExcluded and NoiseLike peaks (optional)

- Once peaks labeled as "AutoExcluded" and "NoiseLike" are confirmed to be noise, you can make them invisible so that only protein peaks of interest are shown in the Peak Table
- Edit Peak Tag and uncheck box for "Visible"
- Can always recheck "Visible" box to display peaks again
- Hiding noise peaks makes it easier to select the target protein peaks when AutoTag is not used

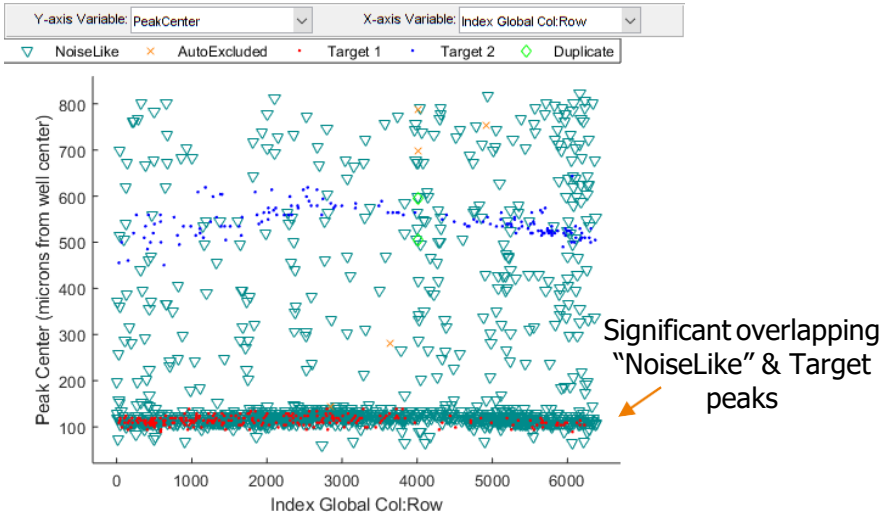


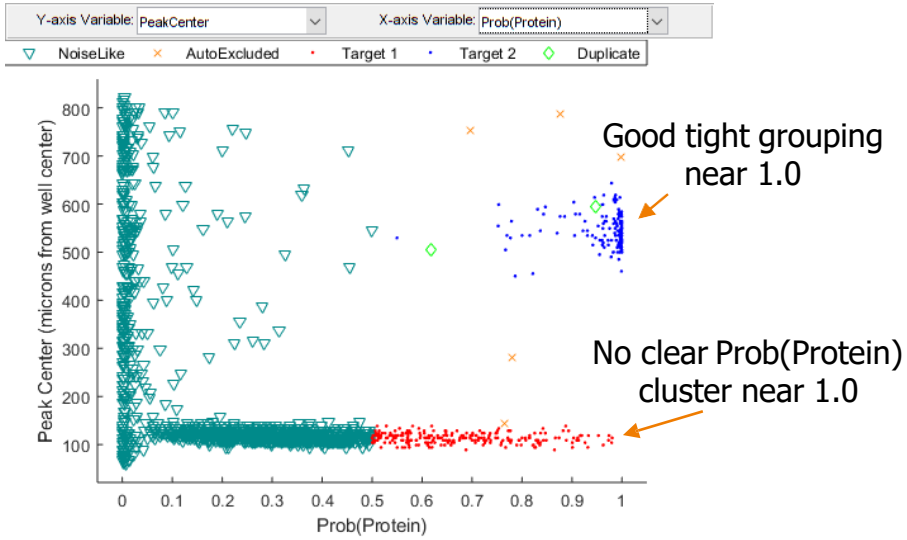
Using Prob(Protein) to inspect marginal peaks



- Prob(Protein) can be plotted from the peak table and is Scout's score of how likely a peak is a real protein peak vs. noise
- Prob(protein) < 0.5 is considered noise
- Tight grouping near Prob(Protein) = 1.0 indicates good Auto Tagging
- Can inspect any peaks near Prob(Protein) = 0.5 using the Inspect function

(optional) Troubleshooting neural net peak detection





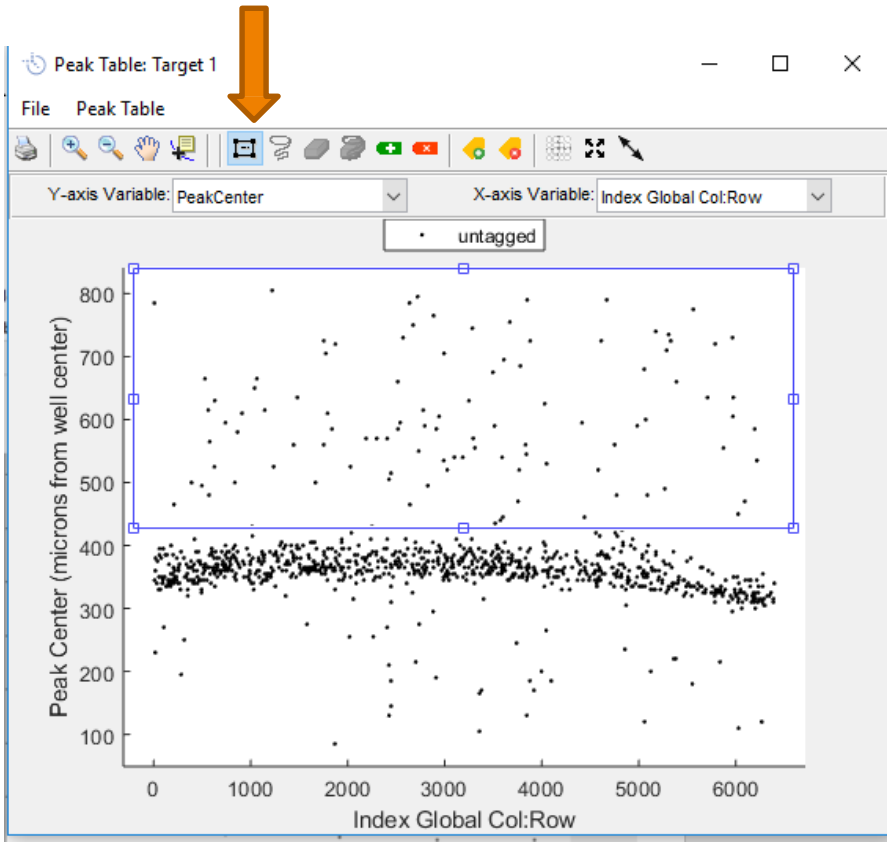
- After running Auto Tag with Neural Net, if you observe a significant number of "NoiseLike" peaks clustered with target peaks, plot Peak Center vs. Prob(Protein) in peak table
- Tight grouping near Prob(Protein) = 1.0 indicates neural net is working
- If no clear grouping by Prob(Protein) is observed, neural net is not recognizing your peak shape. Use Auto Tag without neural net and/or use manual peak curation methods.

Advanced peak curation

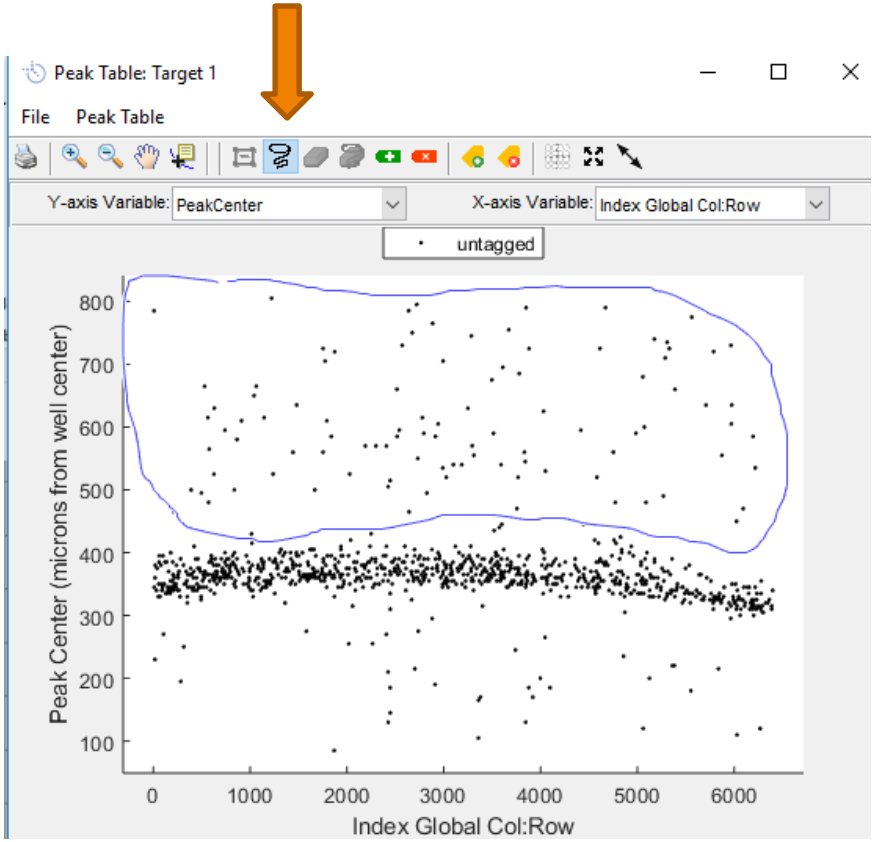
- If the AutoTag feature doesn't work well for your experiment, numerous other advanced peak curation tools exist to select & label peaks in the peak table in a bulk fashion
  - Plot peaks in Peak Table using variety of peak variables (e.g., Peak Center, PeakFillFactor)
  - Identify & select outlier peaks
  - Tag outlier peaks as "Excluded"
  - Inspect questionable peaks in the image

Selecting & Labeling Peaks in the Peak Table

Box selection tool

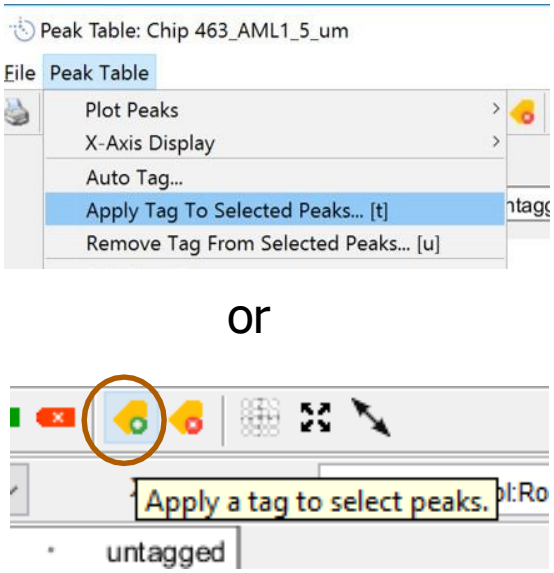


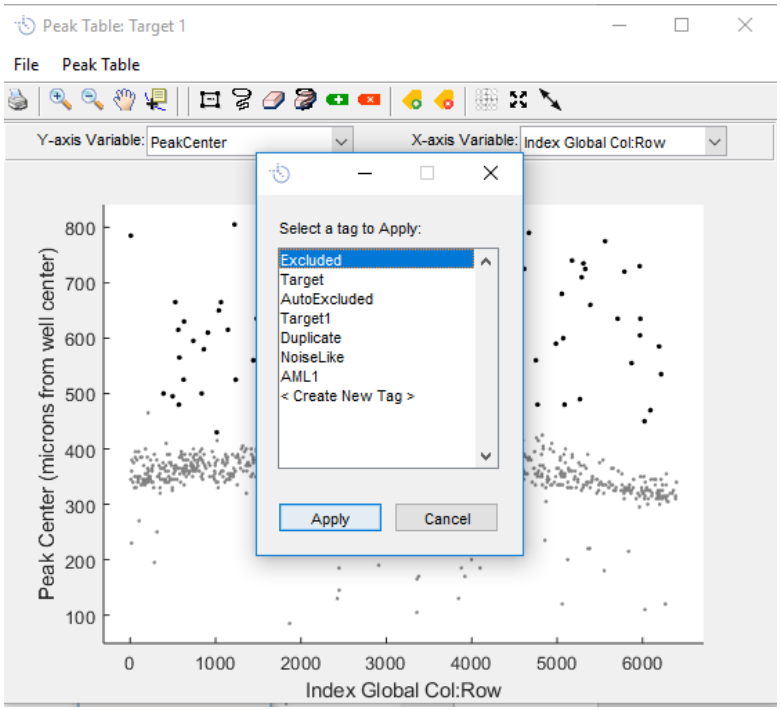
Lasso selection tool



Selecting & Labeling Peaks in the Peak Table

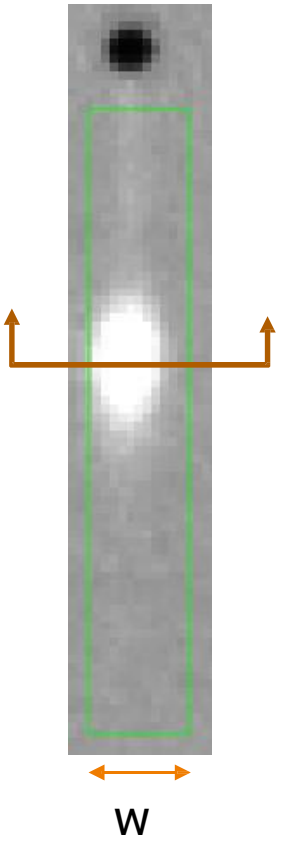
- Apply an existing tag or create a new tag to apply to the selected peaks



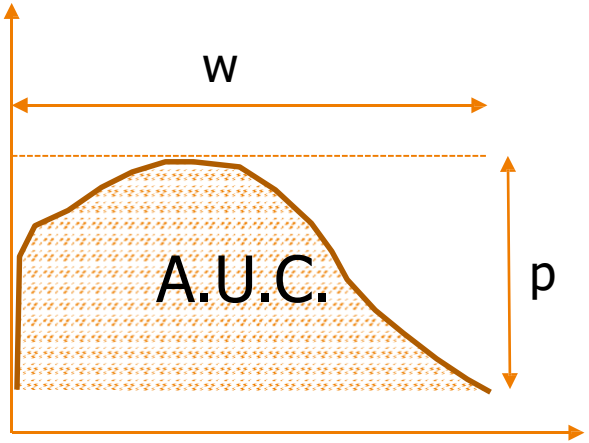


PeakFillFactor

- A measure of how wide the band is in the lane



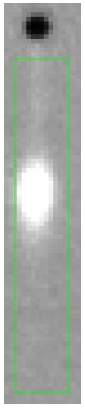
$$\text{Peak Fill Factor} = \frac{\text{A. U. C.}}{\text{w p}}$$

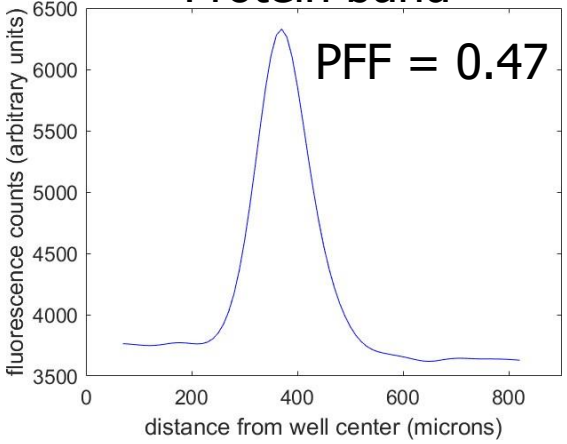


A.U.C. = area under curve

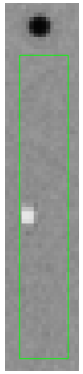
Identifying noise peaks by plotting PeakFillFactor on the Peak Table

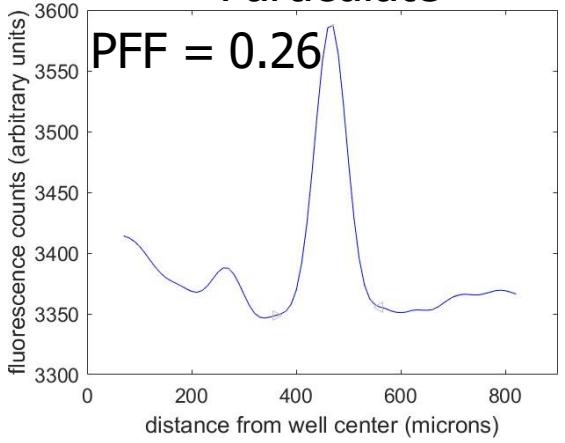
Protein band

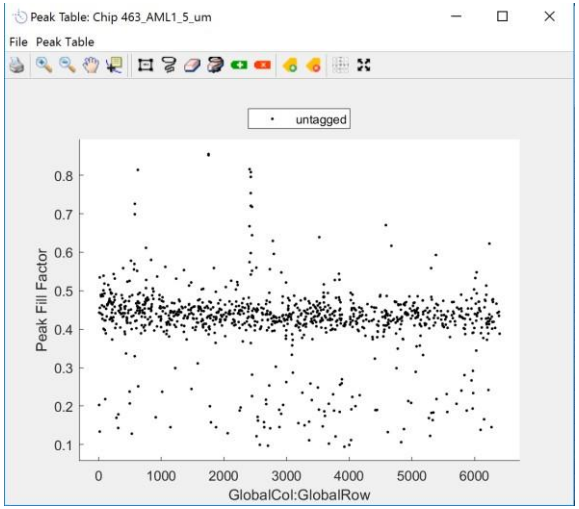




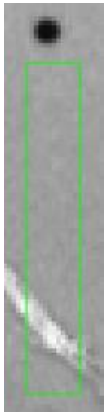
Particulate

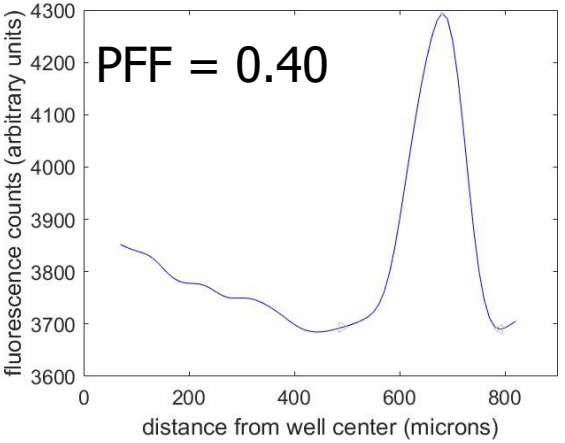






Fiber

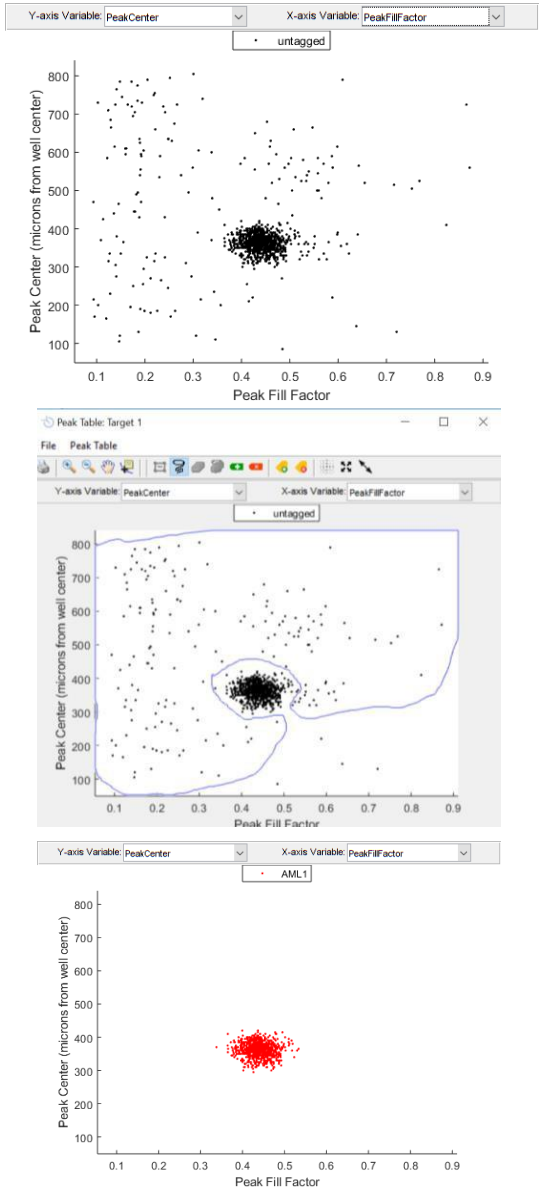




- Good noise peak exclusion by combining PeakFillFactor and Peak Center location

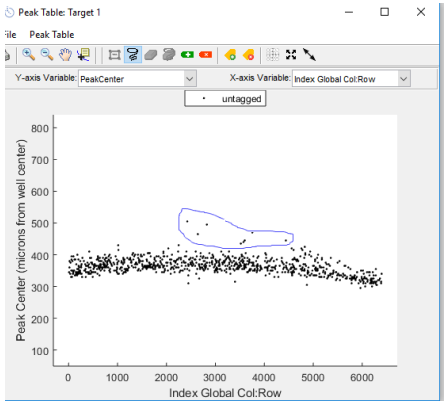
Suggested Advanced Protocol for exclusion of noise peaks

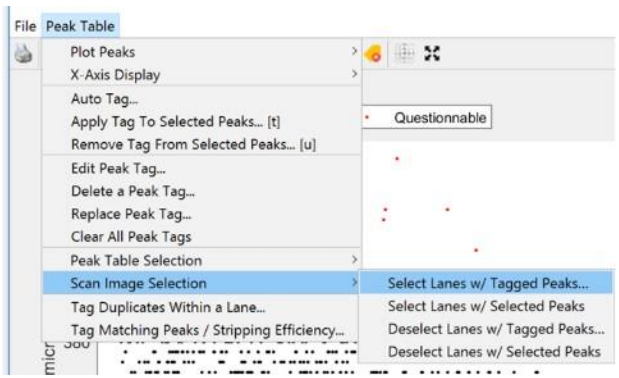
- Using Peak Table, plot Peak Center vs. Peak Fill Factor
- Look for a cluster and use Lasso tool select & exclude non-clustered noise peaks
- Label any questionable peaks as "questionable" and inspect in scan image using Inspect function
- If goal is to have a quick look at your data, just exclude peaks at extremes up to peak cluster and proceed with visualization

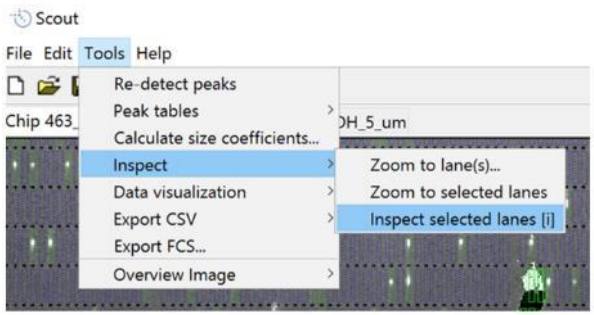


Using **Inspect** Function to Review Questionable Peaks

- Select peaks and create/apply "questionable" tag
- Select lanes containing "questionable" peaks in the image and examine using Inspect feature to make sure they are noise
- Deselect all peaks in Peak Table
- Toggle through lane images
  - If peak is noise, select in Peak Table
  - If peak is real, do not select in Peak Table
- Change tag for all selected peaks in Peak Table from "Questionable" to "Excluded"
- Refresh Peak Table after any protein peaks are labeled







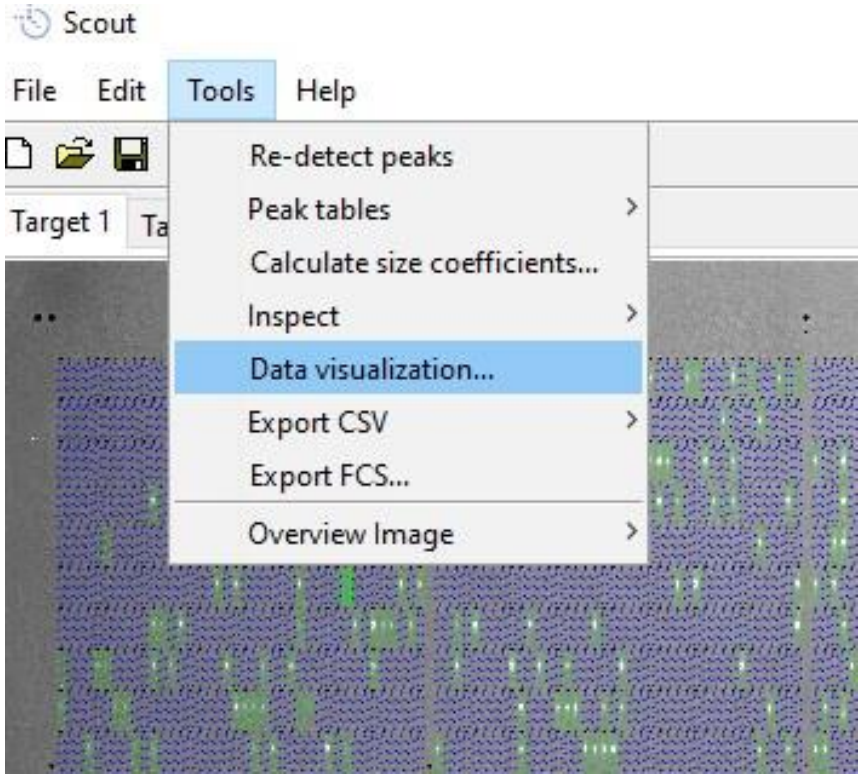
Rejecting Lanes vs. Excluding Peaks

- Rejecting lanes removes all peaks detected in that lane from the peak table and subsequent analysis
  - Use only if the lane is damaged and unusable
- Excluding peaks in the peak table labels only those peaks and does not impact analysis of other peaks in that lane
  - Use to remove specific noise peaks from analysis while allowing other peaks detected in that lane to be accepted

Data Visualization

Data visualization tools

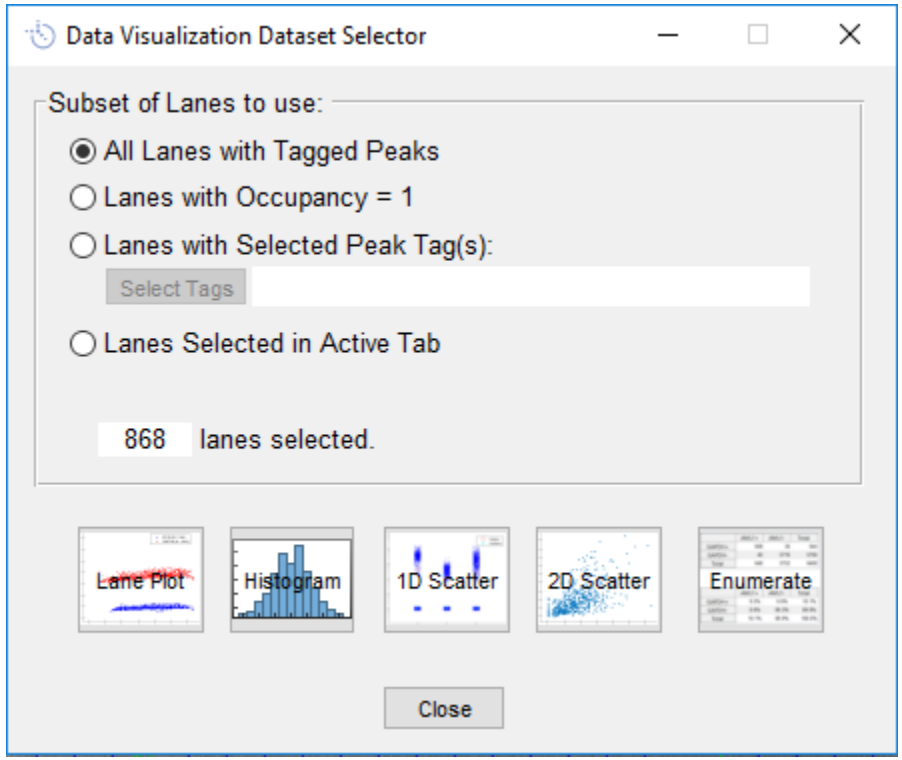
Tools -> Data visualization



Visualization GUI

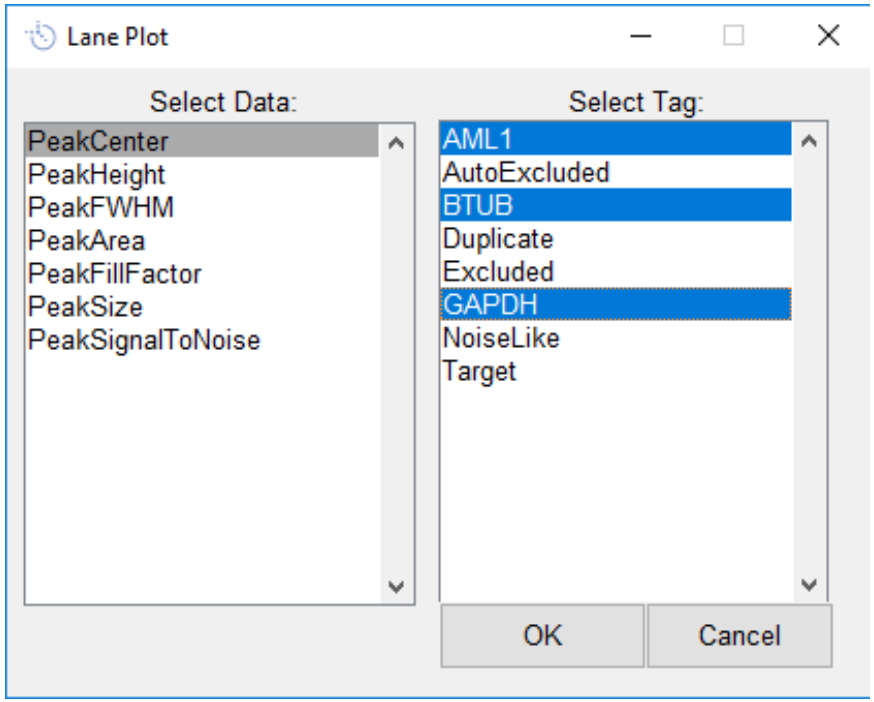
1. Define which lanes to include in analysis

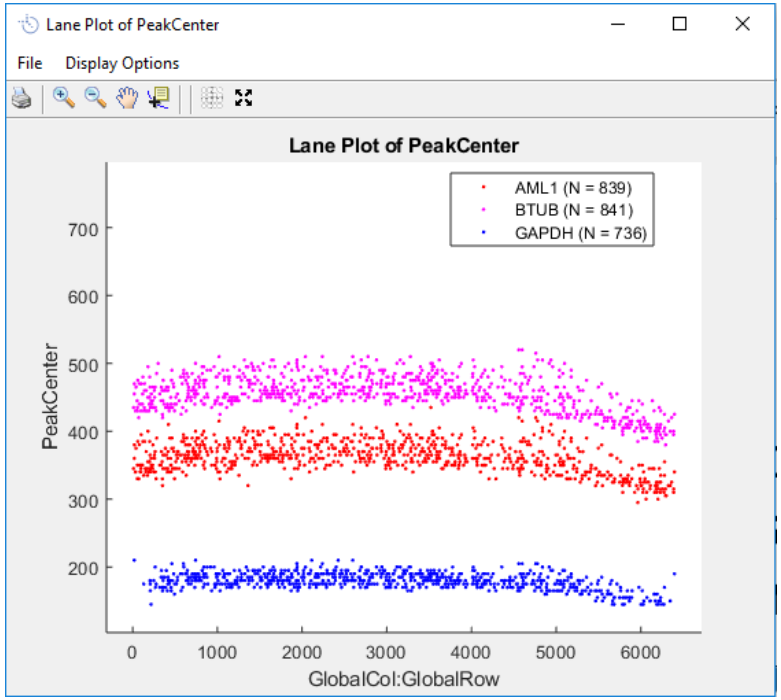
2. Click on the desired plot type



Lane Plot

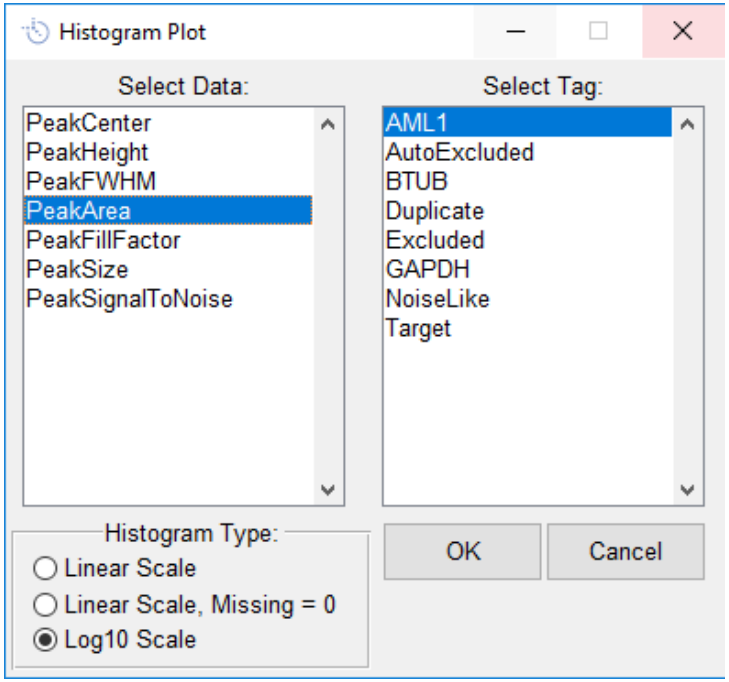
- Similar to Peak Table view (shows location of peaks in each single-cell separation) but can show peaks from multiple scans at once

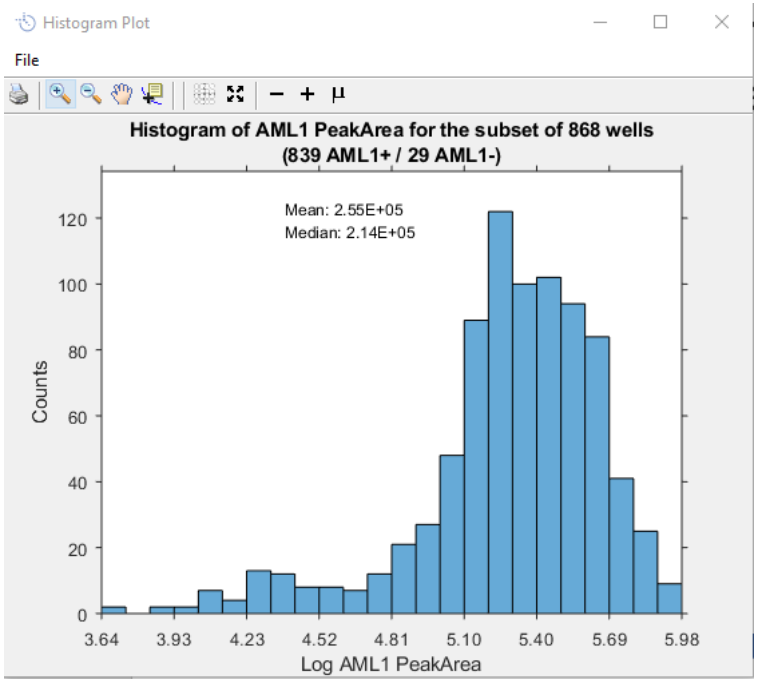




Histogram

- Shows how protein expression varies across sample



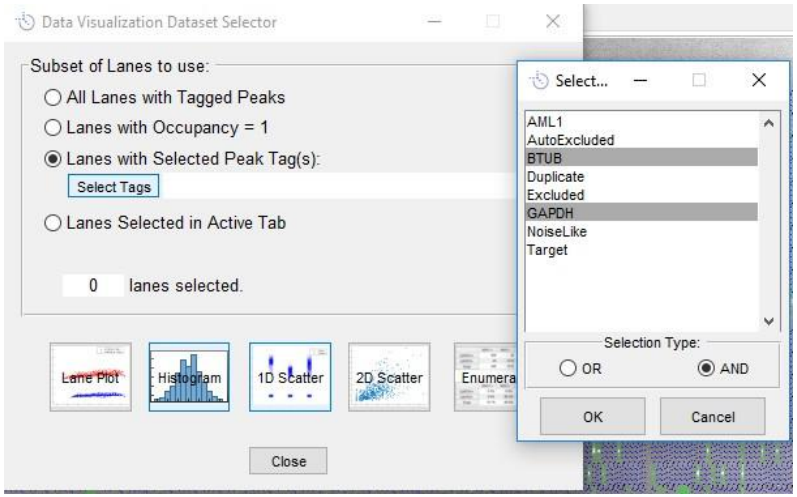


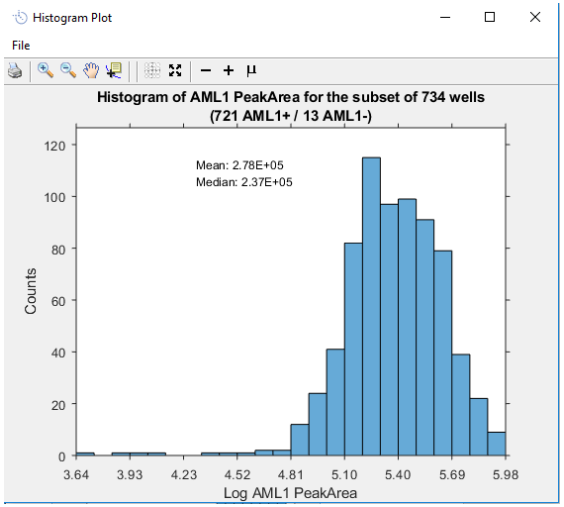
"my target varies by 10-fold across my sample meaning that some cells have 10-times more of my target than others"

Histogram of target expression within cell subpopulations

Example: To create a histogram of AML1 expression only in BTUB+/GAPDH+ cells:

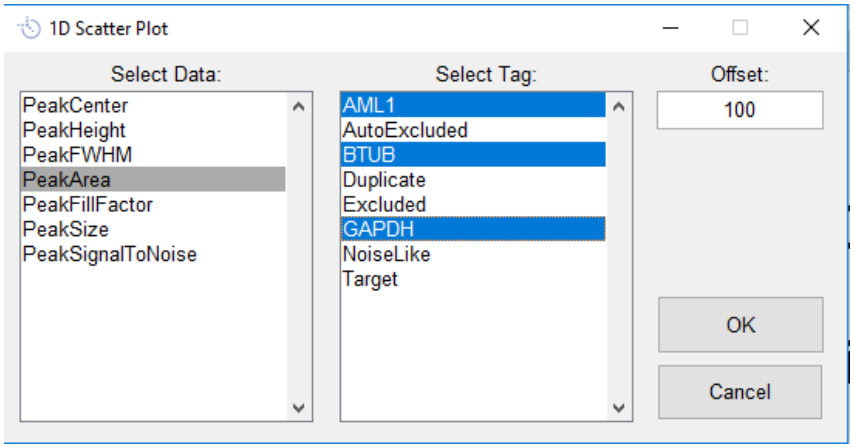
- Visualize lanes with BTUB and GAPDH peaks
- Create a histogram of AML1 peak areas

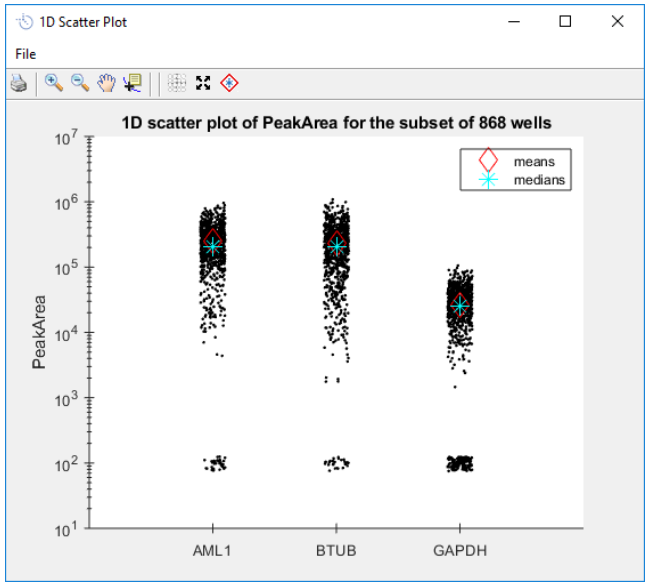




1D Scatterplot

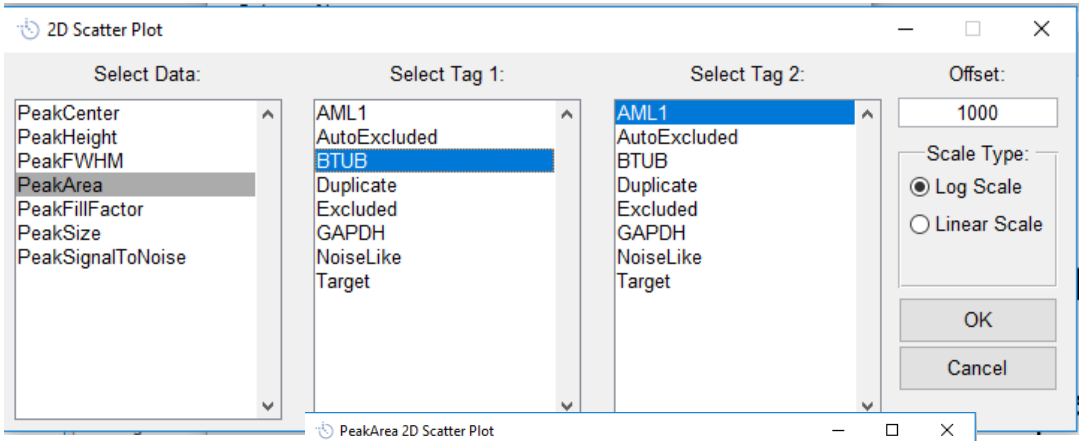
- Another way to show how protein expression varies across samples where each point represents Peak Area from a single-cell
- If no peak area is detected in a plotted lane, Scout will plot a peak at the specified Offset amount

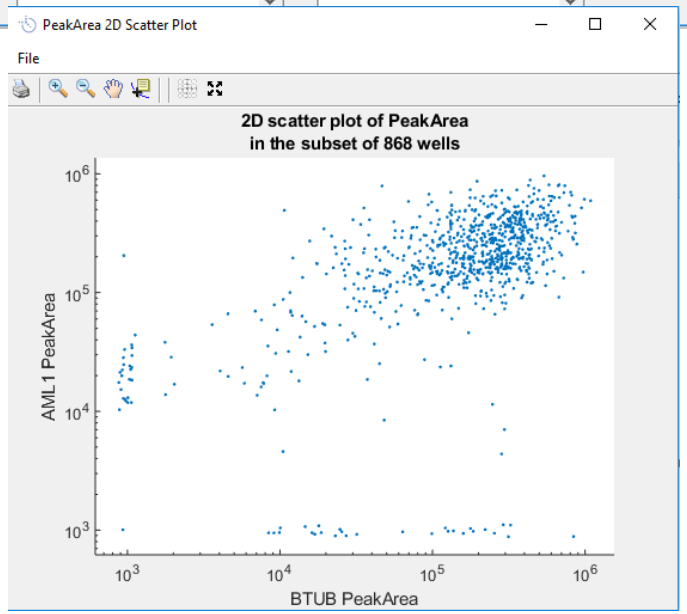




2D Scatterplot

- Identifies subpopulations of cells in data

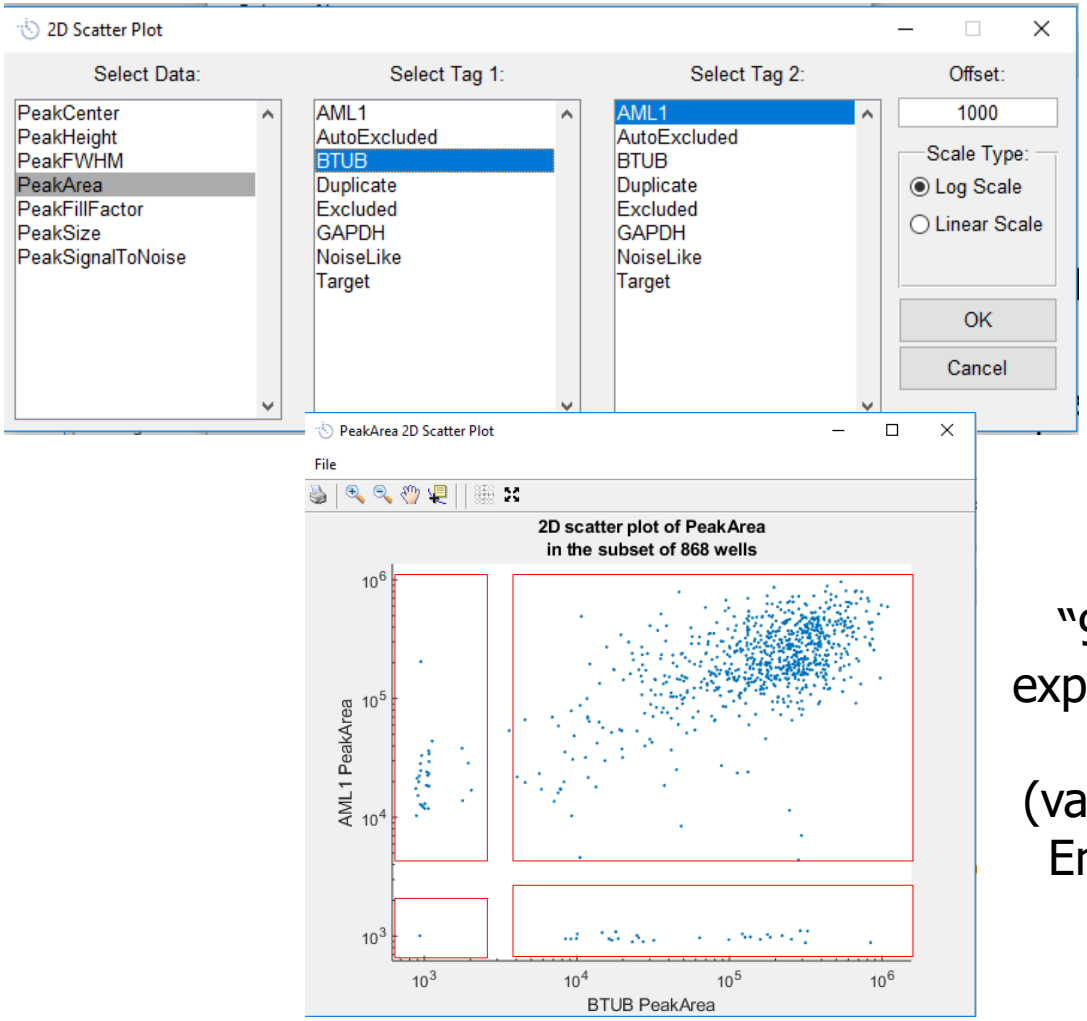




Offset determines the value to plot cells that have no detectable Peak Area for that target

2D Scatterplot

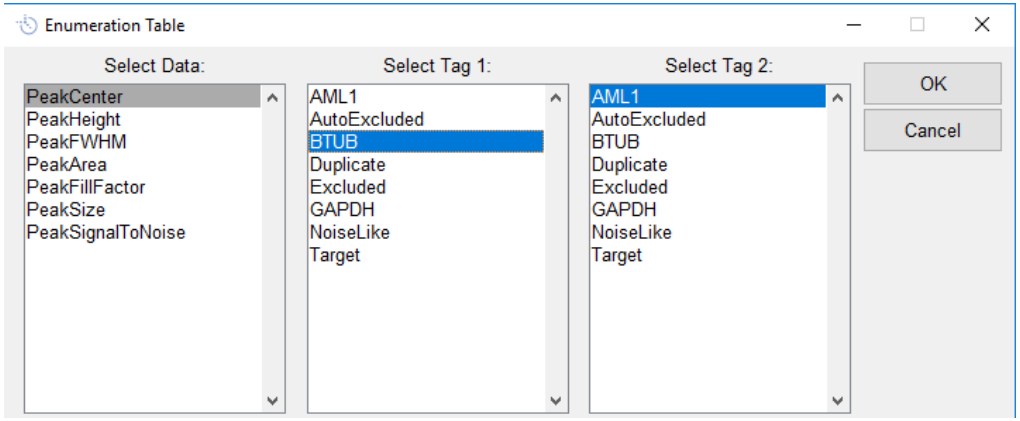
- Identifies subpopulations of cells in data



"94% of my cells express both Target 1 & Target 2"
(value is pulled from Enumeration plot)

Enumeration Table

- Measures % of cells that are in a specific subpopulation



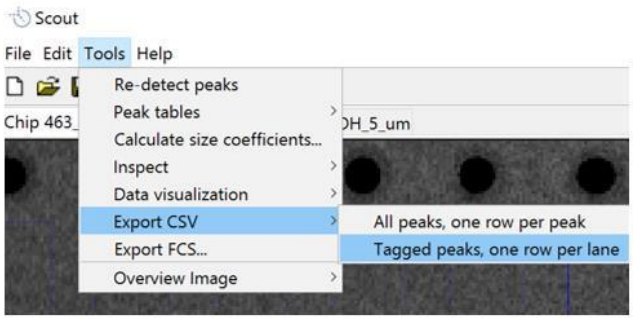
	AML1+	AML1-	Total
BTUB+	813	28	841
BTUB-	26	1	27
Total	839	29	868

	AML1+	AML1-	Total
BTUB+	93.7%	3.2%	96.9%
BTUB-	3.0%	0.1%	3.1%
Total	96.7%	3.3%	100.0%

"94% of my cells express both AML1 & BTUB"
or
"3.2% of my cells express only BTUB"

Export to .csv file for further analysis

- Scout can export peak data to .csv file for further analysis in Excel or other statistical analysis software packages
  - Each row is one lane (one single-cell separation)
  - Columns contain information for each peak detected in each single-cell separation (e.g., Peak Area, peak center, average background signal for each lane)



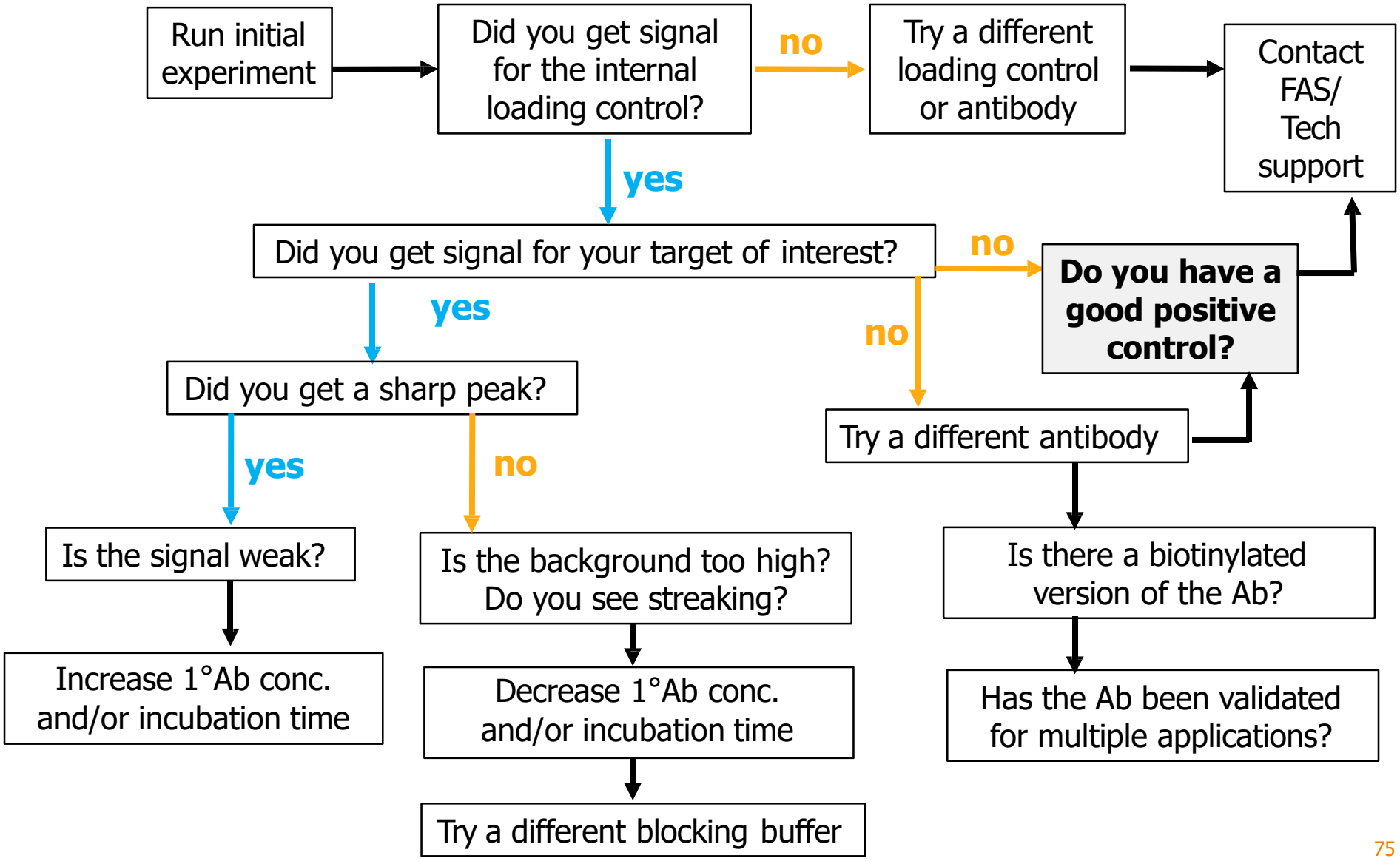
Block	Row	Column	LaneIndex	PeakCenter_Target	PeakHeight_Target	PeakFWHM_Target	PeakArea_Target
15	1	13	5613	405	2713.284191	85	275366.938
15	1	18	5618	395	545.7197048	105	61486.59261
15	1	20	5620	400	854.043649	85	80947.22122
15	1	26	5626	390	1585.09526	80	140930.5406
15	1	30	5630	400	1270.507378	100	137172.2104
15	1	36	5636	400	924.9011454	85	84462.80908
15	2	3	5643	375	881.1730159	105	102461.9676
15	2	14	5654	410	7506.546749	80	670227.7801
15	2	15	5655	395	1642.256325	100	194515.1096
15	2	16	5656	405	423.3557081	120	44959.08005
15	2	17	5657	410	2267.653581	85	213971.4297
15	2	38	5678	400	907.7824125	75	70155.69164
15	3	19	5699	400	670.6625786	105	76414.58695
15	4	35	5755	405	434.2805976	110	54547.41022

Export to .fcs file

- Peak Area data can also be exported to a .fcs file for visualization by flow cytometry software
- Exported .fcs files can be read by FlowJo, etc.

Interpreting the results

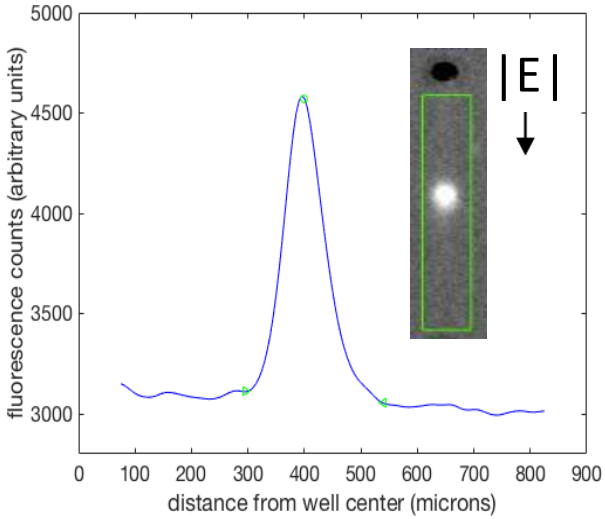
Interpreting the results



Interpreting the results

How can I be confident that the signal for my target of interest is real?

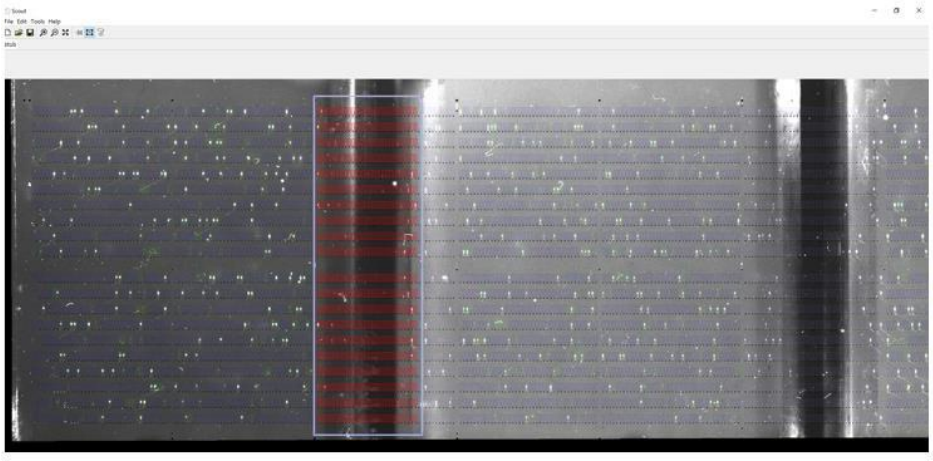
- You observe a peak that is sharp and robust
- You observe a peak in your positive control, but not in your negative control
- The peak is detected at the predicted MW
- Multiple Abs against your target give the same peak



Advanced Analysis

Analyzing 3-Plex Probing Chamber Data

1. Launch Scout software
2. Under the File menu, add first scanned image for your chip
3. Register first scanned image using auto-registration or manual alignment
4. Scout automatically identifies all the lanes in the image and all the peaks in each lane using default settings
5. Reject regions of the chip located between each probing chamber region that were not probed by highlighting the regions, right clicking and marking as "Rejected" (or keyboard shortcut "r").
6. Open & align any other images of that chip
7. Select rejected regions in first tab, apply selected lanes across all tabs and mark them "Reject" across all tabs
8. Optimize peak detection settings, exclude false positive peaks, label protein peaks of interest and visualize data as normal

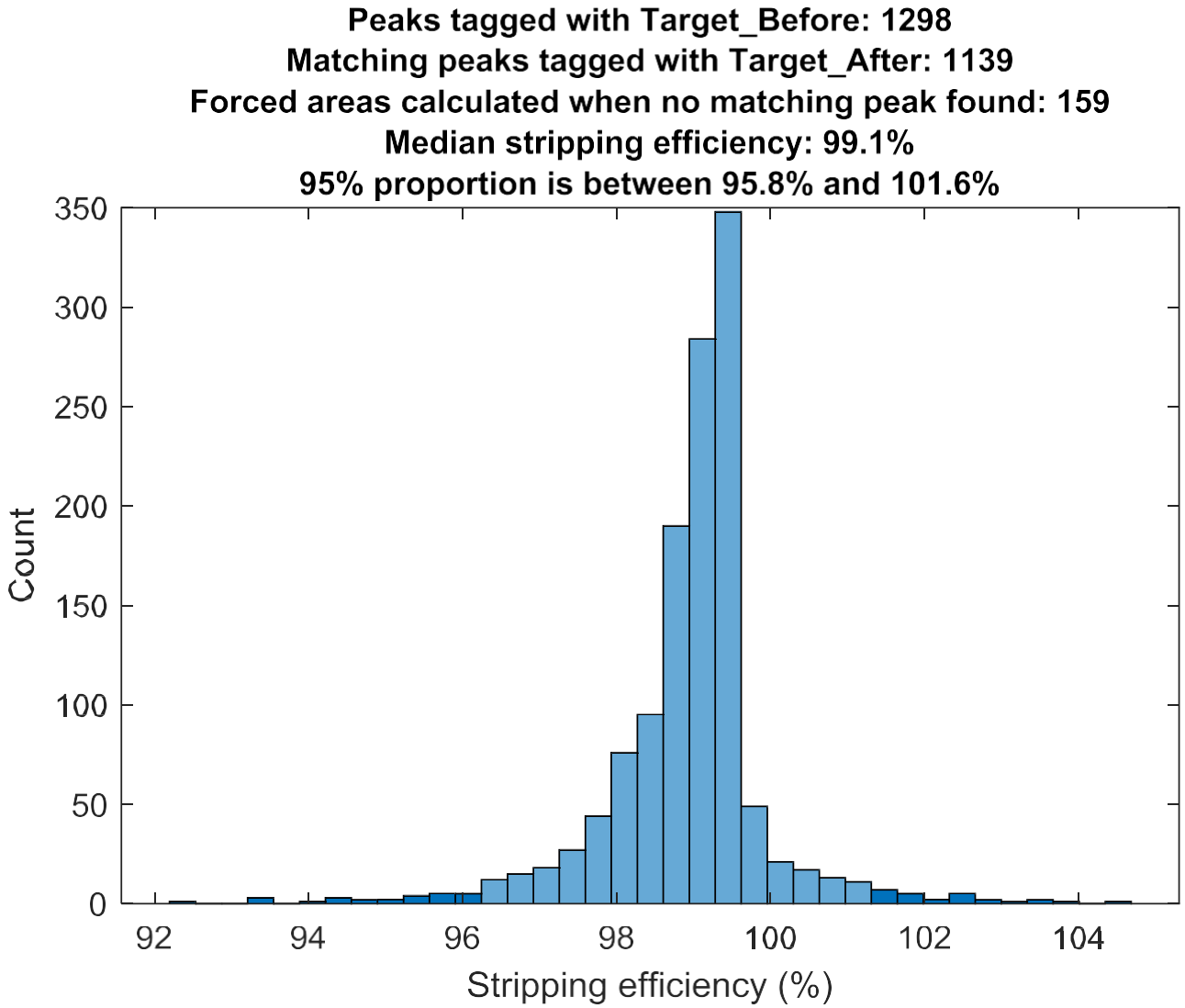


Note: Must use Scout 2.0 or later version to analyze images of scWest chips probed with a 3-plex antibody probing fixture

Calculating Stripping Efficiency using Scout 2.0+

- Scan your chip before and after stripping
- Load and register the 'before' and 'after' images in Scout 2.0+
- For the 'after' scan, adjust the peak SNR threshold to 0.1 (all lanes should turn green)
- Generate peak tables for both images (peak table for 'after' image will be mostly junk peaks)
- On the peak table for the 'before' image, tag your peaks of interest, e.g. "Target_Before"
- On the peak table for the 'after' tab choose command: Peak Table->Tag Matching Peaks / Stripping Efficiency
- Select "Target_Before" to match and create a new tag "Target_After" to apply to the matching peaks
- Accept the default matching tolerance (0.1)
- Choose "Yes" when prompted to perform the stripping efficiency calculation

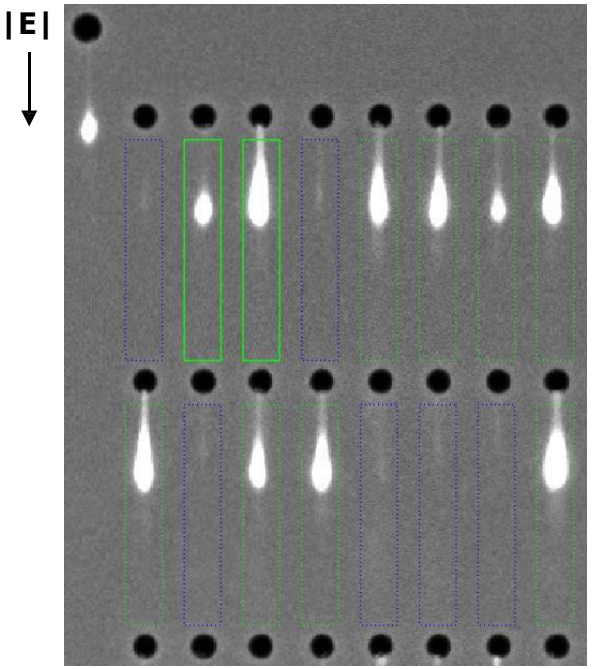
Calculating Stripping Efficiency using Scout 2.0+

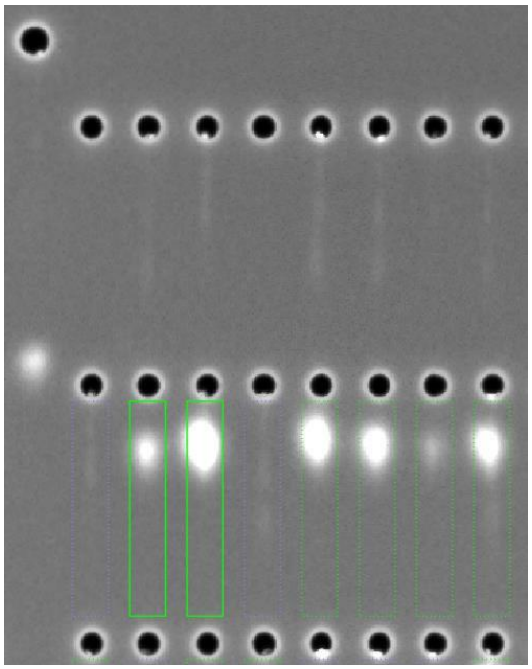


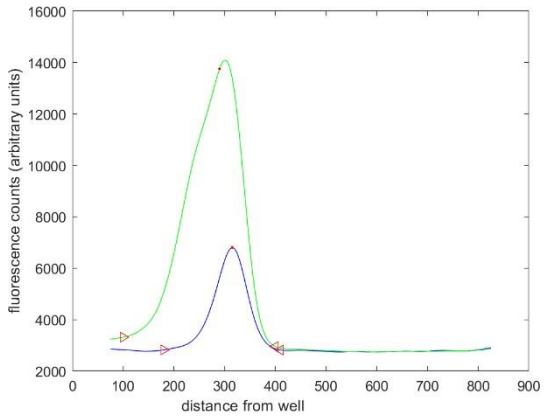
Analyzing peaks that overran the lane

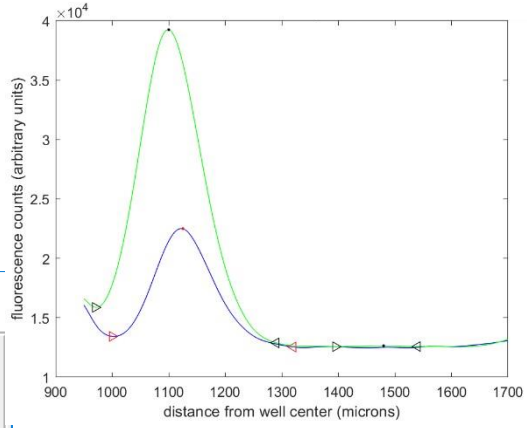
647 channel

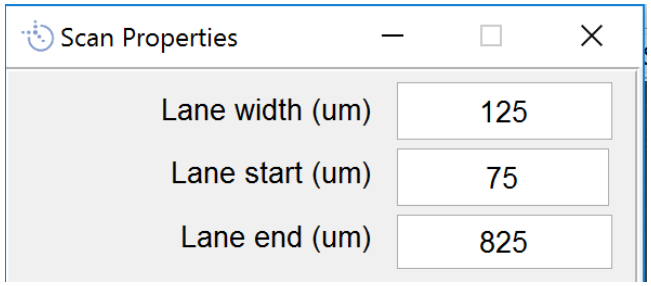
555 channel

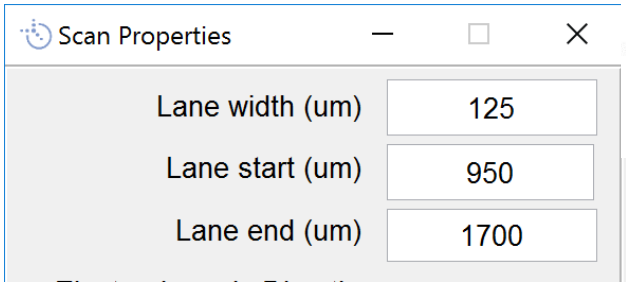








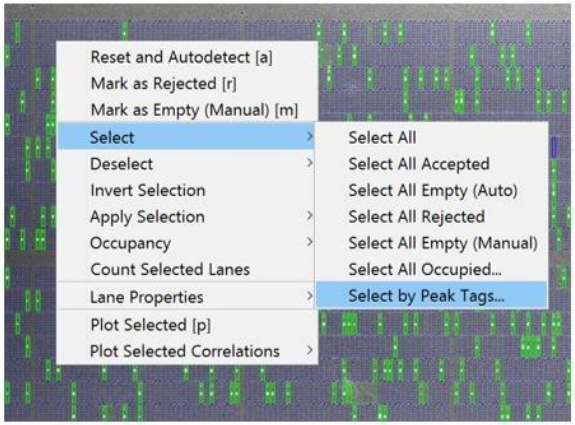


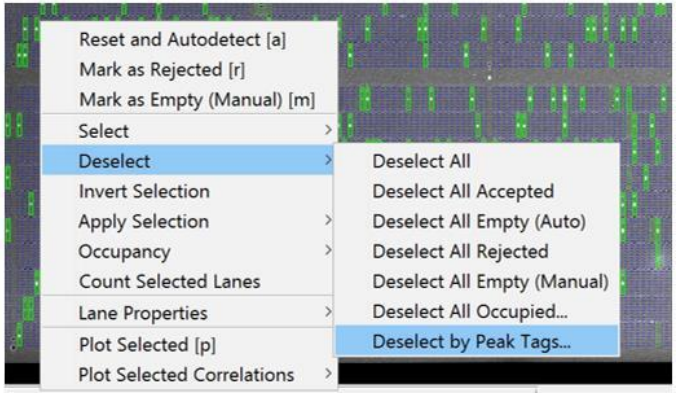


- Change Lane Start & Lane End position in Scan Settings to move lane for each microwell down to 950 micron starting position

Detecting low abundance peaks using Inspect function

1. Detect internal control peaks in color #1. Curate & label internal control peaks using Peak Table
2. Detect target in color #2 using default peak settings. Label detected target peaks using Peak Table.
3. Select internal control lanes in color #2 scan (right click on color #2 scan, Select by Peak Tag)
4. De-select lanes with detected target (right click, Deselect by Peak Tag)
5. Inspect remaining lanes using Inspect Function (Tools > Inspect > Inspect selected lanes or [i])
6. Adjust local peak settings for lanes where target peak is visually identified to detect target peak but peak is not detected by Scout





Normalizing peak area data

- Normalizing peak area data is possible but not typically recommended as it can introduce additional noise to the data
- To normalize peak area data:
  - Export data to .csv file
  - Open up in Excel
  - In new column, divide peak area 1 by peak area 2

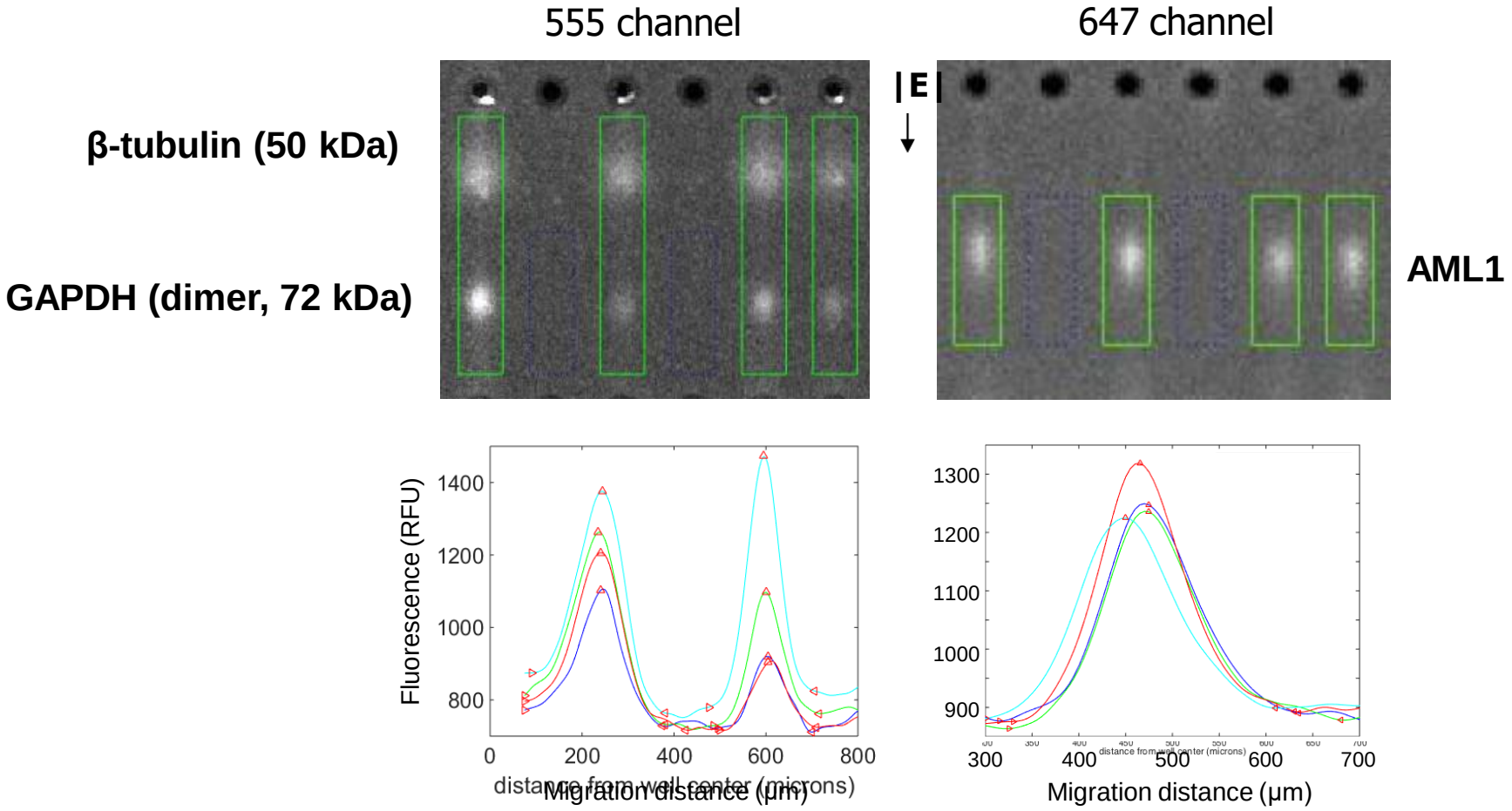
AI	AJ	AK	AL
PeakArea_AML1	PeakArea_BTUB	PeakArea_GAPDH	Normalized Peak Area
87199.08716	30401.40729	57429.98484	=AI12/AJ12
160862.2462	83848.6024	136629.5189	
NaN	26037.305	NaN	
131239.0373	31222.12942	110327.5236	
171740.1886	55498.78628	101781.2065	
92618.55763	29599.32089	89605.72372	
82067.11259	NaN	82673.51102	
88076.13947	33873.77679	83446.42732	
114852.518	NaN	76918.97866	
NaN	NaN	43057.59061	

Molecular weight sizing

- The standard Single-Cell Western workflow provides molecular weight (MW) information for your target relative to an endogenous control protein
- Two alternative approaches allow for absolute molecular weight quantitation on Milo:
  - MW sizing using 2+ endogenous protein controls
  - MW sizing using spiked ladder proteins

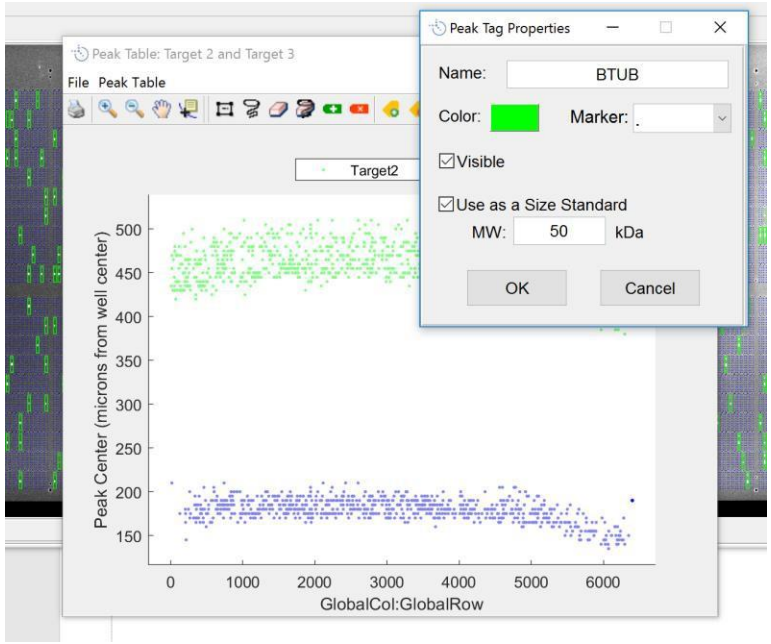
Molecular weight sizing

- Design assay to contain at least two proteins to be used for sizing ladder (e.g., β-tubulin & GAPDH)

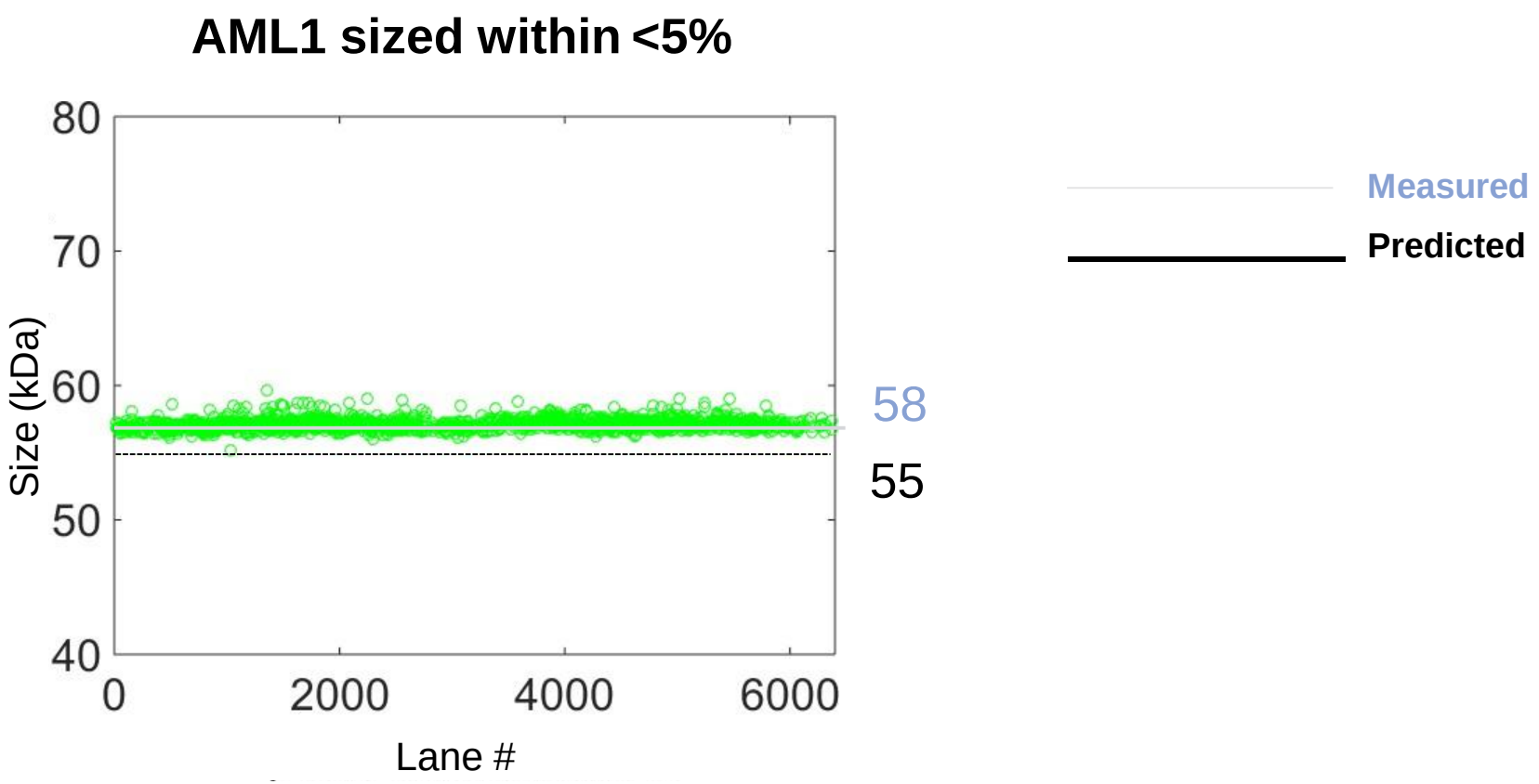


Molecular weight sizing

- Check "Use as a Size Standard" for any peak tag to be used for sizing reference
- Enter molecular weight

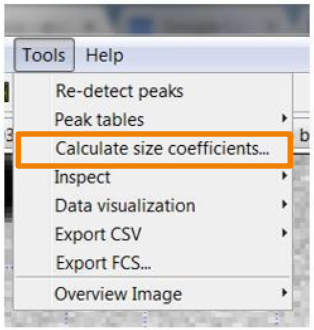


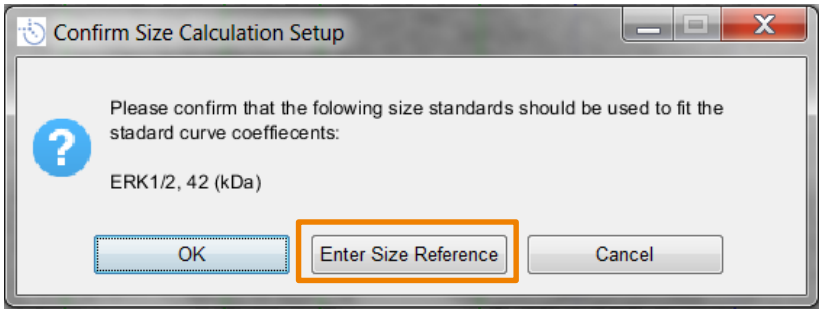
Molecular weight sizing



Correcting for migration variation across chip

- Use one reference protein (e.g., β-tubulin) as a sizing reference
- Tools > Calculate size coefficients > Enter Size Reference:
  - Create peak in center of well (0 microns) and enter 200 kDa for molecular weight
- Will remove migration drift for target(s) of interest





Advanced

Excluding lanes that have been identified as doublet lane in upstream brightfield image

- Visualization tools in Scout will not differentiate between occupancy of 1 or 2.
- To differentiate between occupancy values when plotting peak areas:
  - Click on lanes in image that are known to have doublets and set occupancy to 2 (or more)
  - In the Data Visualization Dataset Selector (Tools > Data Visualization), select Lanes with Occupancy = 1 and create visualization plots as normal
  - Alternatively, export peak area data to .csv file. Exported data includes lane occupancy data in addition to all peak data. Plot data in .csv file for lanes with occupancy of 1.

How to handle peaks with debris on top

- If lane contained a cell but the Peak Area is unreliable due to debris on top of the peak, lane can still be used for enumeration
- If neural net is used, these peaks may be labeled "NoiseLike"
  - Select peak and tag it "peak-dust" (for example)
  - Tag other peaks as usual
  - Scout will enumerate peaks without including "peak-dust" in enumeration calculations (similar to rejecting lane entirely)
  - To plot with "peak-dust" included in data, export to .csv and create enumeration table for (Peak OR Peak-Dust) vs. Target of Interest

Advanced

Suggested workflow for noisy images where Auto Tag fails

- Load in internal control image. Detect, curate, and label internal control peaks
- Load in target image. Select lanes that do not contain internal control peaks and mark as “Manually Empty” on target image. Apply selection across all scans and mark as “Manually Empty”
- Peaks detected in Manually Empty lanes will not be shown in Peak Table
- Then curate & label remaining target peaks which will only be detected in lanes containing an internal control peak

A simple, automated workflow for high quality images

1. Read all images in using auto registration. Peaks will be detected using default settings
2. Generate Peak Table for each scan
3. Run Auto Tag function for each Peak Table
4. Label peaks for protein targets of interest
5. Visualize data

More information?

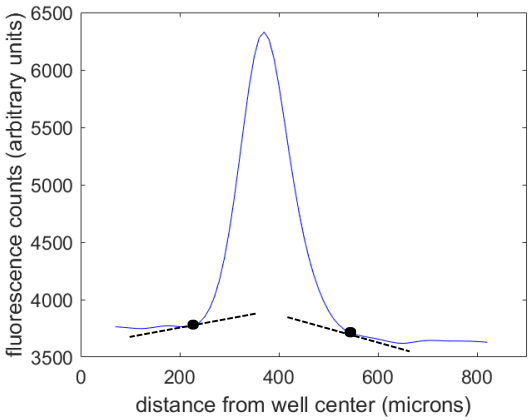
Please contact:

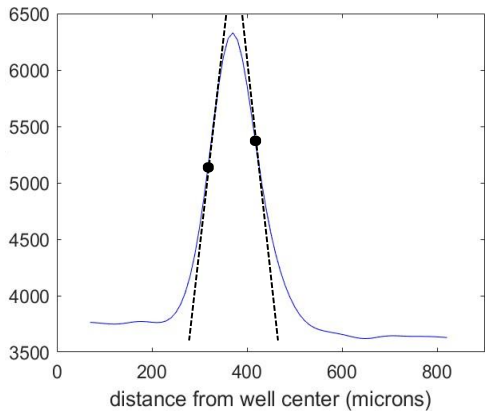
support@proteinsimple.com

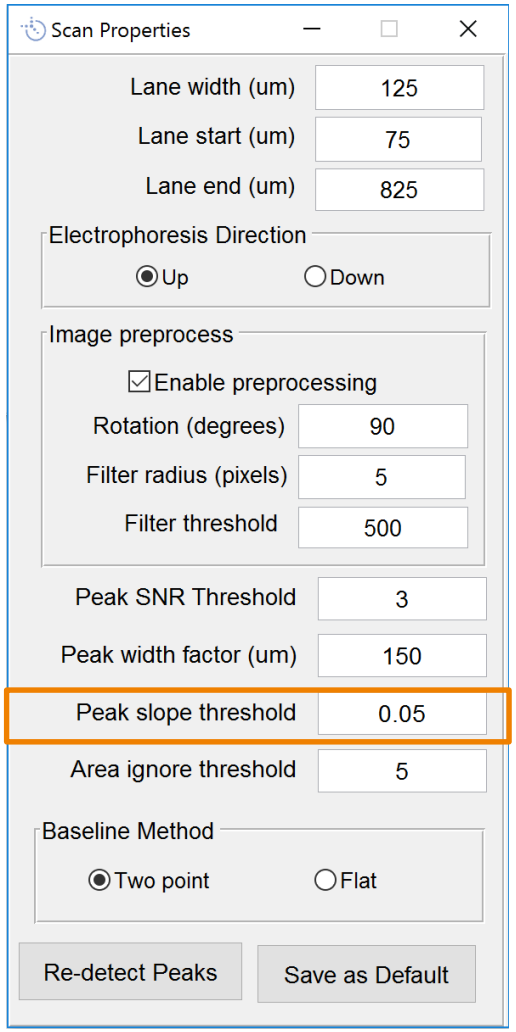
Appendix

Adjusting Peak Slope Threshold

- Peak detection algorithm finds location where slope reaches a specified fraction of the maximum slope (peak slope threshold, e.g. 5%)
- Generally leave as default setting
- Increasing peak slope threshold will bring Peak Start and Peak End point closer to peak center

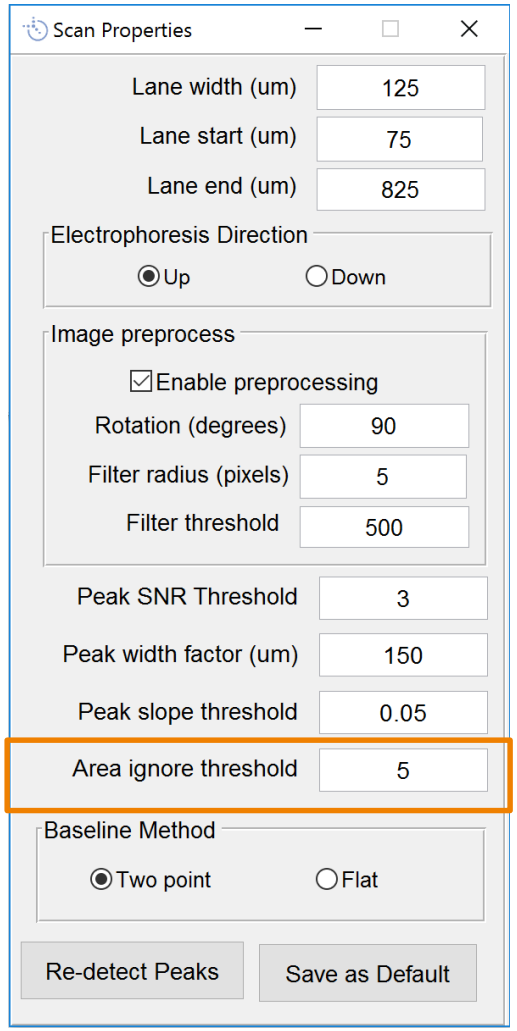






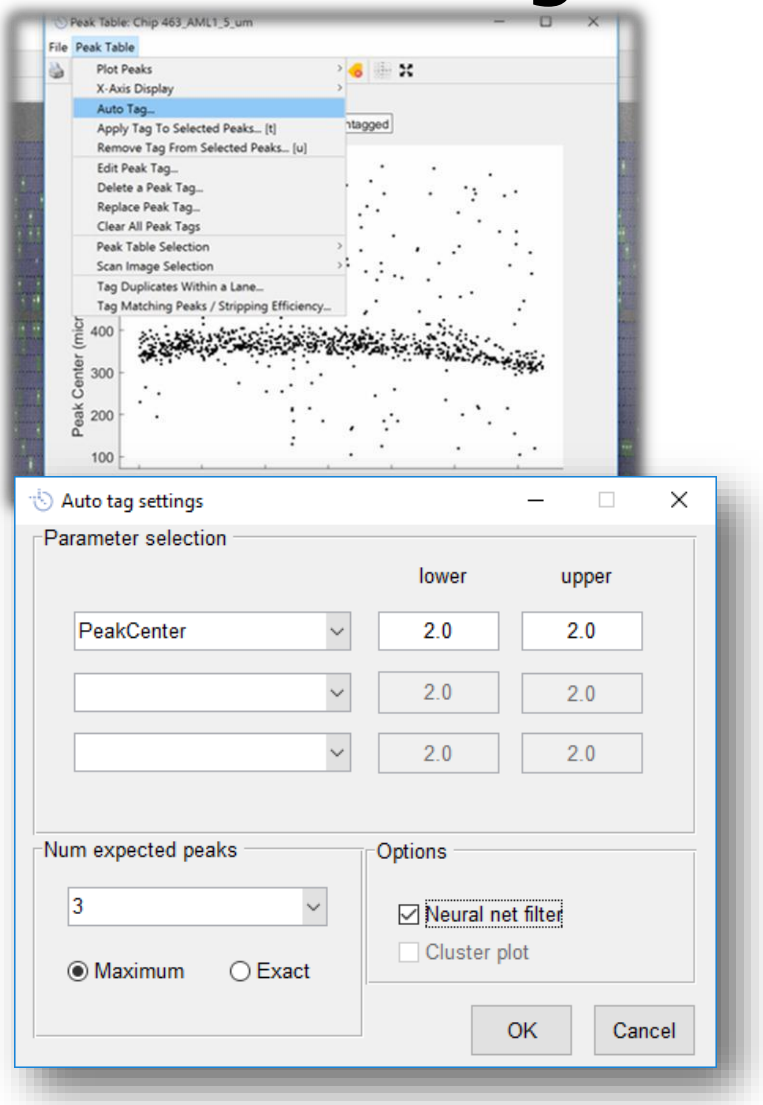
Adjusting Area Ignore Threshold

- Scout will not return peak if sum of Peak Area for all detected peaks is less than the defined threshold value



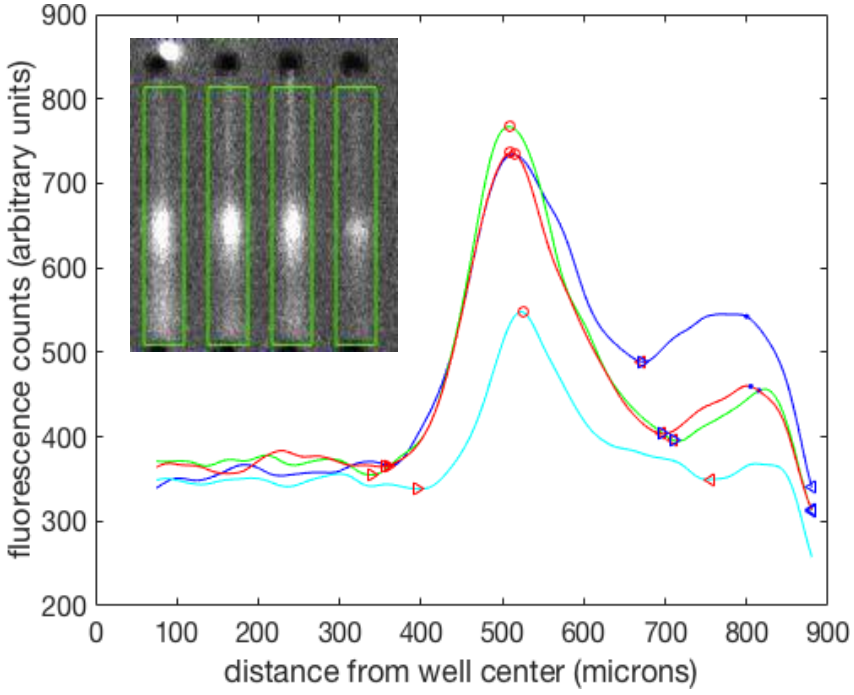
Automated Peak Curation: Auto Tag

- Upper & lower values for parameter selection are what define what is an outlier in the K-means clustering algorithm
- Value is the multiple applied to the difference between 25th and 75th percentile of the distribution of the peak property

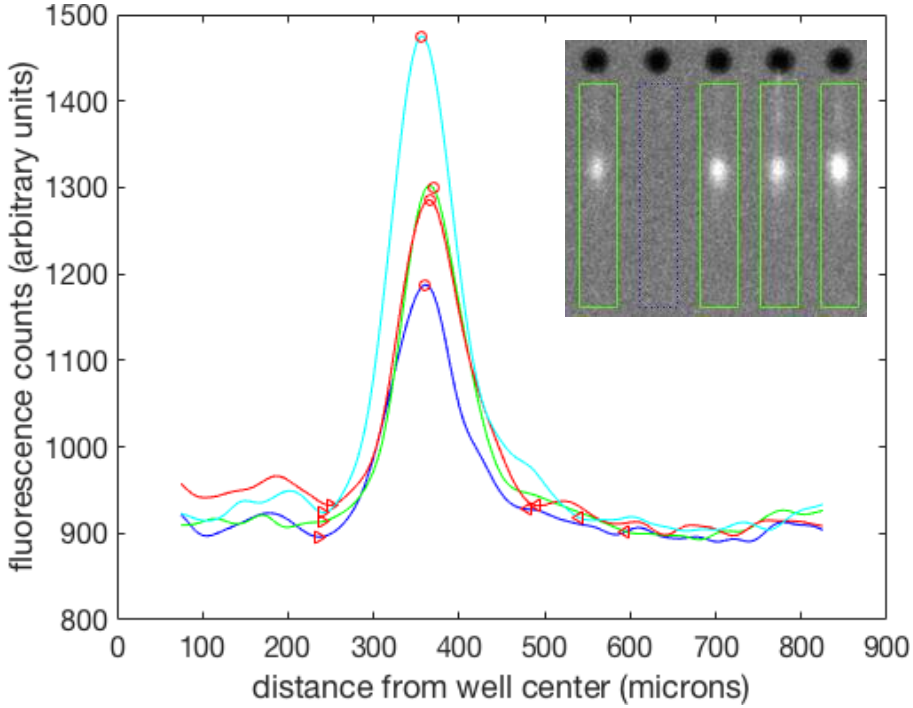


Assay optimization

High Background Signal



Ideal Signal (low background)



|E|

Lowering antibody concentration can reduce background lysate signal