

ClonePix 2 Training Guide

Interpreting Imaging Statistics



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Index

- Index
- [Chapter Purpose](#)
- [Introduction to Imaging Statistics](#)
- [Which Colonies Are Eligible for Picking?](#)
- [Commonly Used Imaging Statistics: Interior Mean Intensity](#)
- [Commonly Used Imaging Statistics: Exterior Mean Intensity](#)
- [Commonly Used Imaging Statistics: Sum Total Intensity](#)
- [Commonly Used Imaging Statistics: Normalized Intensity](#)
- [Which Statistic\(s\) Do I Choose for Defining Picking Crite...](#)
- [Support Resources](#)



Chapter Purpose

The purpose of this module is to guide the user to develop a deeper understanding of the imaging statistics generated by the ClonePix 2 Picking Software and apply this knowledge to select the most relevant criteria for successful picking of clones of interest.

This guide does not include detailed descriptions around sample preparation, setting up a pick run, or instrument maintenance. Please refer to corresponding modules for details on these topics.



Introduction to Imaging Statistics

- For each picking run, the **ClonePix 2 Colony Picking** software automatically **analyzes images** captured of your samples and **calculates**:
 - Fluorescence**: 13 different **fluorescence intensity statistics** for each **fluorophore** measured in each colony.

Statistic	Description	Category	Unit
Exterior Area	The area of the pixels outside the feature boundary in the local vicinity of the feature	Intensity	mm ²
Exterior Mean Intensity	The arithmetic mean intensity of all the pixels outside the feature boundary in the local vicinity of the feature	Intensity	
Exterior Geo Mean Intensity	The geometric mean intensity of all the pixels in the feature	Intensity	
Exterior Median Intensity	The median intensity of all the pixels outside the feature boundary in the local vicinity of the feature	Intensity	
Exterior Total Intensity	The total intensity of all the pixels outside the feature boundary in the local vicinity of the feature	Intensity	
Interior Intensity SD	The standard deviation of the intensity of the pixels in the feature	Intensity	
Interior Mean Center Intensity	The mean intensity of the nine pixels at the center of the feature	Intensity	
Interior Mean Intensity	The arithmetic mean intensity of all the pixels in the feature	Intensity	
Interior Geo Mean Intensity	The geometric mean intensity of all the pixels in the feature	Intensity	
Interior Median Intensity	The median intensity of all the pixels in the feature	Intensity	
Interior Total Intensity	The total intensity of all the pixels in the feature	Intensity	
Normalized Intensity	The total intensity of the feature divided by the primary area	Intensity	
Sum Total Intensity	The sum total intensity of the feature (interior and exterior)	Intensity	



Introduction to Imaging Statistics

- For each picking run, the ClonePix 2 Colony Picking software automatically analyzes images captured of your samples and calculates:
 - Fluorescence:** 13 different fluorescence intensity statistics for each fluorophore measured in each colony.
 - White Light (Prime Configuration):** 40 different statistics for each colony, including colony area, morphology, proximity, and location coordinates.

Statistic	Description	Category	Unit
Actual X	The X co-ordinate of the center of the feature in mm	Position	mm
Actual Y	The Y co-ordinate of the center of the feature in mm	Position	mm
Area	The area covered by the feature (excluding 'child' features) in square mm	Morphology	mm ²
Axis Ratio	The ratio of the minimum and maximum radiuses of the feature measured from 0 (very elongated) to 1 (a perfect circle)	Morphology	
Block	The identifier of the block of features this feature is associated with	General	
Compactness	A measure of how compact the feature is, measured from 0 (not compact) to 1 (a perfect circle)	Morphology	
Deposit Barcode	Barcode of the plate the colony has been deposited in	Information	
Deposit Well	The well the colony has been deposited in	Information	
Edge Excluded	Whether the colony center lies within the exclusion zone	Position	
Feature ID	A unique identifier	General	
Group	Group the feature is assigned to	General	
Image Column	The column the image containing the feature is in	Position	
Image Row	The row the image containing the feature is in	Position	
Intensity SD	The standard deviation of the intensity of the pixels in the feature	Intensity	
Manual Group	Whether the feature was manually added to its current group	General	
Mean Center Intensity	The mean intensity of the nine pixels at the center of the feature	Intensity	
Mean Intensity	The mean intensity of all the pixels in the feature	Intensity	
Median Intensity	The median intensity of all the pixels in the feature	Intensity	
Perimeter	The length of the perimeter of the feature in mm	Morphology	mm
Picked	Flag to signify if the colony has been picked.	Information	
Pixel Area	The area covered by the feature (excluding 'child' features) in pixels	Morphology	pixels
Pixel Perimeter	The length of the perimeter of the feature in pixels	Morphology	pixels
Pixel Radius Max	The maximum radius of the feature in pixels	Morphology	pixels
Pixel Radius Min	The minimum radius of the feature in pixels	Morphology	pixels
Pixel Radius SD	The radius standard deviation of the feature in pixels	Morphology	pixels
Pixel Total Area	The total area of the feature (including 'child' features) in pixels	Morphology	pixels
Pixel X	The X co-ordinate of the center of the feature in pixels relative to the top left of the image	Position	pixels
Pixel Y	The Y co-ordinate of the center of the feature in pixels relative to the top left of the image	Position	pixels
Proximity	The distance to the closest neighboring colony in the same image	Morphology	mm
Radius Max	The maximum radius of the feature in mm	Morphology	mm

Statistic	Description	Category	Unit
Radius Min	The minimum radius of the feature in mm	Morphology	mm
Radius SD	The radius standard deviation of the feature in mm	Morphology	mm
Saturated Percentage	The percentage of saturated pixels in the feature	Intensity	%
Saturated Pixels	The number of saturated pixels in the feature	Intensity	
Selected	Whether the feature is currently selected	General	
Source Barcode	The barcode of the plate that the feature is in	Position	
Source Well	The well the feature is in	Position	
Total Area	The total area of the feature (including 'child' features) in square mm	Morphology	mm ²
Volume Equivalent	The volume of a sphere having the same cross-sectional area as the feature in cubic mm	Morphology	mm ³
Well Index	A numerical annotation of the Source Well for graphical presentation	Position	



Introduction to Imaging Statistics

- For each picking run, the ClonePix 2 Colony Picking software automatically analyzes images captured of your samples and calculates:
 - Fluorescence: 13 different fluorescence intensity statistics for each fluorophore measured in each object.
 - White Light (Prime Configuration): 40 different statistics for each object identified, including colony area, morphology, proximity, and location coordinates.
- Statistics** calculated for each **individual colony** can be reviewed by clicking on a **colony** in the **image**, then clicking on the **Statistics** tab at the **Results Review** step of the **Pick Run** process.
- Clicking to select a **statistic** in the displayed **list** will call up a specific **description/definition** of that **parameter** below the frame.



The screenshot displays the ClonePix 2 software interface. On the left, a fluorescence image shows several colonies, with one colony highlighted by a red circle and a blue square. Below the image, the text '5808 (8%)' is visible. On the right, a statistics panel is open, showing a list of parameters for the selected colony. The 'FITC 1500ms Intensity' section is expanded, showing various intensity statistics. The 'General' section shows 'Block: 12', 'Feature ID: 823', 'Group: Too Small', 'Manual Group: False', and 'Selected: False'. The 'Intensity' section shows 'Intensity SD: 3,264.765', 'Mean Centre Intensity: 9,741.889', 'Mean Intensity: 5,626.423', 'Median Intensity: 7,837', 'Saturated Percentage: 0.000%', and 'Saturated Pixels: 0'. The 'Morphology' section shows 'Area: 0.041mm²', 'Axis Ratio: 0.772', 'Compactness: 0.854', and 'Perimeter: 0.774mm'. A red box highlights the 'FITC 1500ms Exterior Mean Intensity' section, which contains the text: 'The arithmetic mean intensity of all the pixels outside the feature boundary in the local vicinity of the feature'. At the bottom of the statistics panel, the 'Statistics' tab is selected.

FITC 1500ms Intensity	
[FITC 1500ms] Exterior Mean Inter	1,501.353
[FITC 1500ms] Exterior Median Int	1,137
[FITC 1500ms] Exterior Total Inten	1,989,293.000
[FITC 1500ms] Interior Intensity St	5,848.058
[FITC 1500ms] Interior Mean Centri	9,266.778
[FITC 1500ms] Interior Mean Inten	9,695.519
[FITC 1500ms] Interior Median Inte	10,024
[FITC 1500ms] Interior Total Inten	504,167.000
[FITC 1500ms] Normalized Intensity	48,182.131
[FITC 1500ms] Sum Total Intensity	2,505,470.826

General	
Block	12
Feature ID	823
Group	Too Small
Manual Group	False
Selected	False

Intensity	
Intensity SD	3,264.765
Mean Centre Intensity	9,741.889
Mean Intensity	5,626.423
Median Intensity	7,837
Saturated Percentage	0.000%
Saturated Pixels	0

Morphology	
Area	0.041mm²
Axis Ratio	0.772
Compactness	0.854
Perimeter	0.774mm

[FITC 1500ms] Exterior Mean Intensity
The arithmetic mean intensity of all the pixels outside the feature boundary in the local vicinity of the feature

Acquisition | Detection | Non-cellular | Groups | **Statistics**



Which Colonies Are Eligible for Picking?

- The **Ungated** group represents the **pool of colonies** that are **eligible for picking**.

The screenshot displays a software interface for colony selection. On the left, a list of criteria is shown with corresponding colored circles and counts:

- All Undiscarded Features (white circle)
- Edge Excluded [328] (red circle)
- Too Big [0] (purple circle)
- Too Small [282] (blue circle)
- Irregular 1 [0] (orange circle)
- Irregular 2 [4] (yellow circle)
- Proximity [8] (pink circle)
- Ungated [11]** (green circle, highlighted with a black box)

On the right side of the list, there are two buttons: "Increase Priority" and "Decrease Priority".

Below the list, there is a "Group" section with the following controls:

- Name: Ungated (text input field)
- Hidden:
- Colour: [Green color swatch] Colour... (button)
- Remove (button)

At the bottom of the interface, there is a navigation bar with tabs: "Non-cellular", "Groups" (selected), and "Statistics".



Which Colonies Are Eligible for Picking?

- The **Ungated** group represents the **pool of colonies** that are **eligible for picking**.
- Based on a **pre-defined subset** of imaging **statistics**, these colonies have **passed** all of the **default criteria for size, regularity of shape (compactness/axis ratio), and proximity to other colonies or the well edge** – i.e. those that were **not** classified in the **groups** higher in the list.

The screenshot displays a software interface for colony selection. At the top, a list of groups is shown under the heading 'All Undiscarded Features'. The groups are: 'Edge Excluded [328]' (red), 'Too Big [0]' (purple), 'Too Small [282]' (blue), 'Irregular 1 [0]' (orange), 'Irregular 2 [4]' (yellow), 'Proximity [8]' (pink), and 'Ungated [11]' (green). A red bracket on the right side of the list groups the first six items and is labeled 'Excluded'. A black box with a white arrow points to the 'Ungated [11]' group, and a white box with black text below it says 'Eligible for Picking'. To the right of the list are two buttons: 'Increase Priority' and 'Decrease Priority'. Below the list is a 'Group' configuration section for 'Ungated', which includes a 'Name' field containing 'Ungated', a 'Hidden' checkbox, a color selection area with a green square and a 'Colour...' button, and a 'Remove' button. At the bottom of the interface, there are tabs for 'Non-cellular', 'Groups', and 'Statistics', with 'Groups' currently selected.



Which Colonies Are Eligible for Picking?

- The **Ungated** group represents the **pool of colonies** that are **eligible for picking**.
- These colonies have **passed** all of the **default criteria** for **size, regularity of shape (compactness/axis ratio), and proximity to other colonies or the well edge** – i.e. those that were **not** classified in the **groups** higher in the list.
- From this **Ungated** group you can select a **final group** of colonies to pick by **gating** on the **statistic(s)** that best align with your **goals** for the pick run.

The screenshot displays a software interface for colony selection. At the top, a list of groups is shown under the heading "All Undiscarded Features". The groups are:

- Edge Excluded [328]
- Too Big [0]
- Too Small [282]
- Irregular 1 [0]
- Irregular 2 [4]
- Proximity [8]
- Ungated [111]**

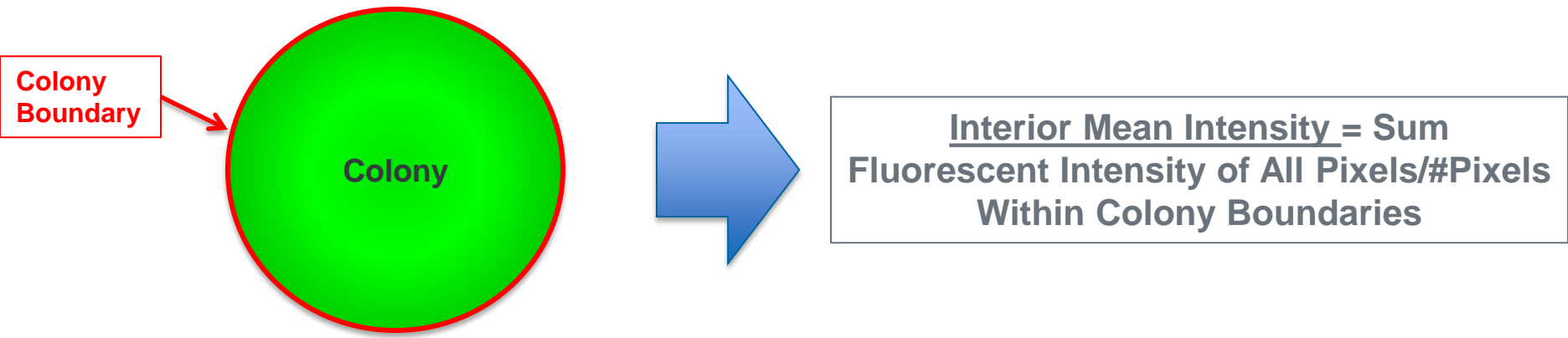
A red bracket on the right side of the list groups the first six items and is labeled "Excluded". A black box with a white arrow points to the "Ungated [111]" group, labeled "Eligible for Picking". To the right of the list are buttons for "Increase Priority" and "Decrease Priority". Below the list is a "Group" configuration panel for the "Ungated" group, which includes a "Name" field, a "Hidden" checkbox, a color selection button labeled "Colour...", and a "Remove" button. At the bottom of the interface, there are tabs for "Non-cellular", "Groups", and "Statistics".

For research use only. Not for use in diagnostic procedures.

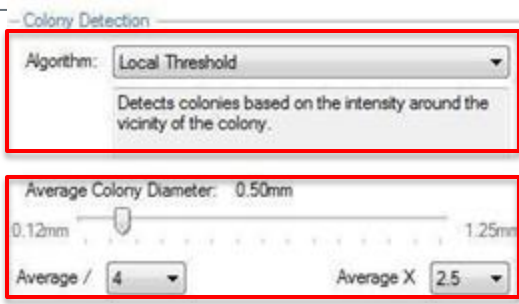
Commonly Used Imaging Statistics: Interior Mean Intensity

While the **ClonePix 2 Clone Picking software** generates a broad array of **statistics**, generally only **4** of these are commonly utilized for **defining** your **colonies to pick**:

- 1) **Interior Mean Intensity:** The arithmetic mean intensity of all the fluorescent pixels in the colony



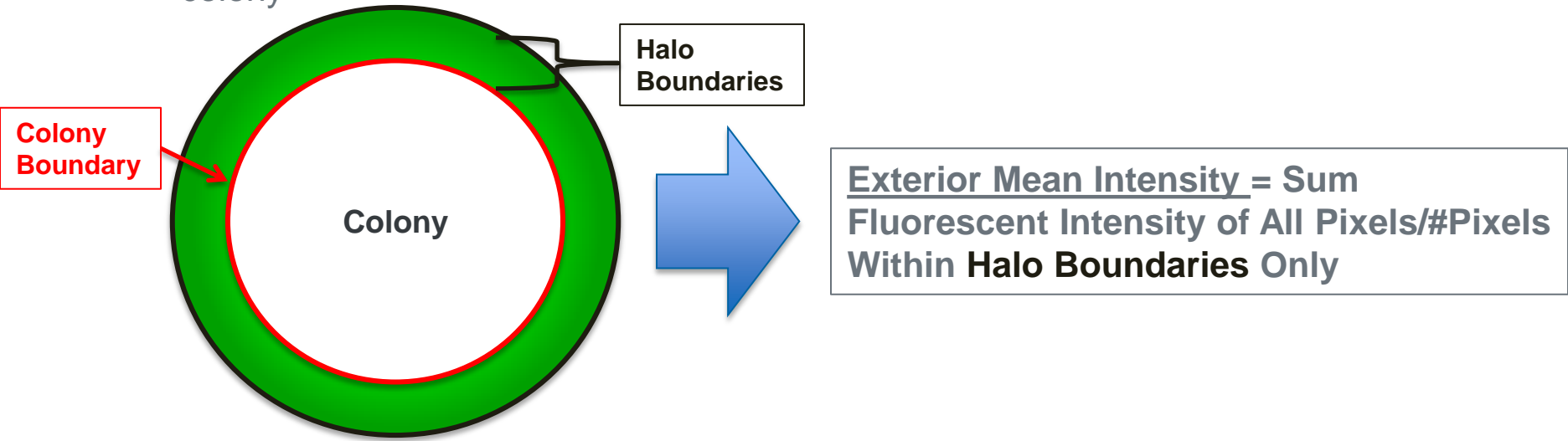
* **Colony boundaries** are determined by the **Colony Detection criteria (Algorithm & Average Colony Diameter settings)** that you set using the white light (TransWL) image of your sample during the **Imaging & Results Review Steps** of your **Pick Run**.



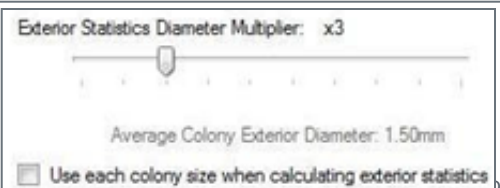
Commonly Used Imaging Statistics: Exterior Mean Intensity

While the ClonePix 2 Clone Picking software generates a broad array of statistics, generally only 4 of these are commonly utilized for defining your colonies to pick:

2) **Exterior Mean Intensity:** The arithmetic mean intensity of the selected fluorophore for all of the pixels outside the colony boundary in the local vicinity of the colony



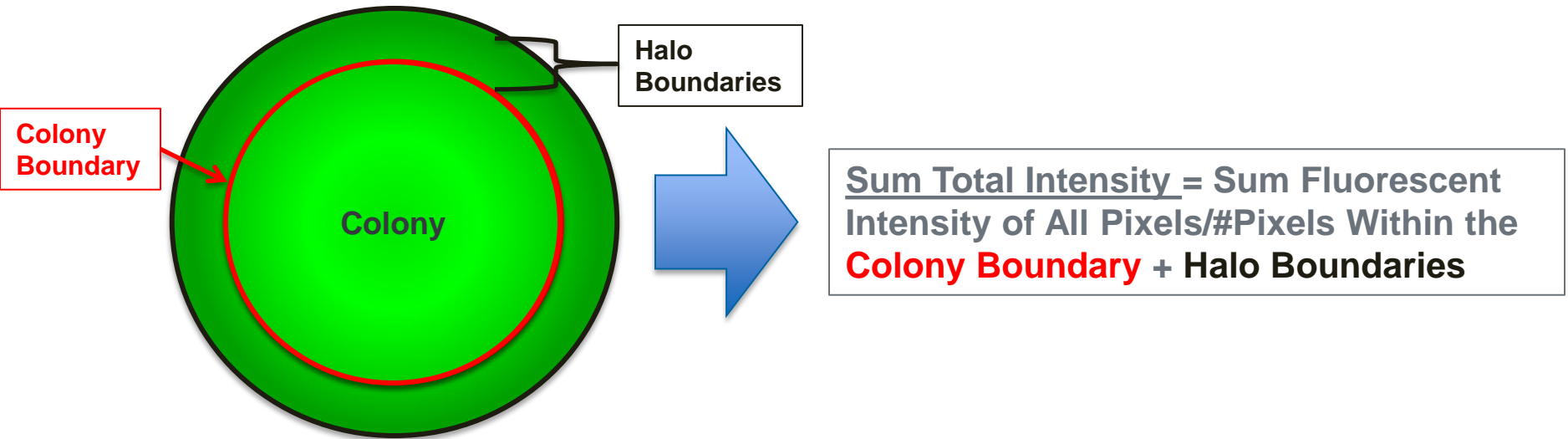
* **Halo Boundaries** are determined by multiplying the **calculated average colony boundary** value by the **Exterior Statistics Diameter Multiplier** (set to x3 by default) value that you set during the **Imaging & Results Review Steps** of your **Pick Run**. If the **Use each colony size when calculating exterior statistics** checkbox is selected, then this value will be calculated based on a **per colony** basis.



Commonly Used Imaging Statistics: Sum Total Intensity

- While the ClonePix 2 Clone Picking software generates a broad array of statistics, generally only 4 of these are commonly utilized for defining your colonies to pick:

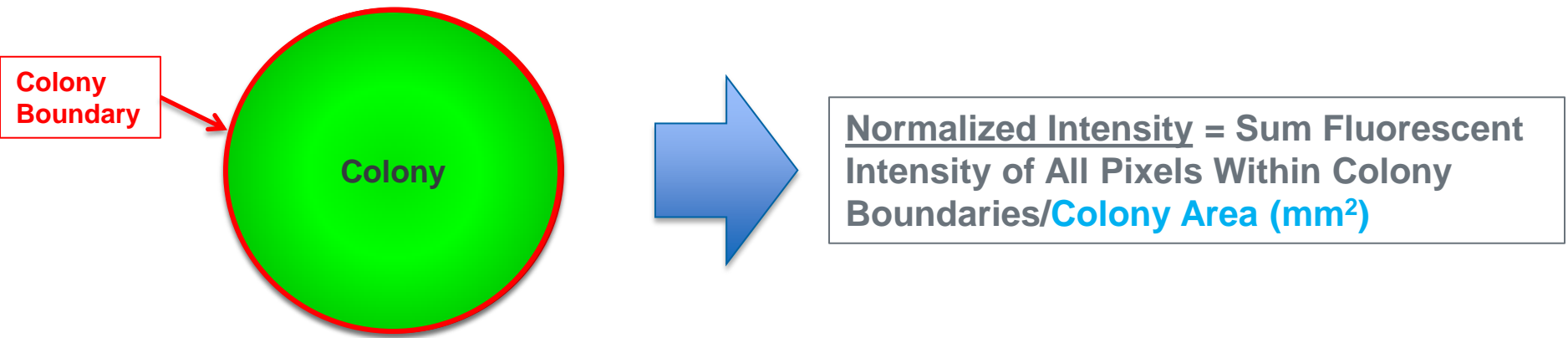
3) **Sum Total Intensity**: The sum total intensity of the selected fluorophore within the colony + halo (interior and exterior)



- See previous slides for detailed information on calculation of **Colony Boundary** and **Halo Boundaries**.

Commonly Used Imaging Statistics: Normalized Intensity

- While the **ClonePix 2 Clone Picking** software generates a broad array of **statistics**, generally only 4 of these are commonly utilized for **defining** your colonies to pick:
 - Normalized Intensity:** The sum total intensity of the selected fluorophore within the colony divided by the colony area.



- Colony Area** is calculated by the software, by multiplying by **pi** (π) by the **colony radius value squared**:

$$A = \pi r^2$$



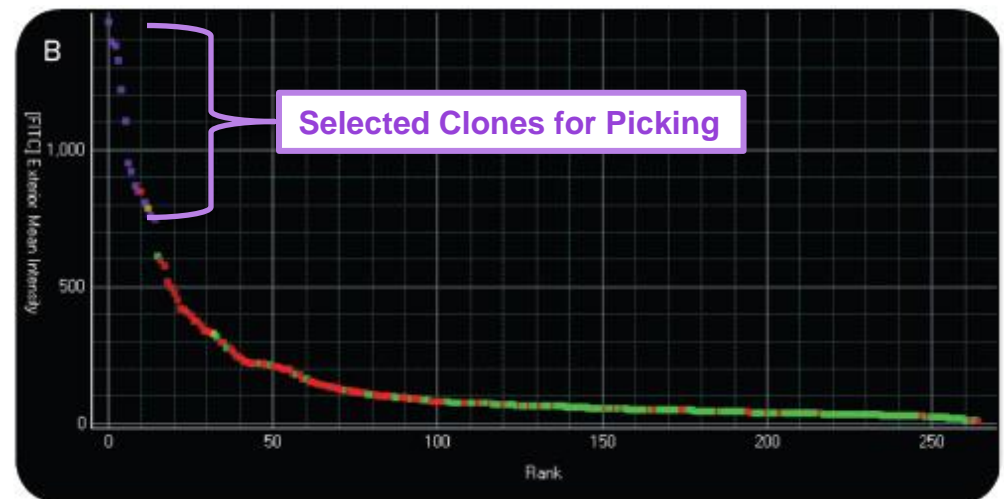
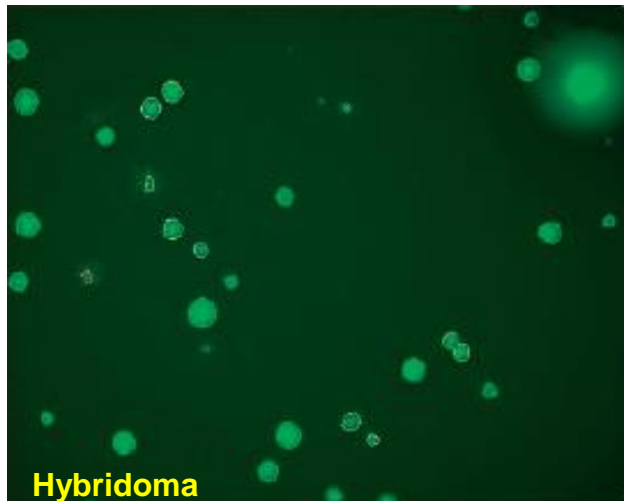
Which Statistic(s) Do I Choose for Defining Picking Criteria?

- **Three** main factors will dictate your best choice of **statistic(s)** to define your **criteria** for colony picking:

1) Application:

a) Hybridomas:

- **First Round Picking:** Select colonies based on **Exterior Mean Intensity** – generally for this step, set a relatively **low threshold** as the goal is to pick **any colonies** that have **significant fluorescent signal/background**.
- **Subcloning:** Select colonies based on **high Exterior Mean Intensity** and **exclude** colonies with **high Interior Mean Intensity/low Exterior Mean Intensity** to avoid clones that are **not** secreting IgG properly (i.e. bound to cell surface).



Which Statistic(s) Do I Choose for Defining Picking Criteria?

- **Three** main factors will dictate your best choice of **statistic(s)** to define your **criteria** for **colony picking**:
 - 1) **Application**:
 - b) **Cell Line Development**: Choose your **statistic for picking** based on the **expected staining pattern** for your **selected antibody** or **expressed protein** in your **cell line**.

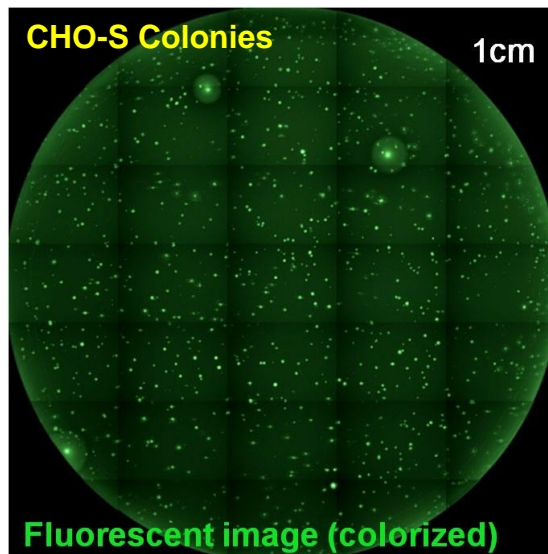
Set your gates **stringently** to select only the **highest expressing clones**. (See the **next section** for more information on considering **staining patterns** when selecting statistics).

Which Statistic(s) Do I Choose for Defining Picking Criteria?

- Three main factors will dictate your best choice of **statistic(s)** to define your **criteria** for colony picking:

2) Staining Pattern:

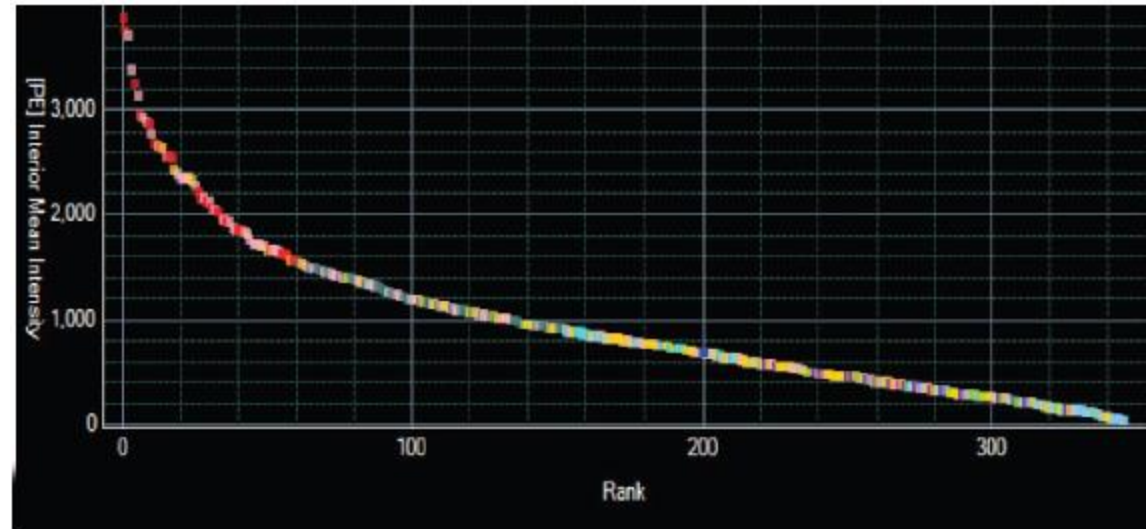
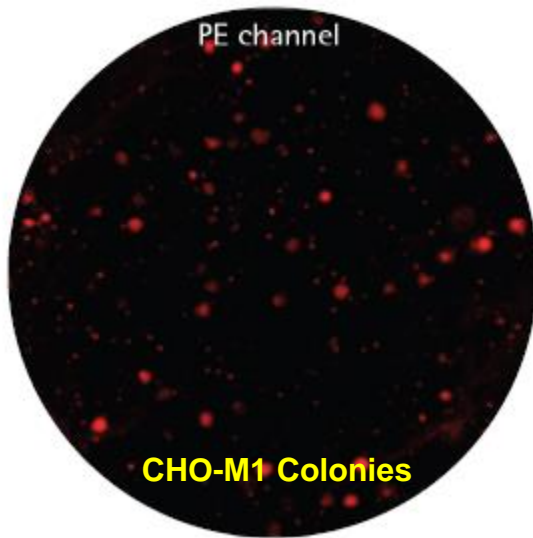
- a) For **Antibody or Expressed Protein Secretion** where a **halo pattern** is expected choose one of the following statistics:
 - **Mean Exterior Intensity** to select colonies based on **fluorescent intensity** of the **halo only** (most common for **hybridomas**)
 - **Sum Total Intensity** to select colonies based on **fluorescent intensity** of the **colony + halo**.



Selecting for High IgG Expression in CHO-S Colonies: Sum Total Intensity was the preferred statistic used to select colonies for picking in the example above.

Which Statistic(s) Do I Choose for Defining Picking Criteria?

- Three main factors will dictate your best choice of **statistic(s)** to define your **criteria** for colony picking:
 - 2) **Staining Pattern:**
 - b) For **GPCR/Cell Surface Protein Expression** where fluorescent signal should only be localized to the **colony surface** choose:
 - **Interior Mean Intensity** to select colonies for picking.



Selecting for M1 GPCR Cell Surface Expression in CHO-M1 Colonies using Interior Mean Intensity.

Which Statistic(s) Do I Choose for Defining Picking Criteria?

- Three main factors will dictate your best choice of **statistic(s)** to define your **criteria for colony picking**:

3) Positive Colony Size Heterogeneity:

- In cases where you observe **significant variability** in **colony area** across colonies with **equivalent Mean Interior Intensity** of your selected fluorophore, choose:
- **Normalized Intensity** to select colonies based on **fluorescent signal normalized to colony area**.
- Set your **gate stringently** to select **larger colonies with high fluorescent signal for picking** and **exclude smaller colonies** that may be **false positives and/or unlikely to survive post-picking**.
- If you are unsure as to how to set your gates appropriately using this statistic, alternatively you may choose to pick your colonies based on **Mean Interior Intensity**, then select **Area** in the **Order By** dropdown in the **Sort Options** section at the **Picking Review** step. You can then monitor viability and expression levels and correlate with colony size.

Support Resources

- Go to the HELP menu within ClonePix 2 Software
- Support and Knowledge Base: <http://mdc.custhelp.com/>
- Request Support: <http://mdc.custhelp.com/app/ask> or via email support@moldev.com
- Technical Support can also be reached by telephone:
 - 1 (800) 635-5577
 - Select options for Tech Support → Biotherapeutics Products → ClonePix Instruments





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