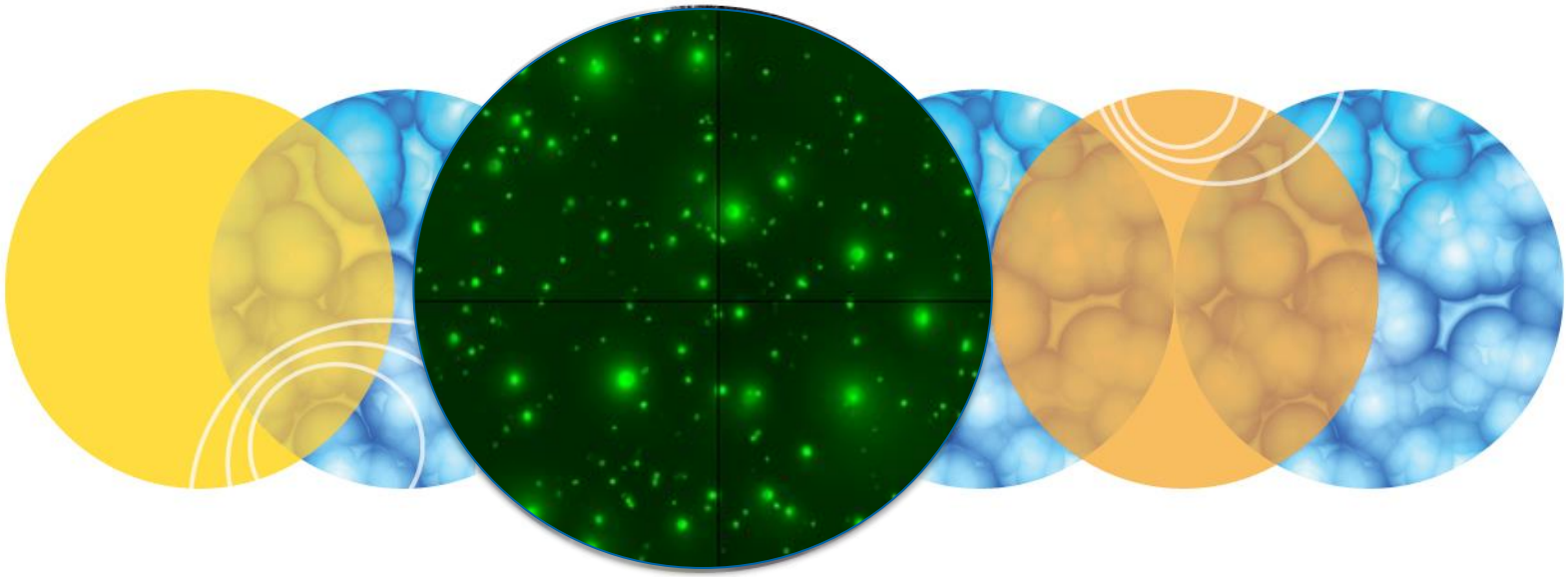

ClonePix 2 Training Guide

Interpreting Imaging Statistics

Date Revised 03/03/2017 Version A

MOLECULAR DEVICES
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Index

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	mm²
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	

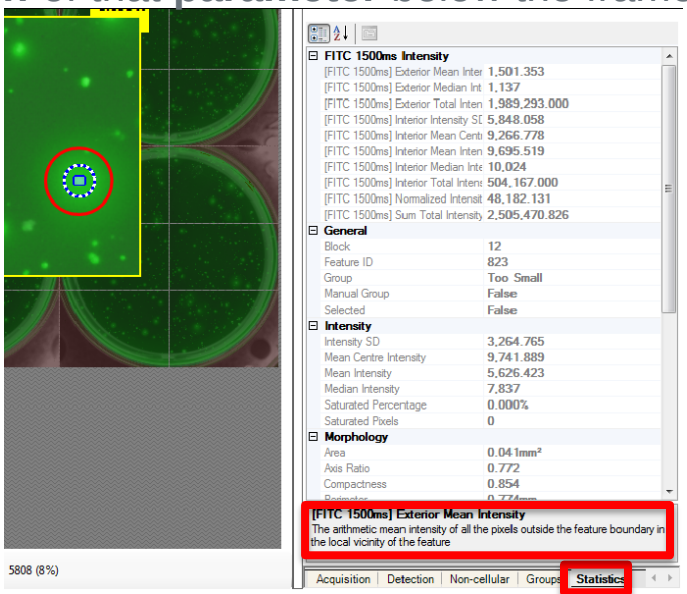
Introduction to Imaging Statistics

- 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
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	

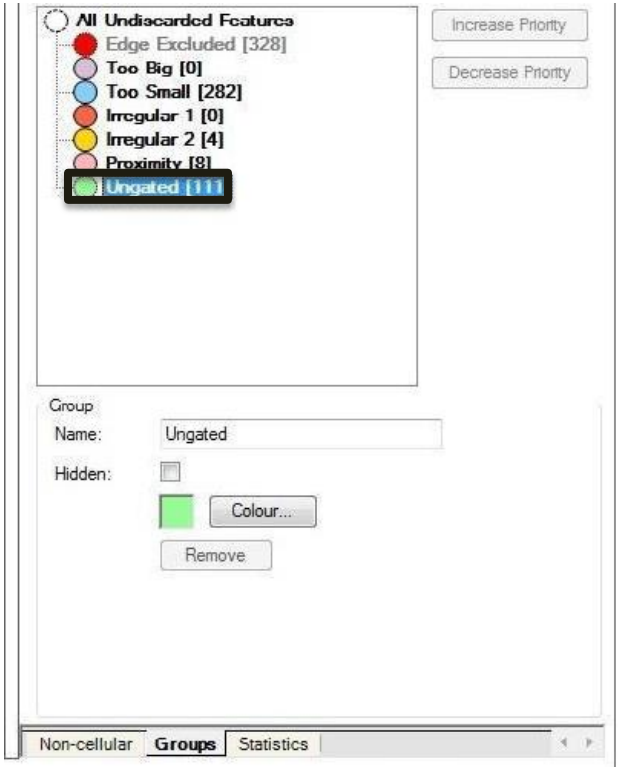
Introduction to Imaging Statistics

- 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.



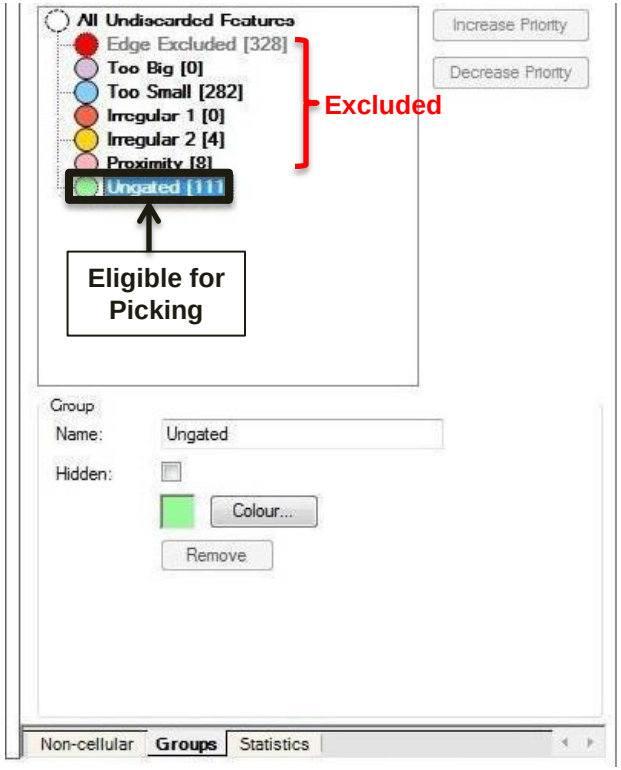
Which Colonies Are Eligible for Picking?

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



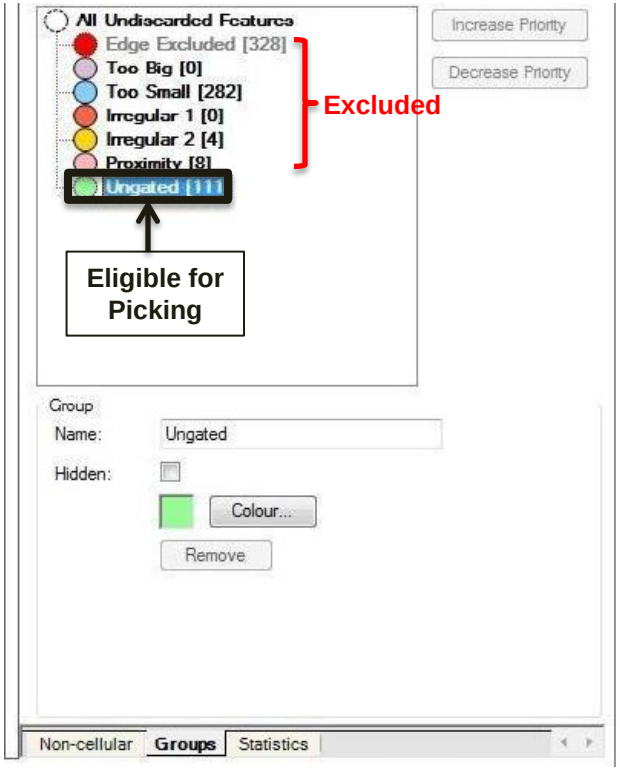
Which Colonies Are Eligible for Picking?

- 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.



Which Colonies Are Eligible for Picking?

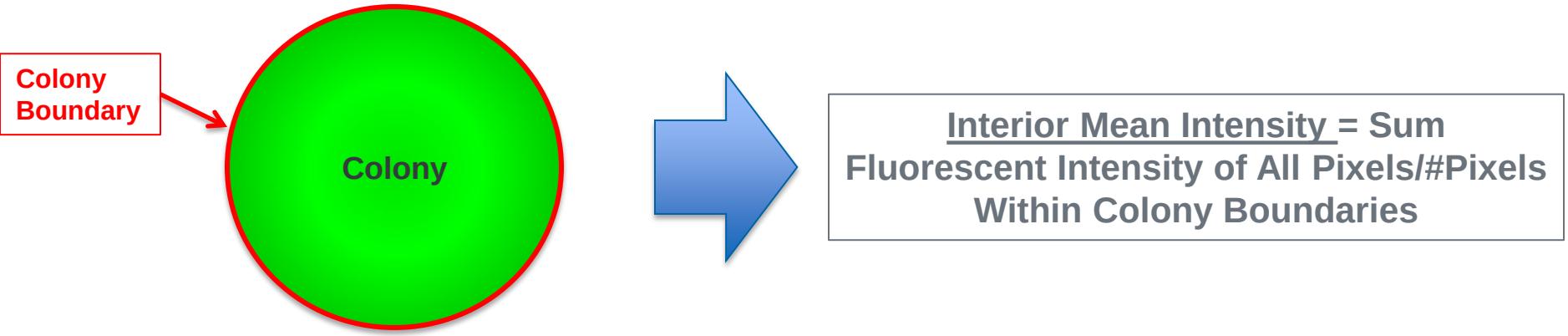
- The **Ungated group** represents the **pool of colonies** that are **eligible for picking.**
- These colonies have **passed** all of the **default criteria** for **size, regularity of shape (compactness/axis ratio), and proximity to other colonies** or the **well edge** – i.e. those that were **not** 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.



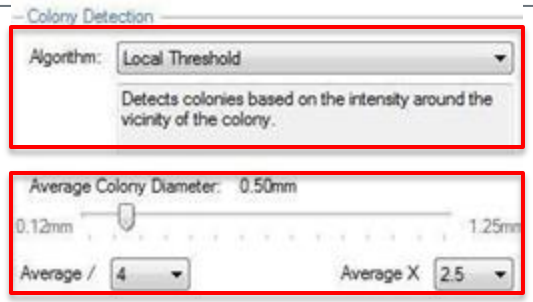
Commonly Used Imaging Statistics: Interior Mean Intensity

- 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



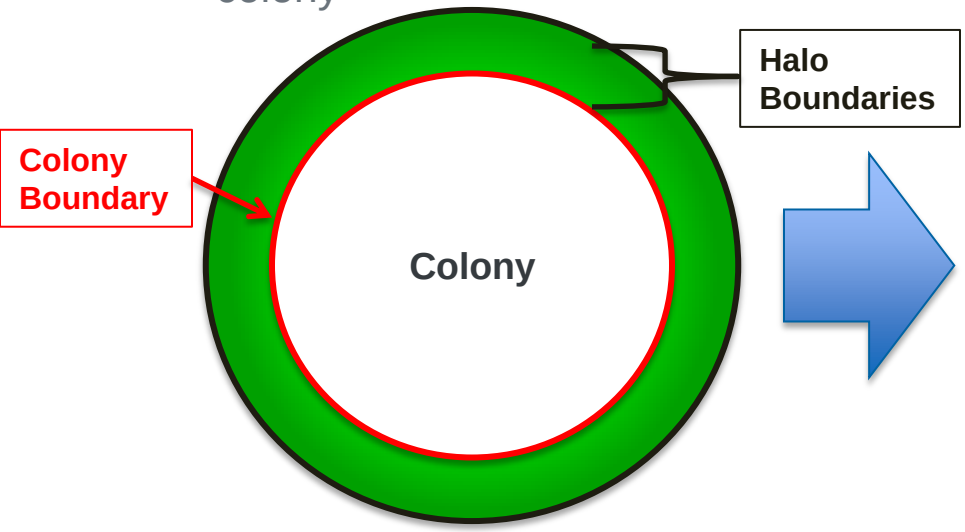
* ***Colony boundaries*** *are determined by the* ***Colony Detection criteria*** *(****Algorithm & Average Colony Diameter settings****) that you set using the white light (TransWL) image of your sample during the* ***Imaging & Results Review Steps*** *of your* ***Pick Run****.*



Commonly Used Imaging Statistics: Exterior Mean Intensity

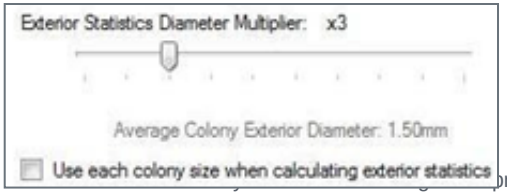
- While the **ClonePix 2 Clone Picking software** generates a broad array of **statistics**, generally only **4** of these are commonly utilized for **defining** your **colonies to pick**:

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



<u>Exterior Mean Intensity</u> = Sum Fluorescent Intensity of All Pixels/#Pixels Within Halo Boundaries Only

* ***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.*

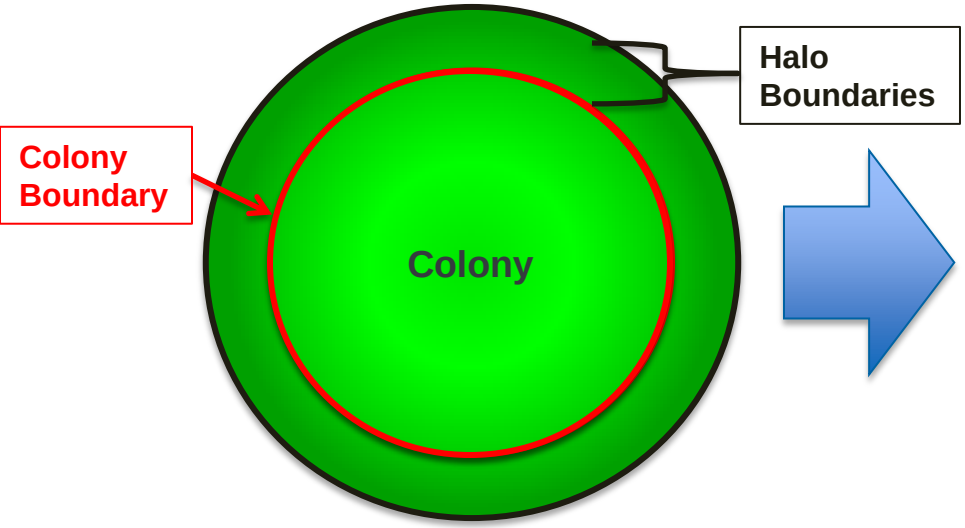


procedures.

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) <u>Sum Total Intensity:</u> The sum total intensity of the selected fluorophore within the colony + halo (interior and exterior)



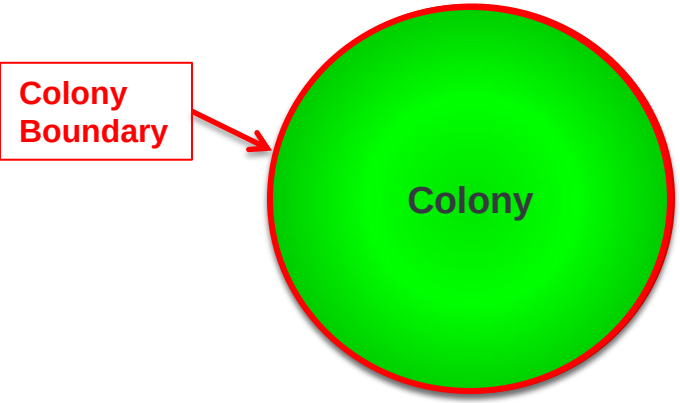
<u>Sum Total Intensity</u> = Sum Fluorescent Intensity of All Pixels/#Pixels Within the Colony Boundary + Halo Boundaries

- *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**:

4) **Normalized Intensity:** The sum total intensity of the selected fluorophore within the colony divided by the colony area.



Normalized Intensity = Sum Fluorescent Intensity of All Pixels Within Colony Boundaries/Colony Area (mm^2)

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

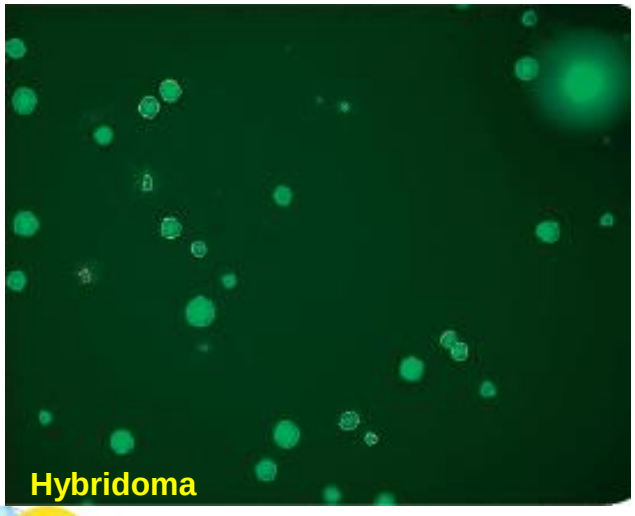
$$A = \pi r^2$$

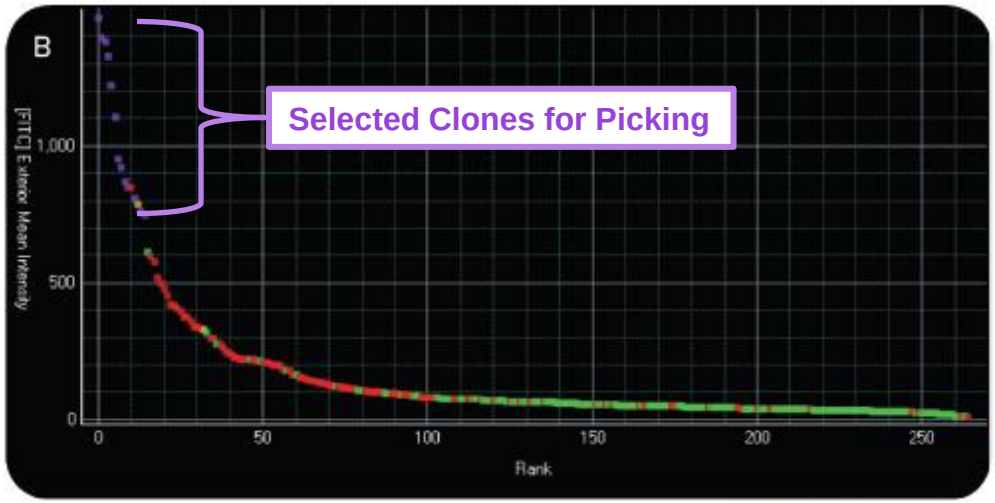
.

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) **<u>Application</u>**:

  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).

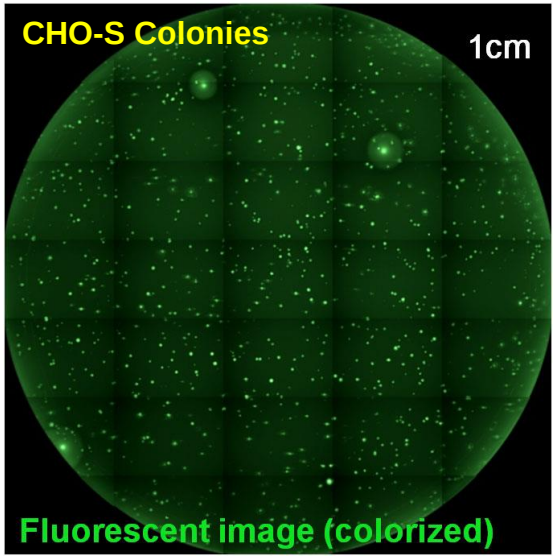
Which Statistic(s) Do I Choose for Defining Picking Criteria?

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

  2) **<u>Staining Pattern</u>**:

  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.*

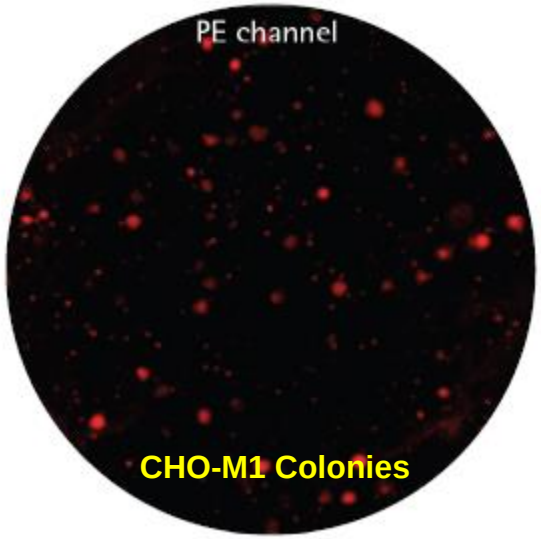
Which Statistic(s) Do I Choose for Defining Picking Criteria?

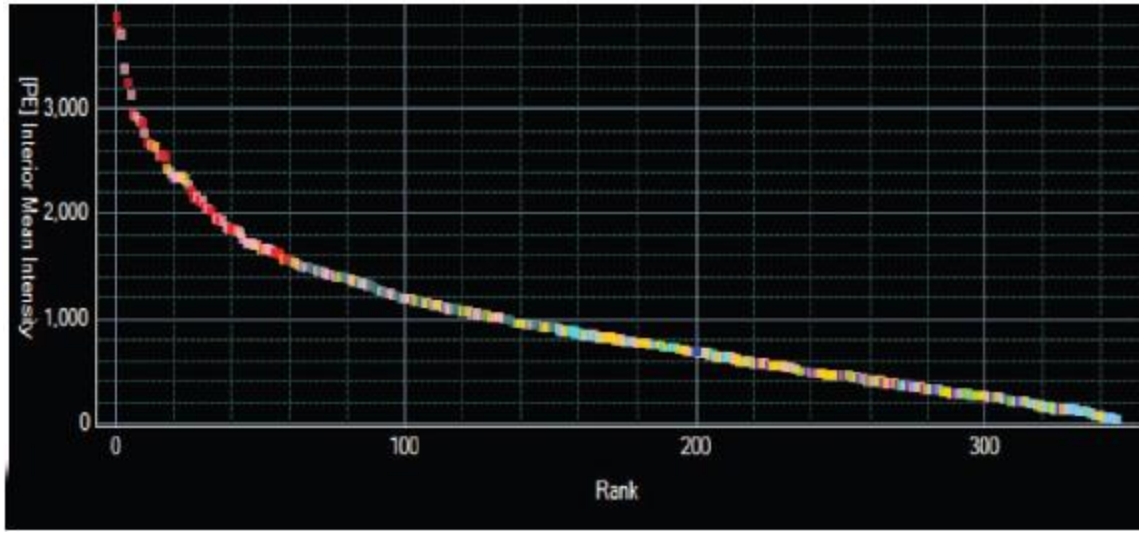
- **Three** main factors will dictate your best choice of **statistic(s)** to define your **criteria** for **colony picking**:

  2) <u>**Staining Pattern**</u>:

     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

MOLECULAR DEVICES

ADVANCING PROTEIN AND CELL BIOLOGY