

# Unmixing Fundamentals: It’s not magic!

By David Rach, Ph.D.
UMGCCC Flow Cytometry Shared Resource

September 15th, 2026

Unmixing Fundamentals: It's not magic!
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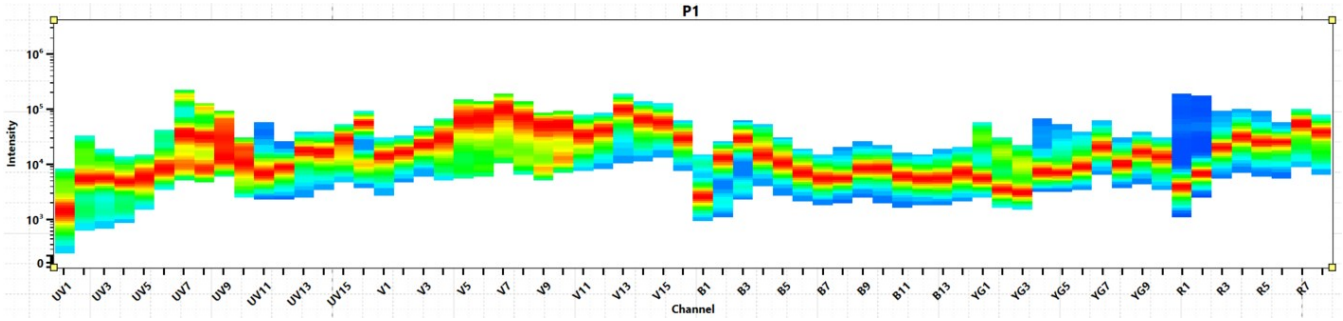
# A spectral cytometer needs three things for successful unmixing:

**Full-stained Samples**



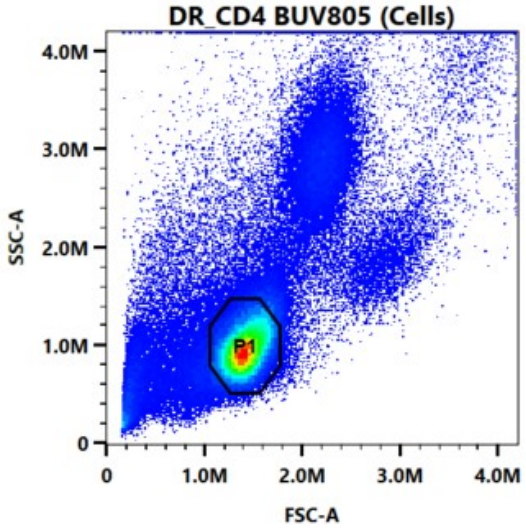

**Single-color Reference Controls**

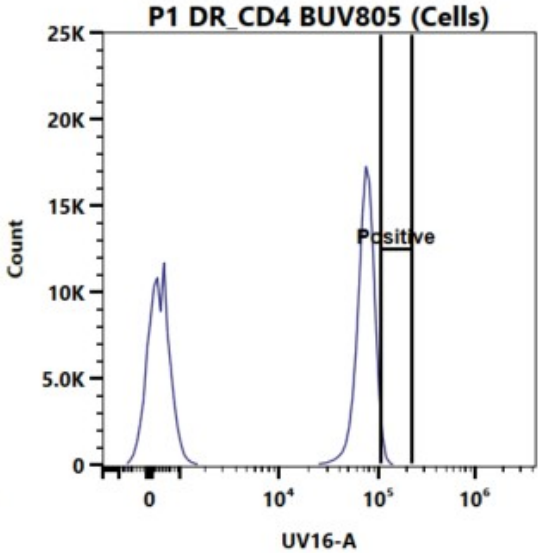
- Yellow Reference Signature
- Blue Reference Signature
- Red Reference Signature
- Violet Reference Signature
- Orange Reference Signature

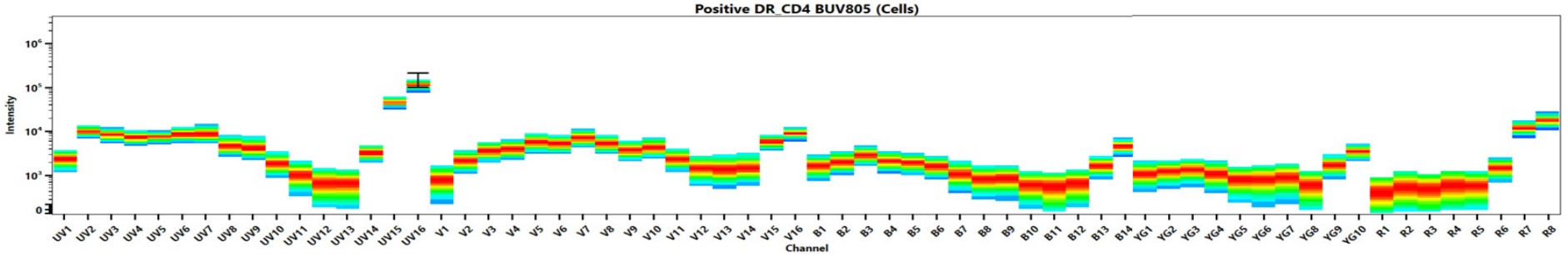
**Unstained Reference Controls**

- Autofluorescence Cell Subset #1
- Autofluorescence Cell Subset #2

# The Unmixing Screen:



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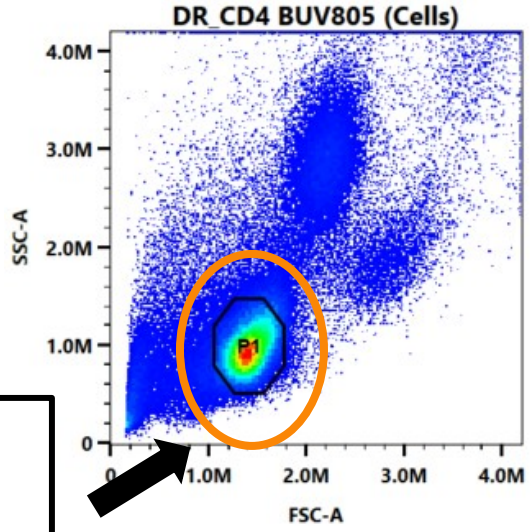
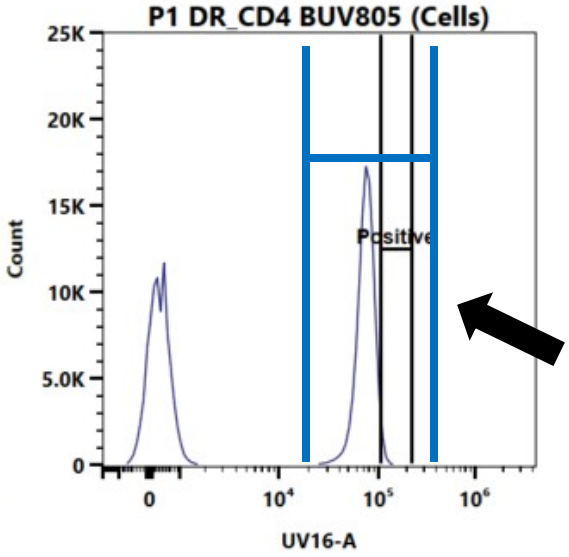
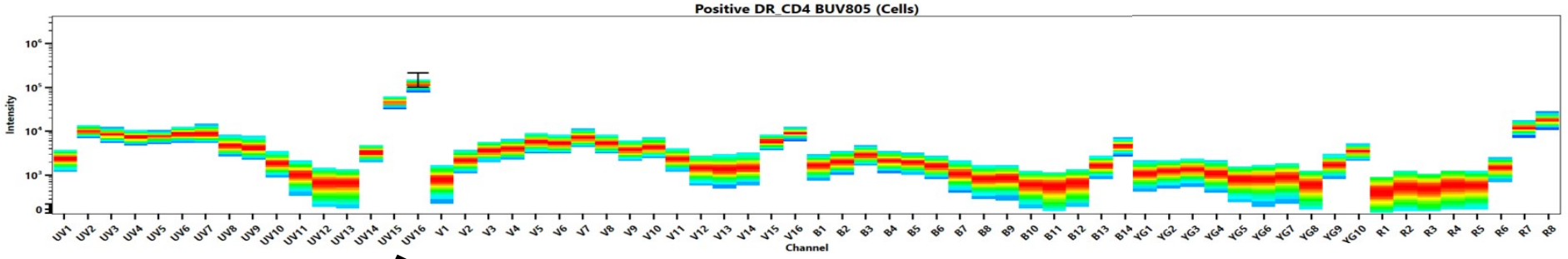



What if I gated like this?

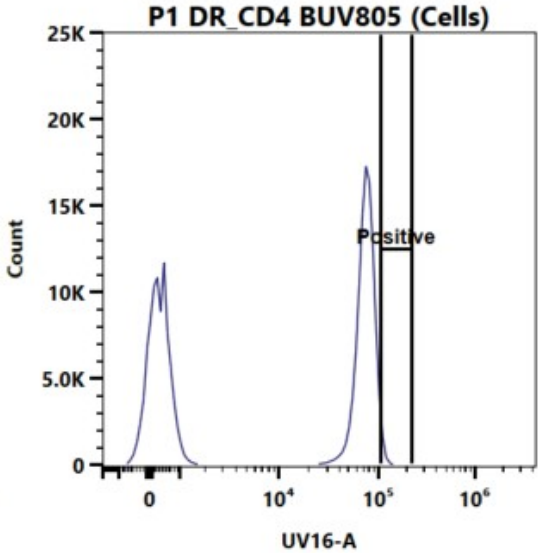



What if I grabbed the entire peak?

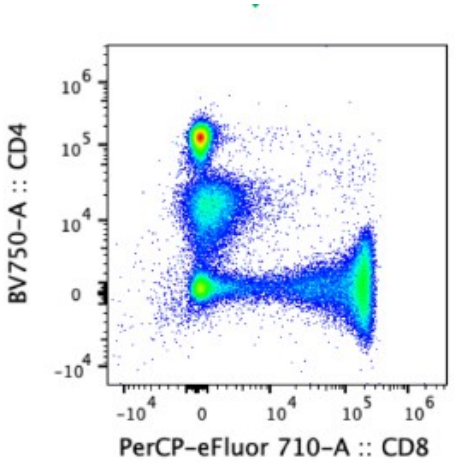



What does this even do again!?!?!? What if I set it on the adjacent detector?

# Rationale:





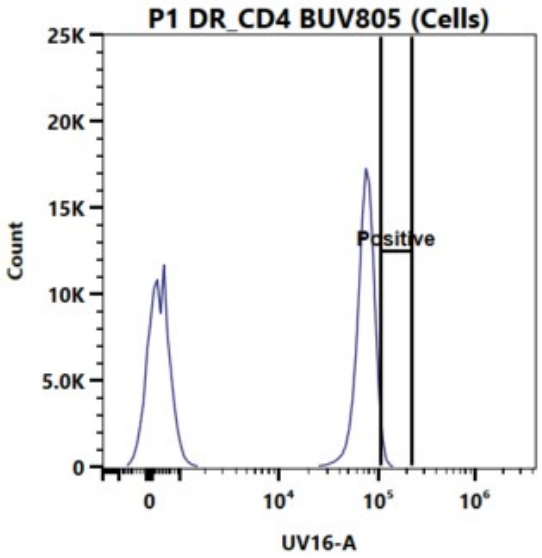

1) Raw Data

2) ???

*Something something least squares*

3) Unmixed Data

# Rationale:

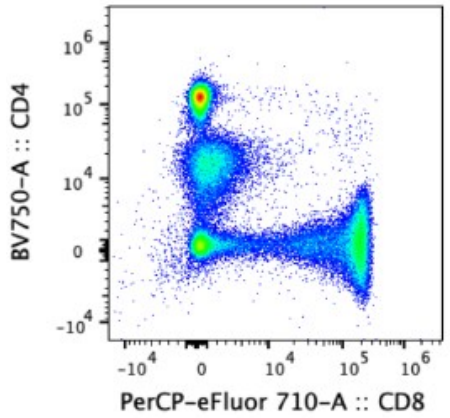


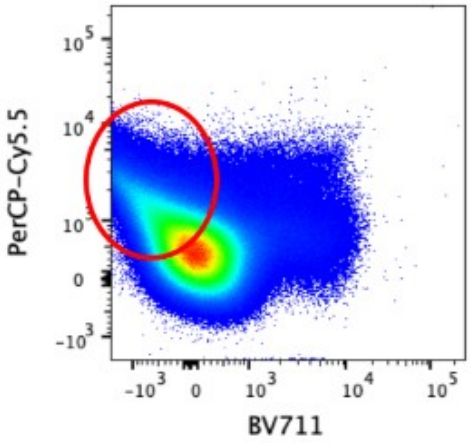

1) Raw Data



2) ???

*Something something least squares*



# Goals:

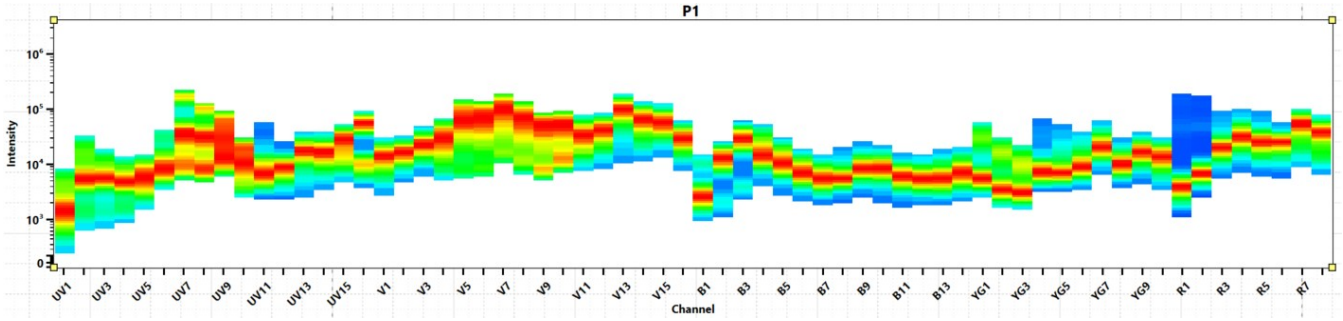


- Provide conceptual understanding
- Demystify some of underlying math
- Help build your own mental model to better troubleshoot issues you may encounter in your own unmixing adventures

Generated by David Rach with Bard, a large language model from Google AI.
Prompt: Computer thinking about how to draw a human.

# A spectral cytometer needs three things for successful unmixing:

**Full-stained Samples**

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**Unstained Reference Controls**

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# Let’s go back to the beginning



Generated by David Rach with Bard, a large language model from Google AI.
Prompt: Lego person jumping into the Back to the Future delorean.

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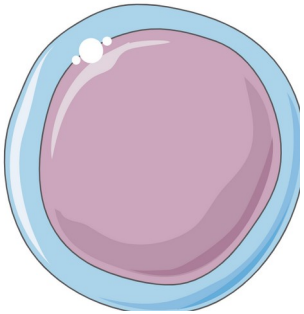
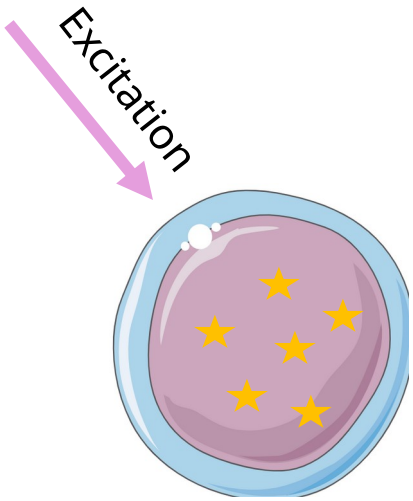
# *In our tube, we have a cell*



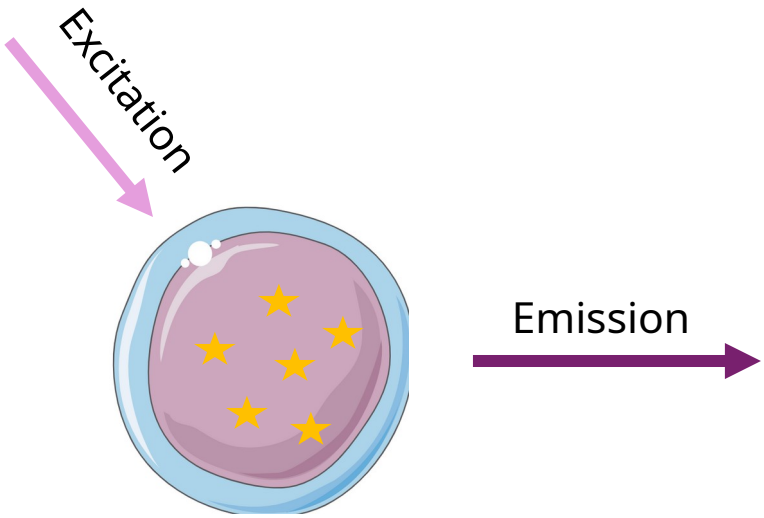
# *With some autofluorescent molecules*



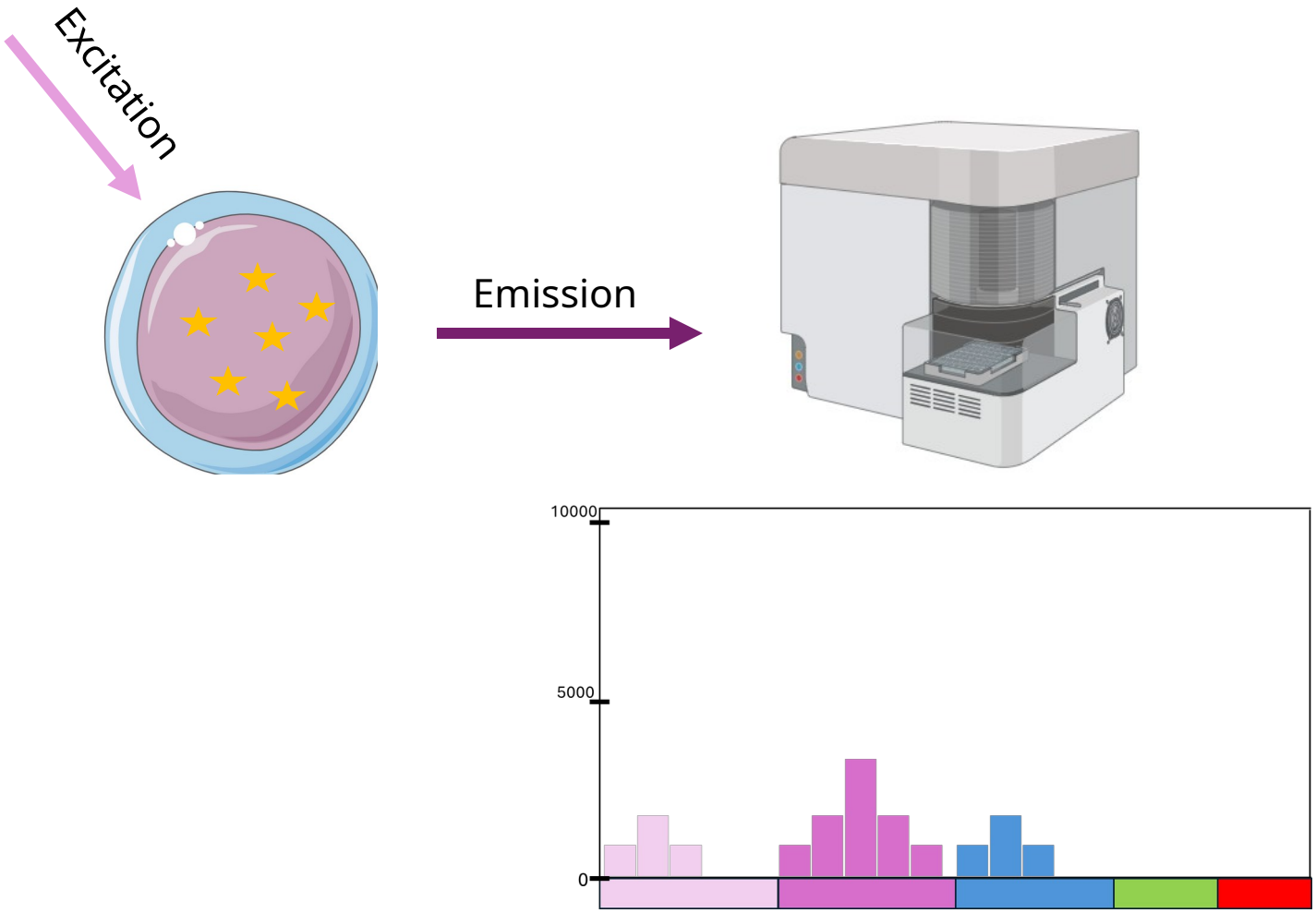
# *Our Aurora shines a laser at it*



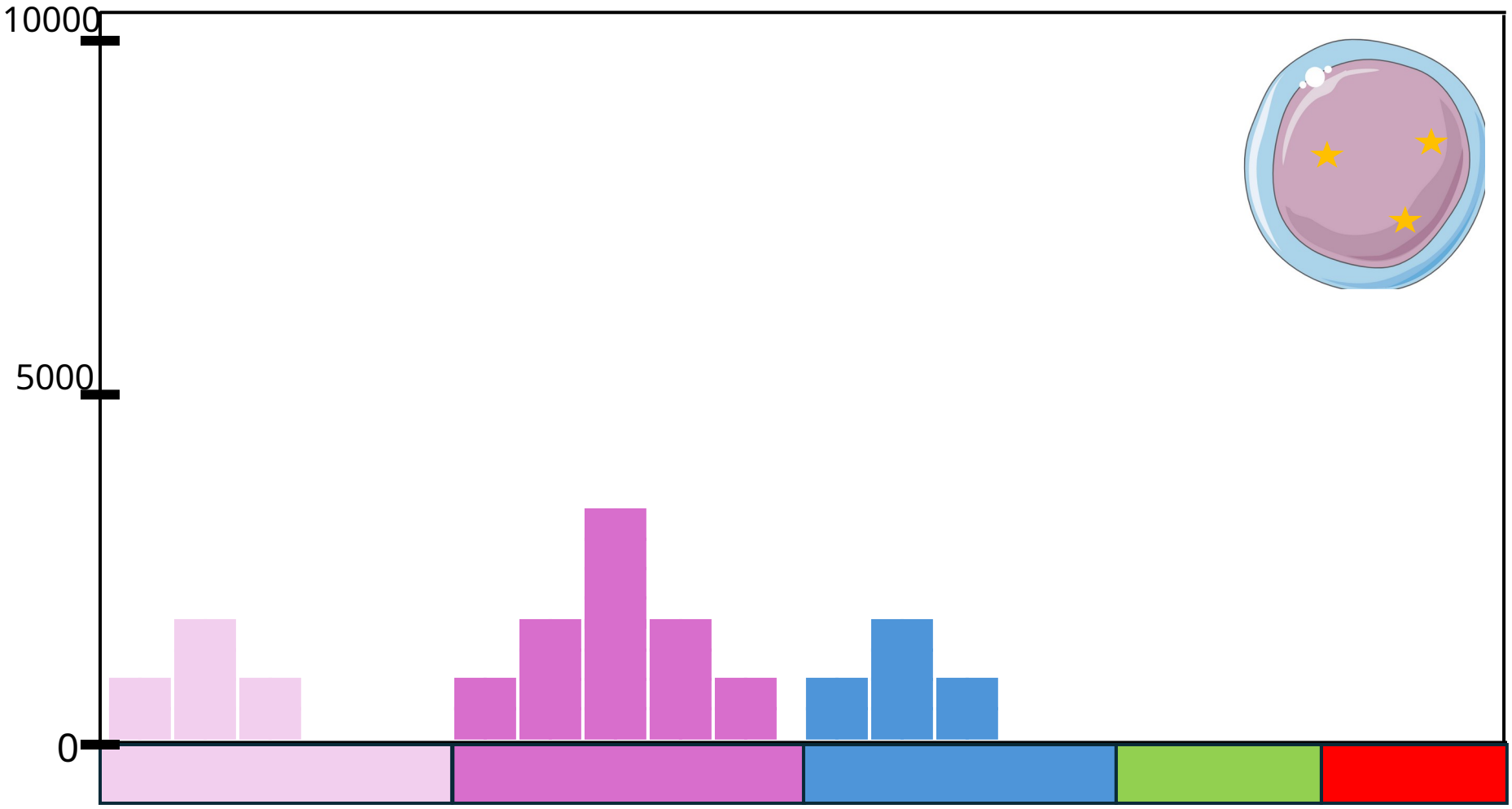
# *The cells molecules emit signal back*



# *Signal is captured across the various detectors*



# Raw Signal



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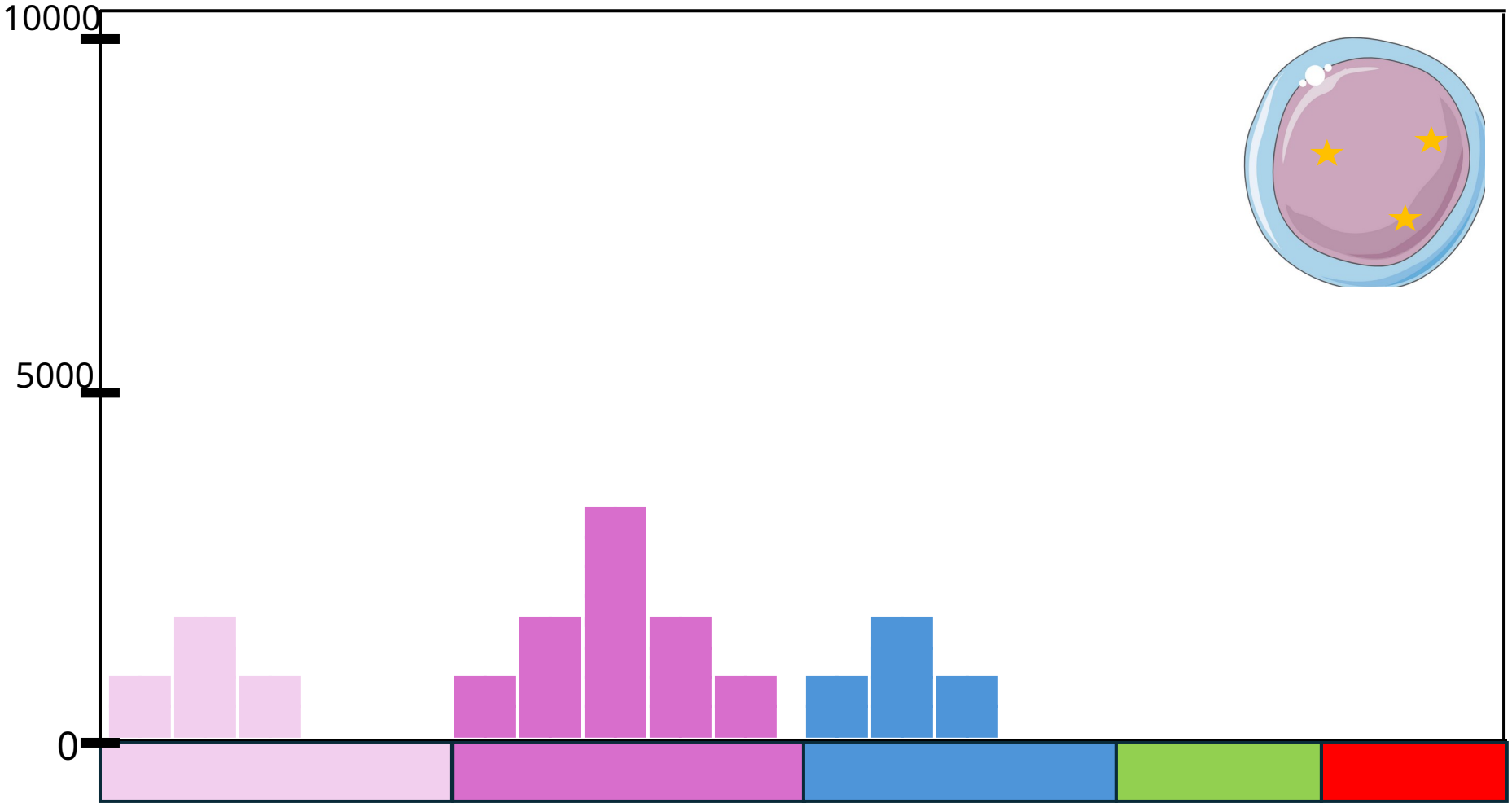


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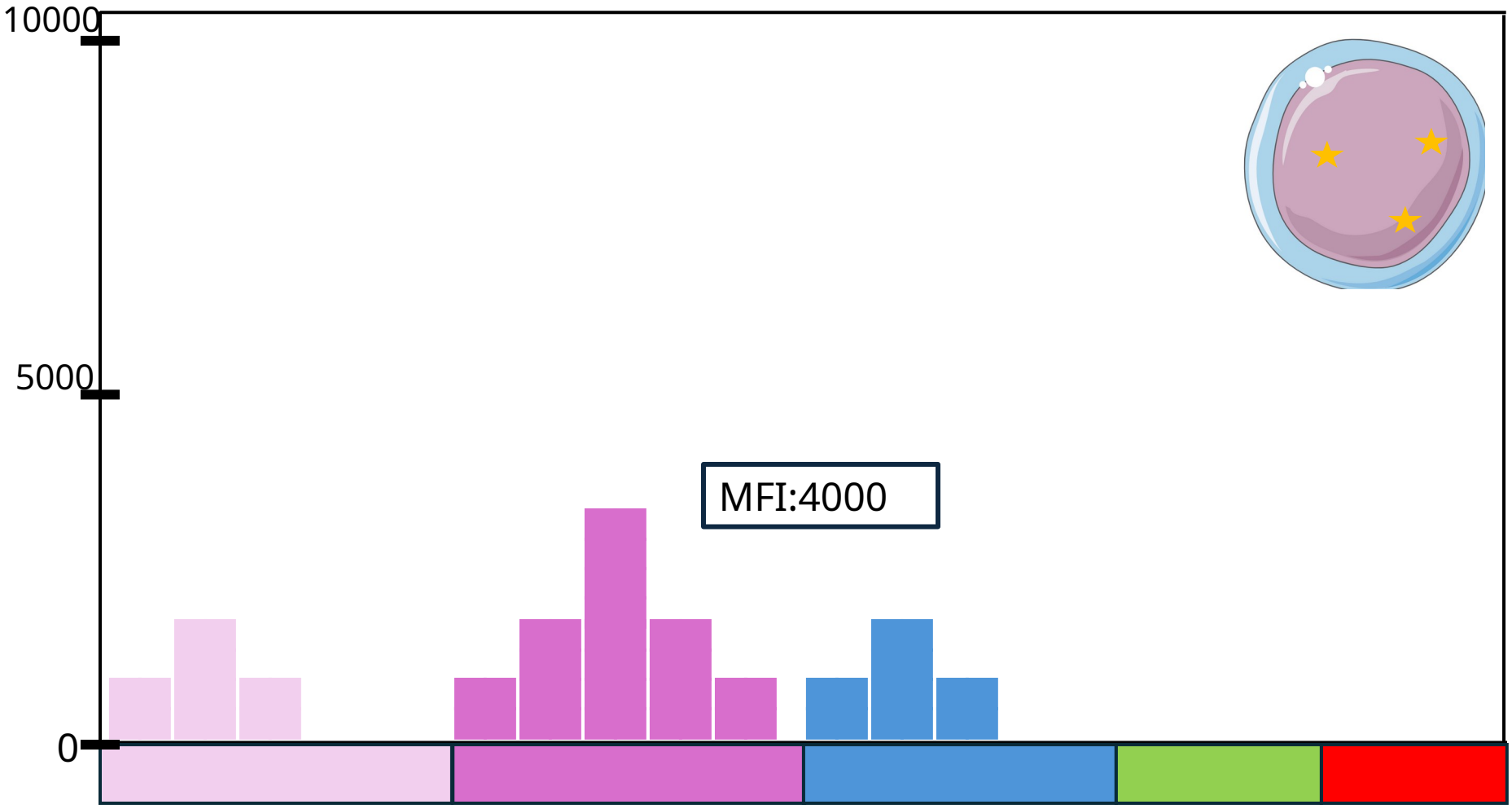
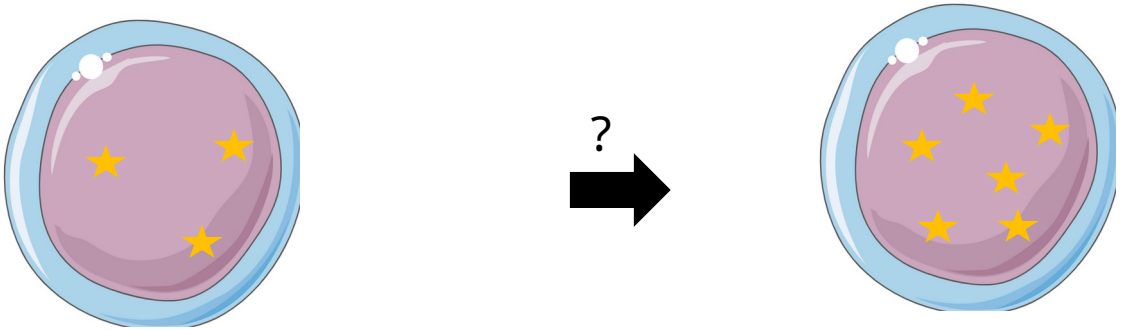
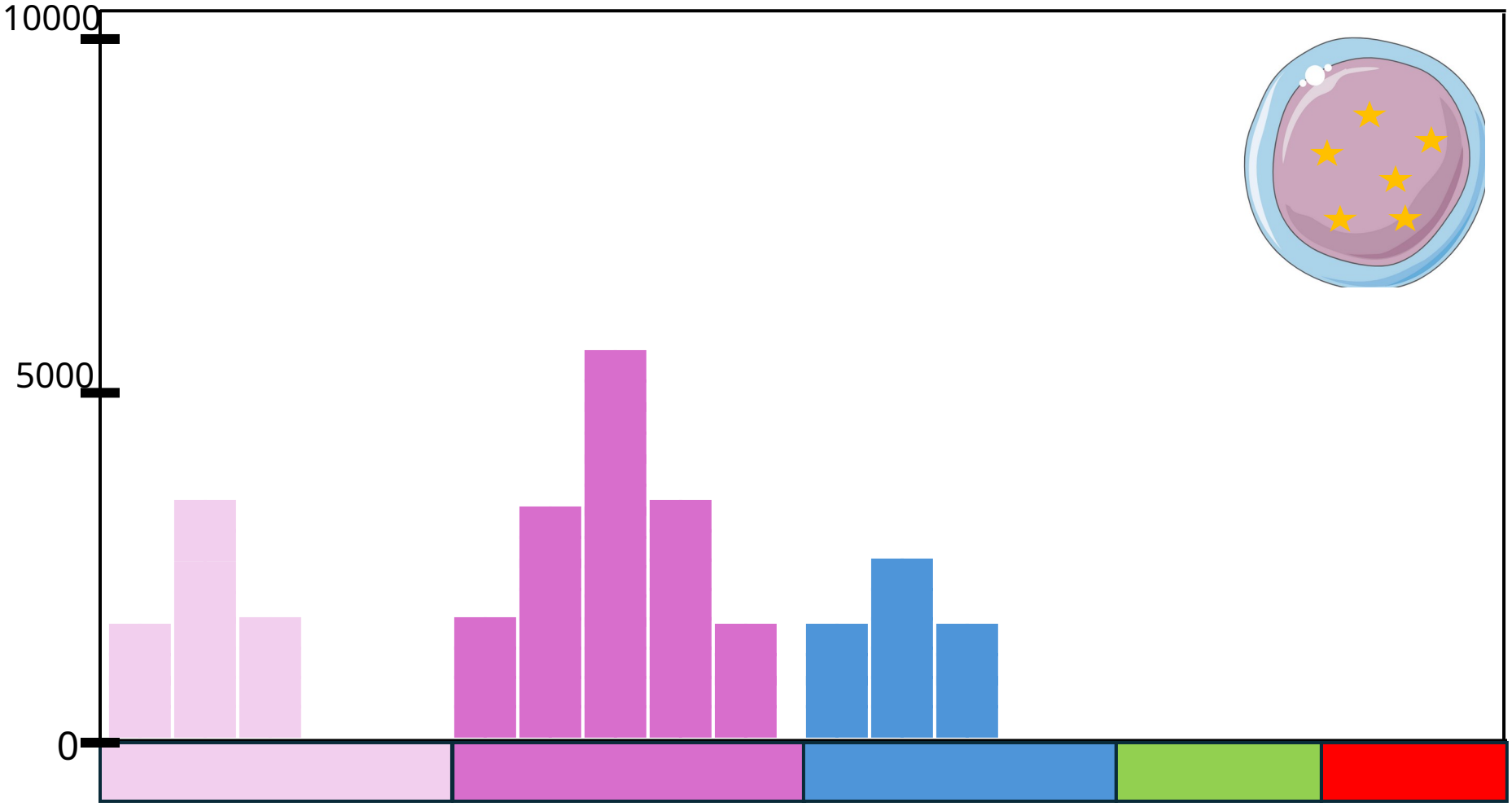
# Raw Signal



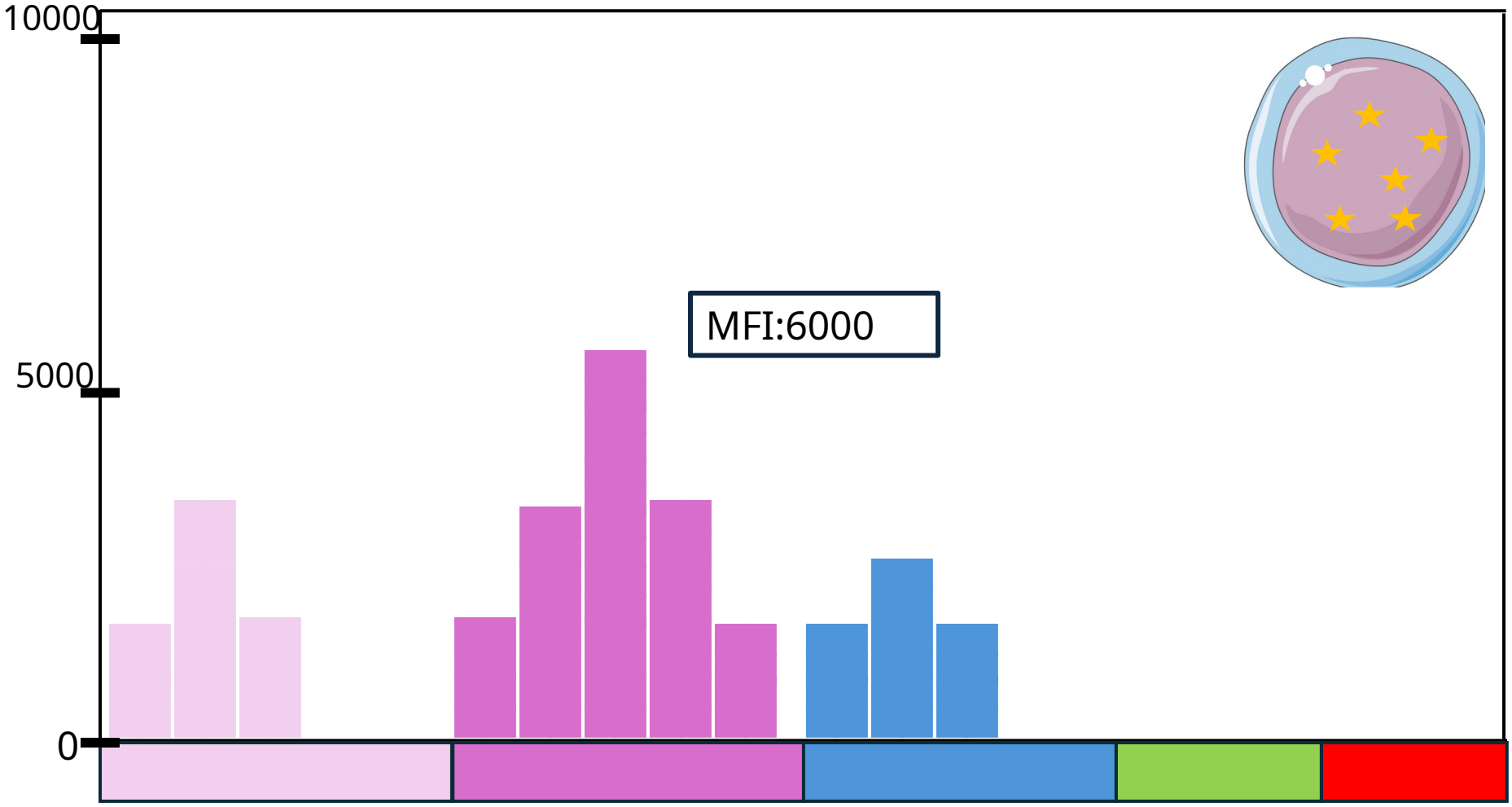
# *What if there were twice as many autofluorescent molecules?*



# *An increase in signal brightness*



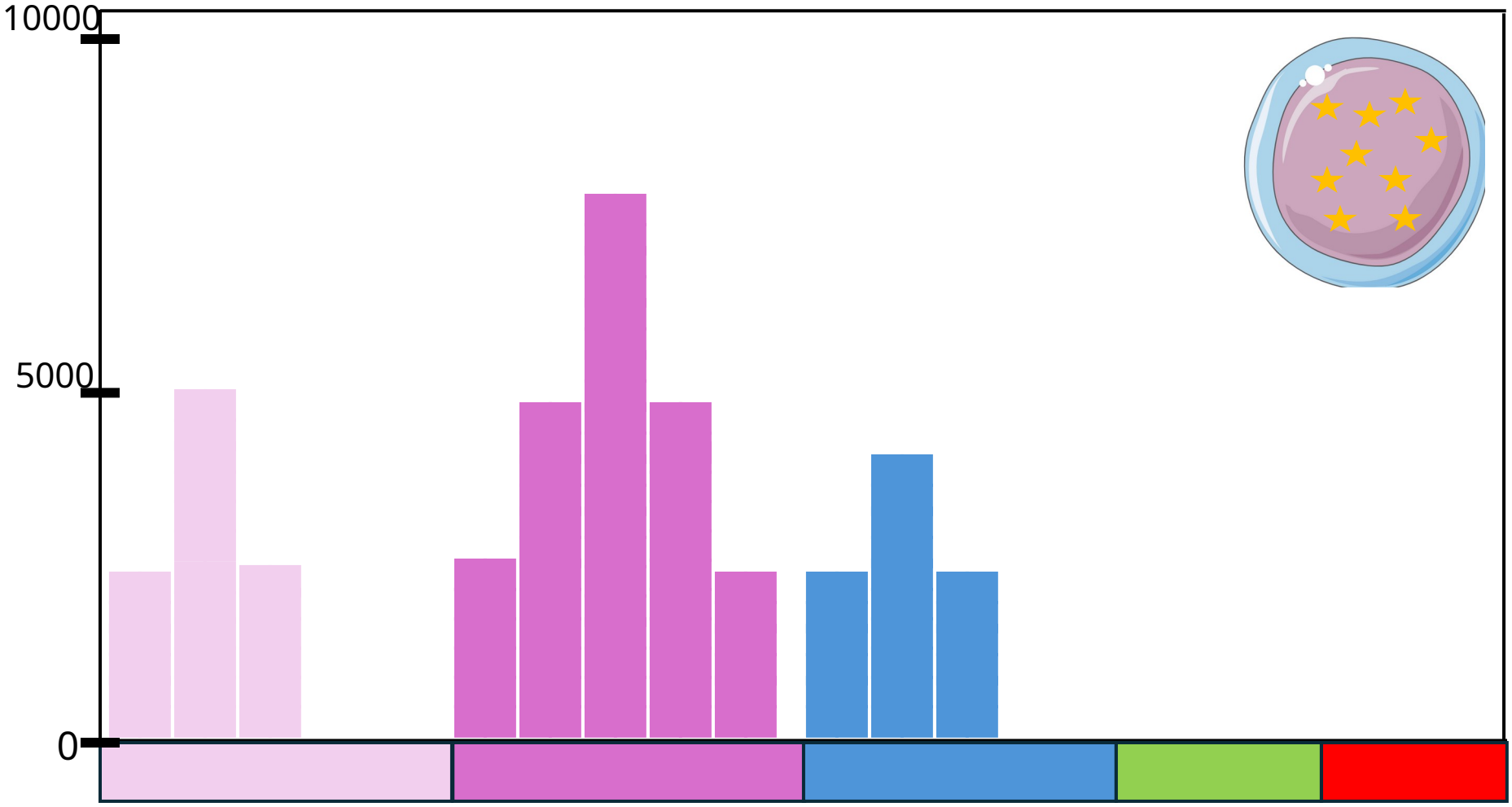
# *An increase in signal brightness*



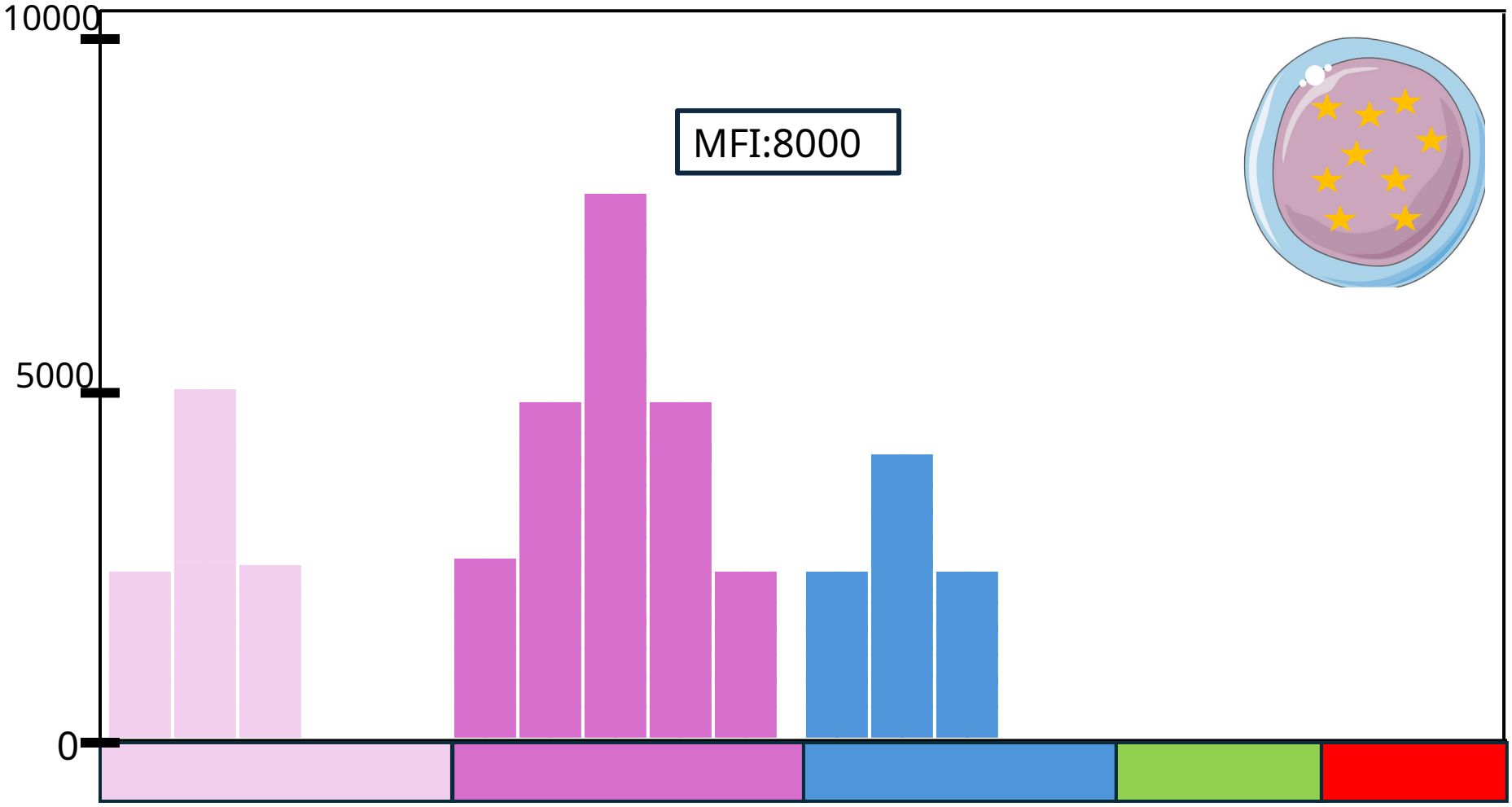
# *What if we increased the autofluorescent molecules again?!?*



# *An even brighter signal!*



# *An even brighter signal!*



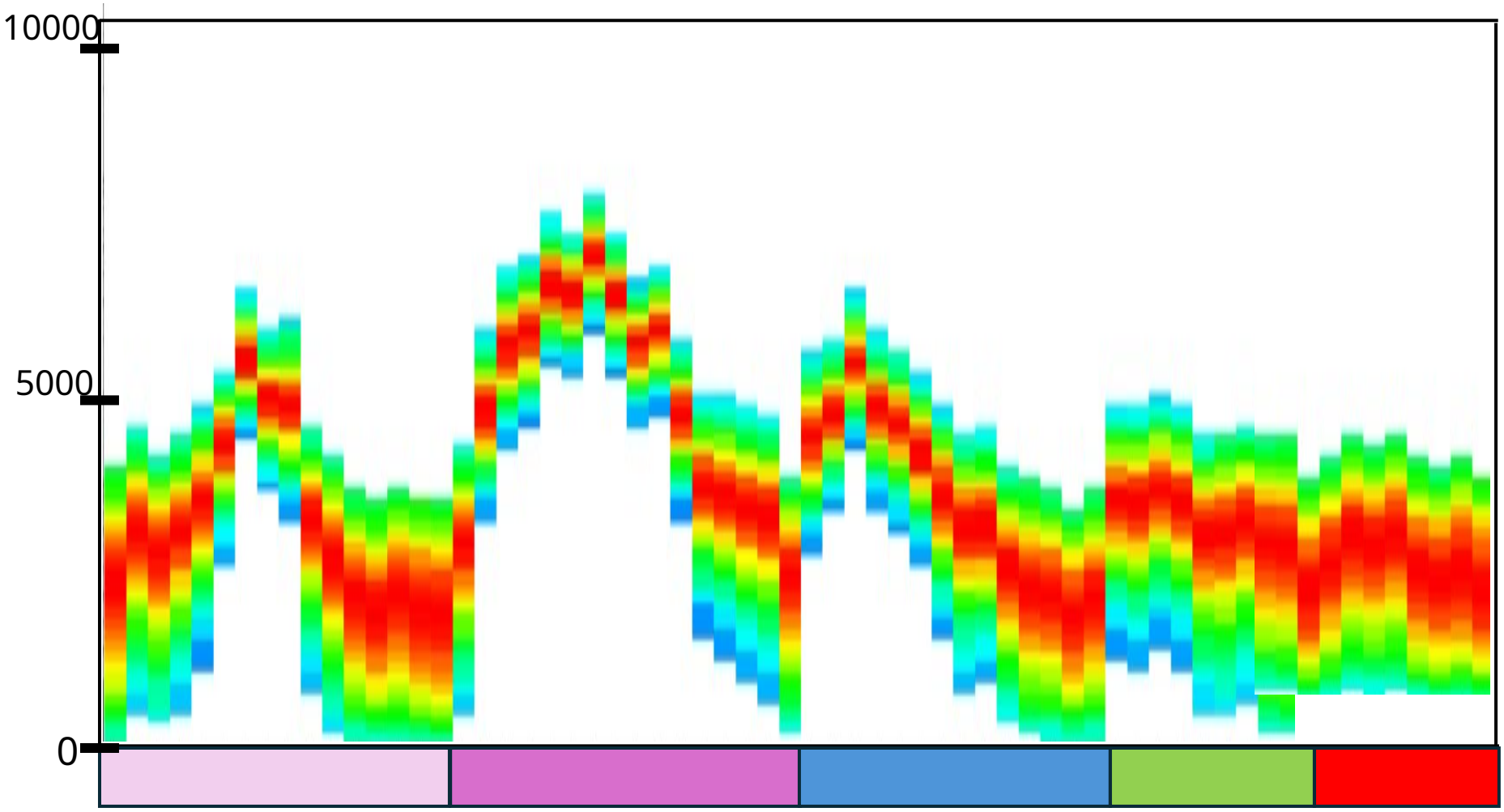
# First essential idea: Brightness



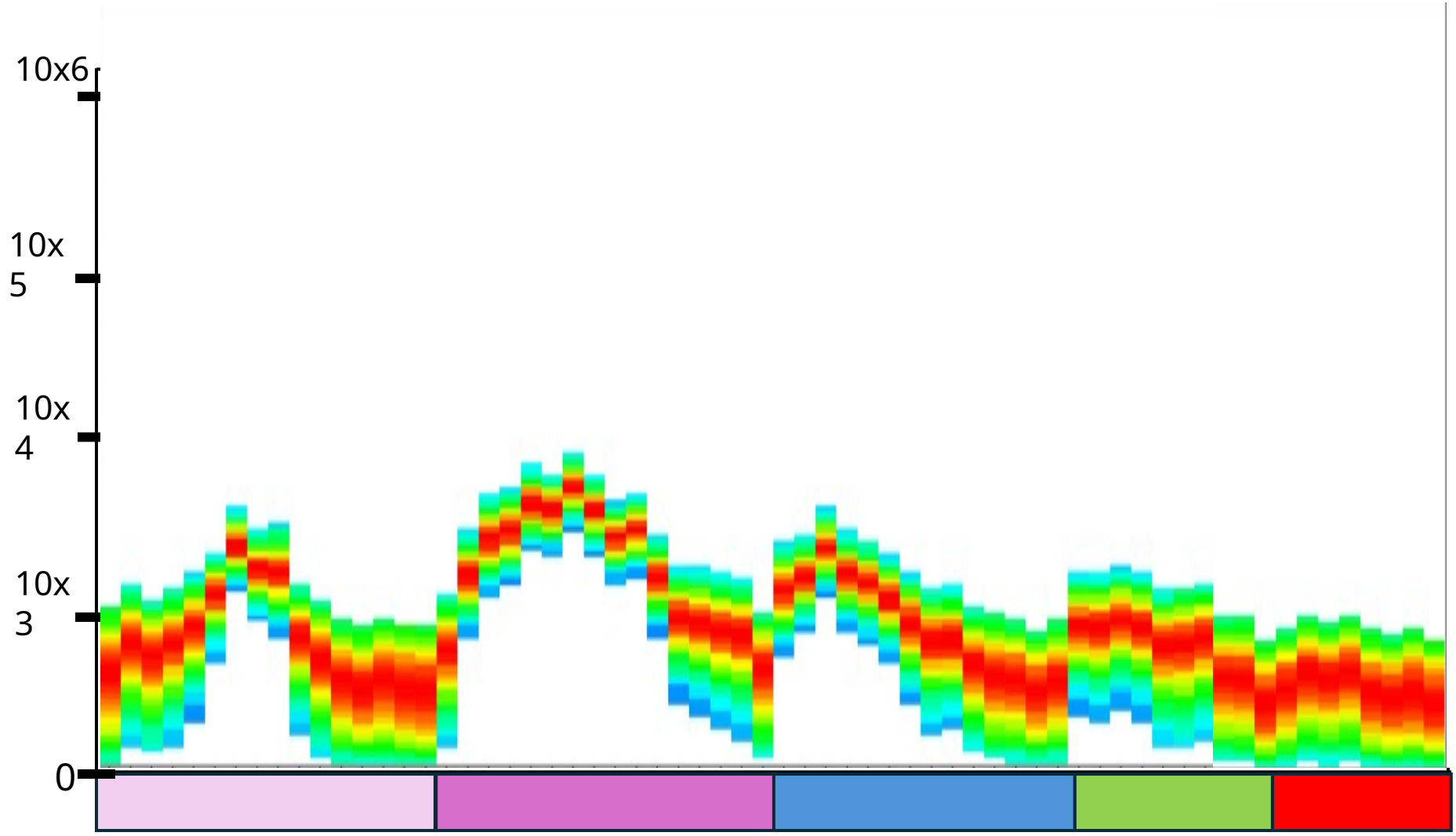
# *As we record more cells*



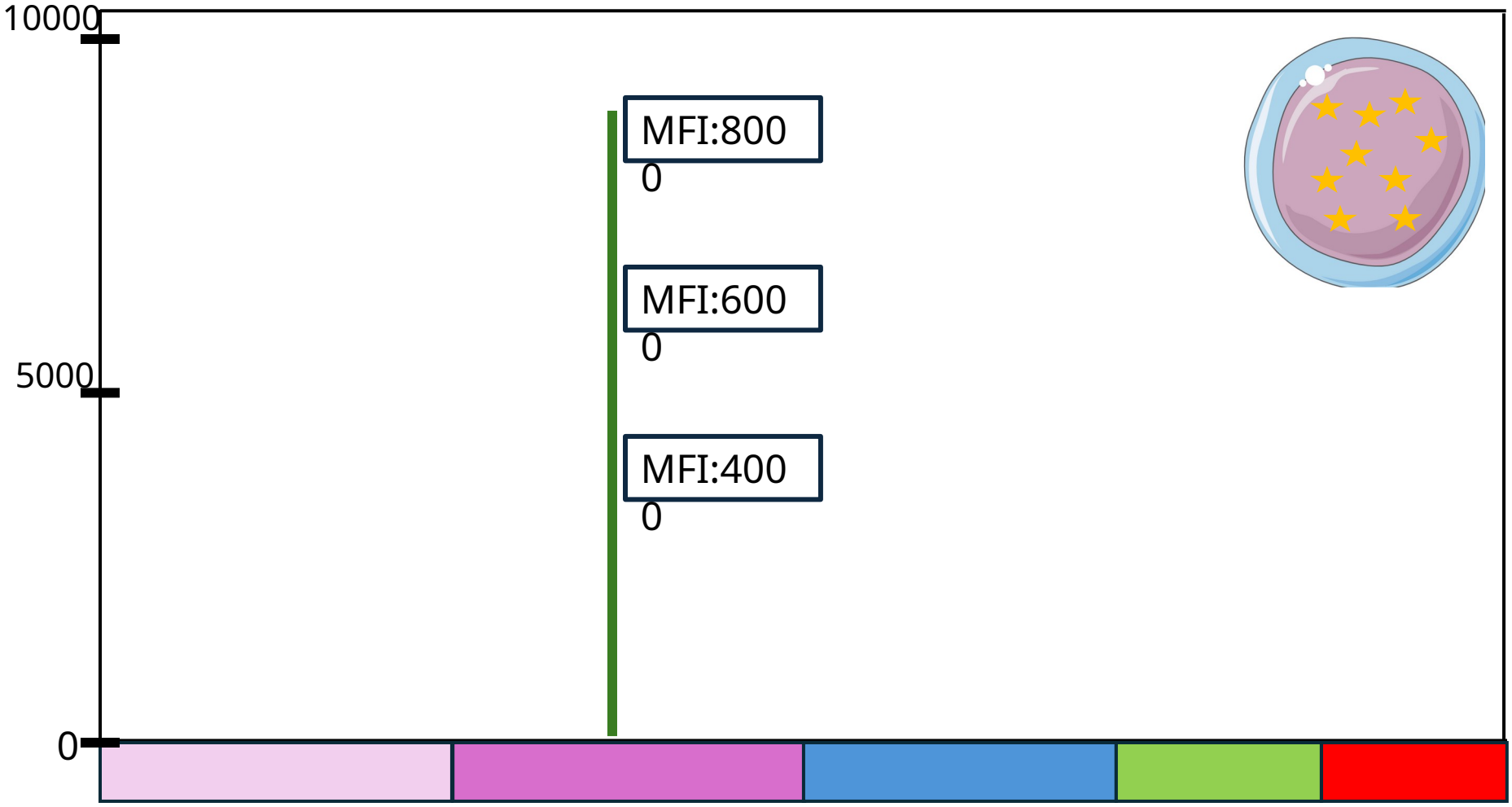
# Accounting for density



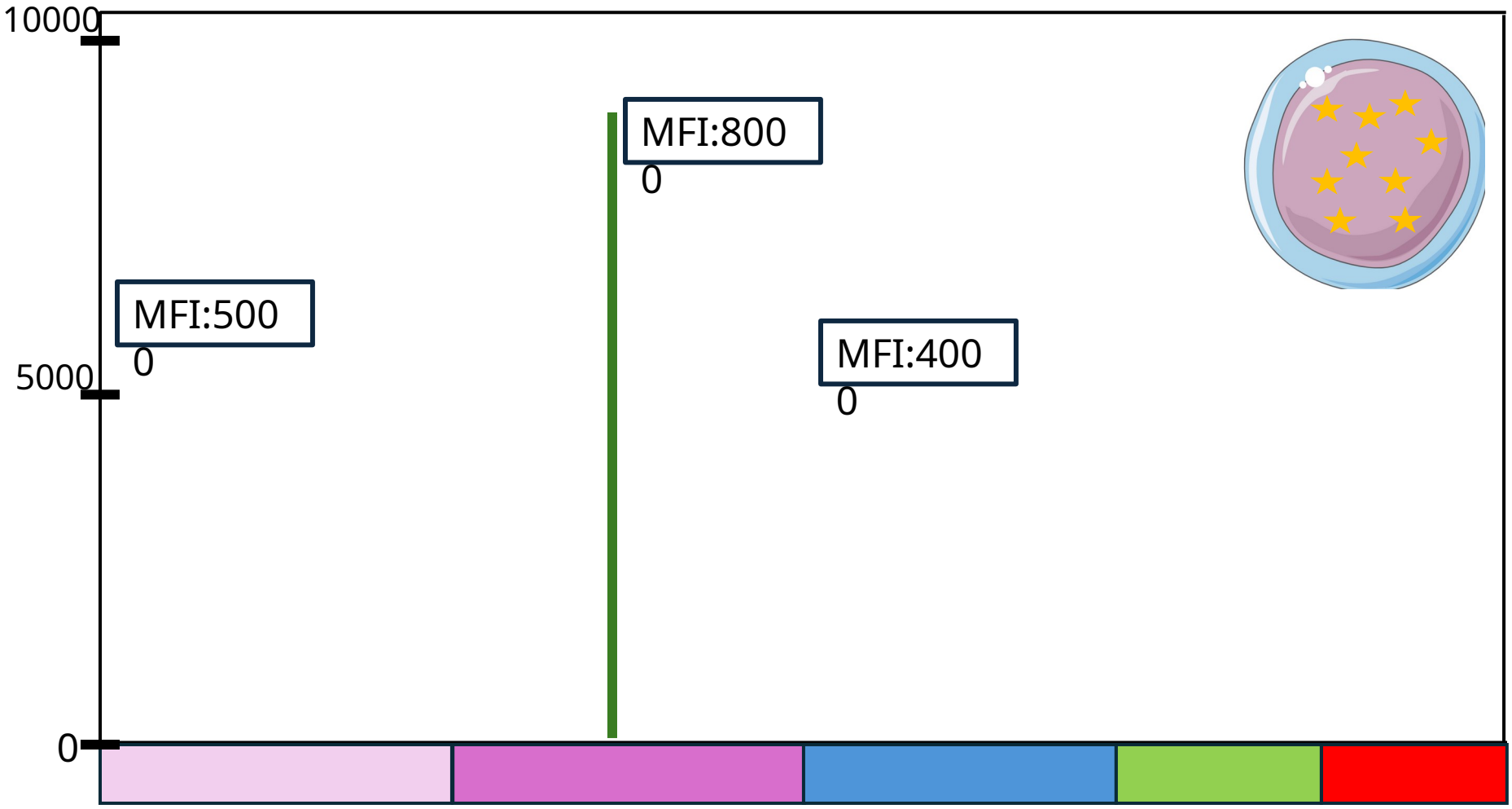
# And transforming to a logarithmic axis



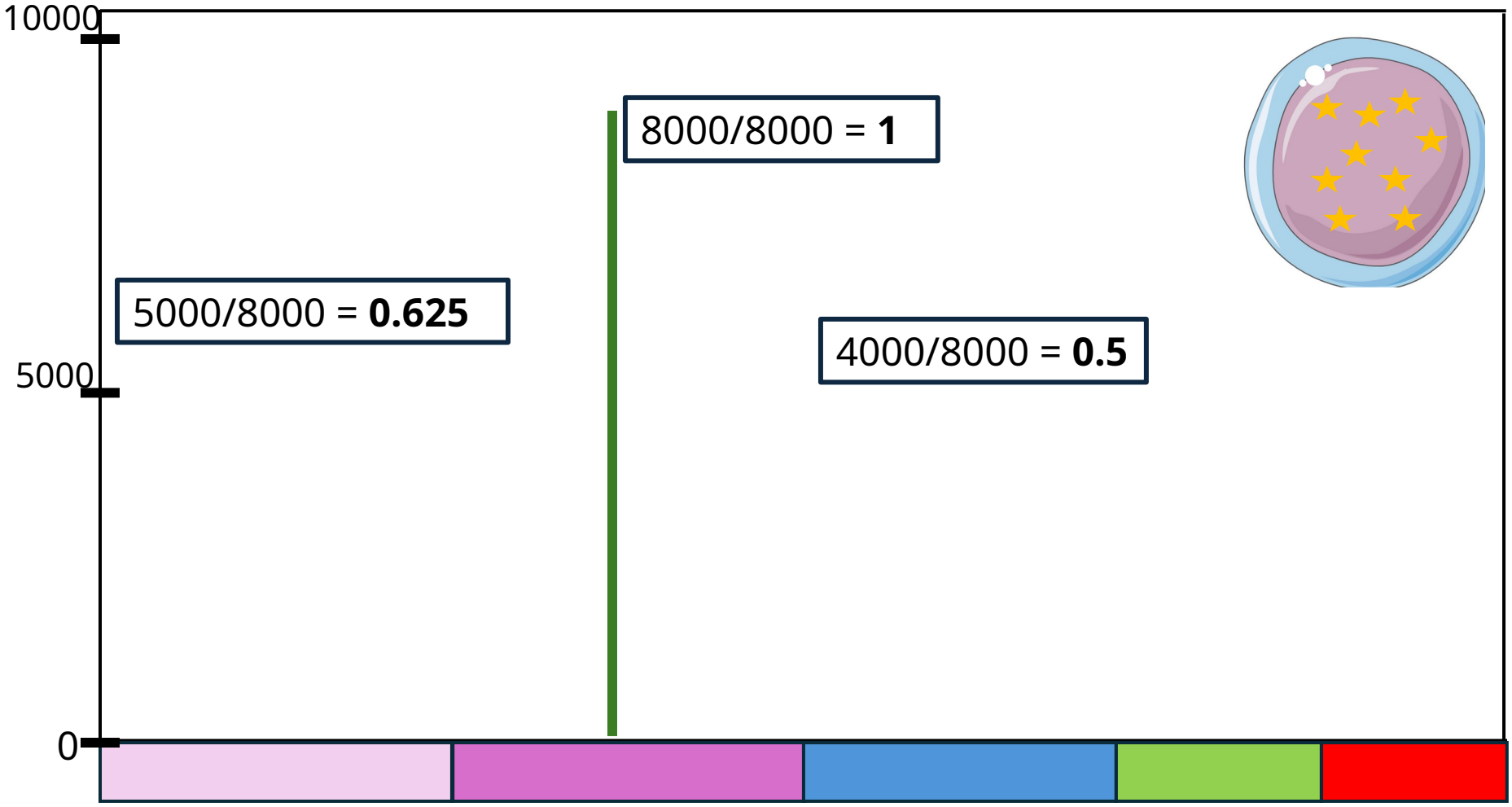
# Different brightness, but are they the same color?



# Normalizing/Scaling by Peak Detector



# Divide each detectors measurement by the peak detectors measurement



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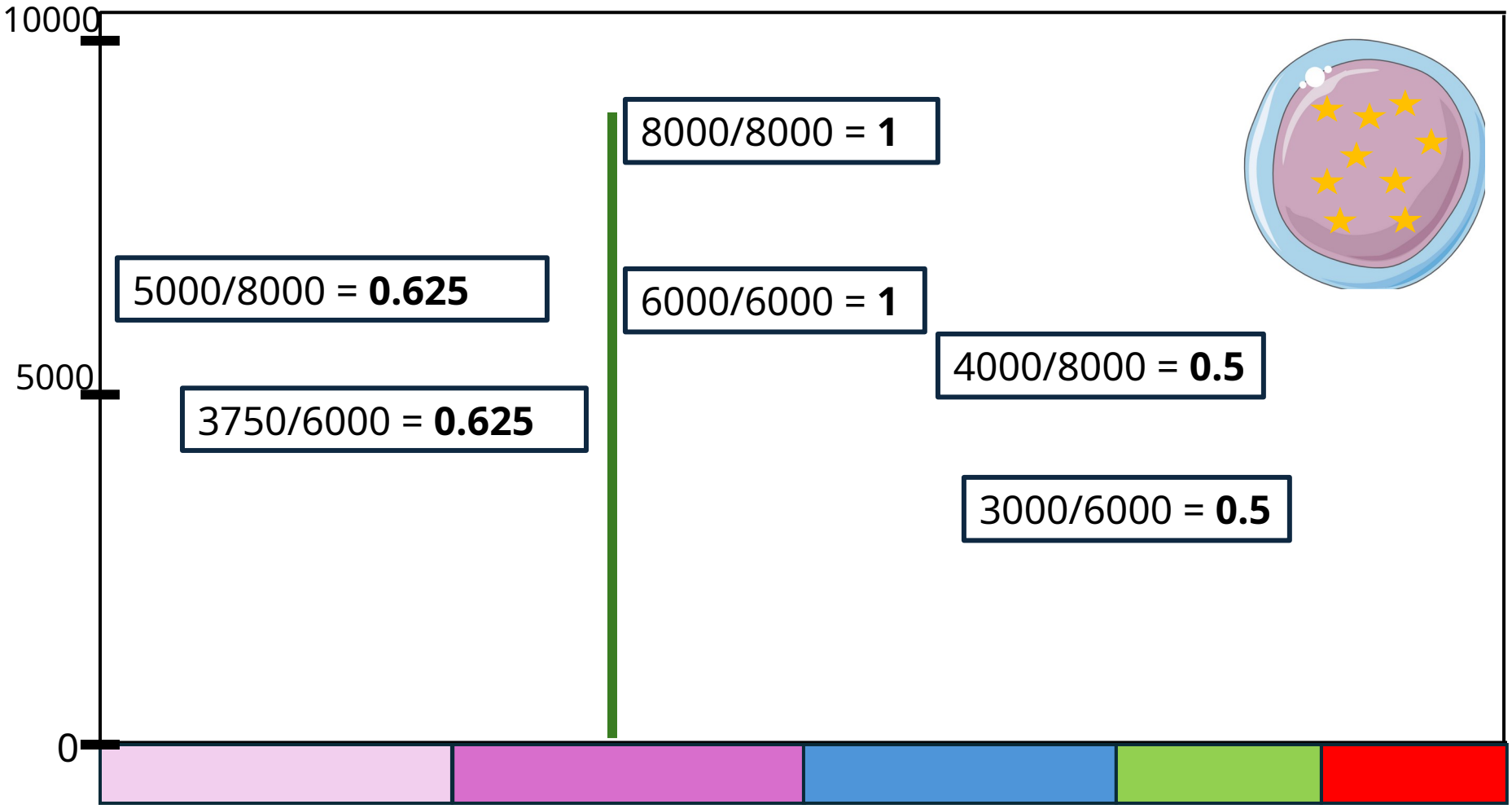
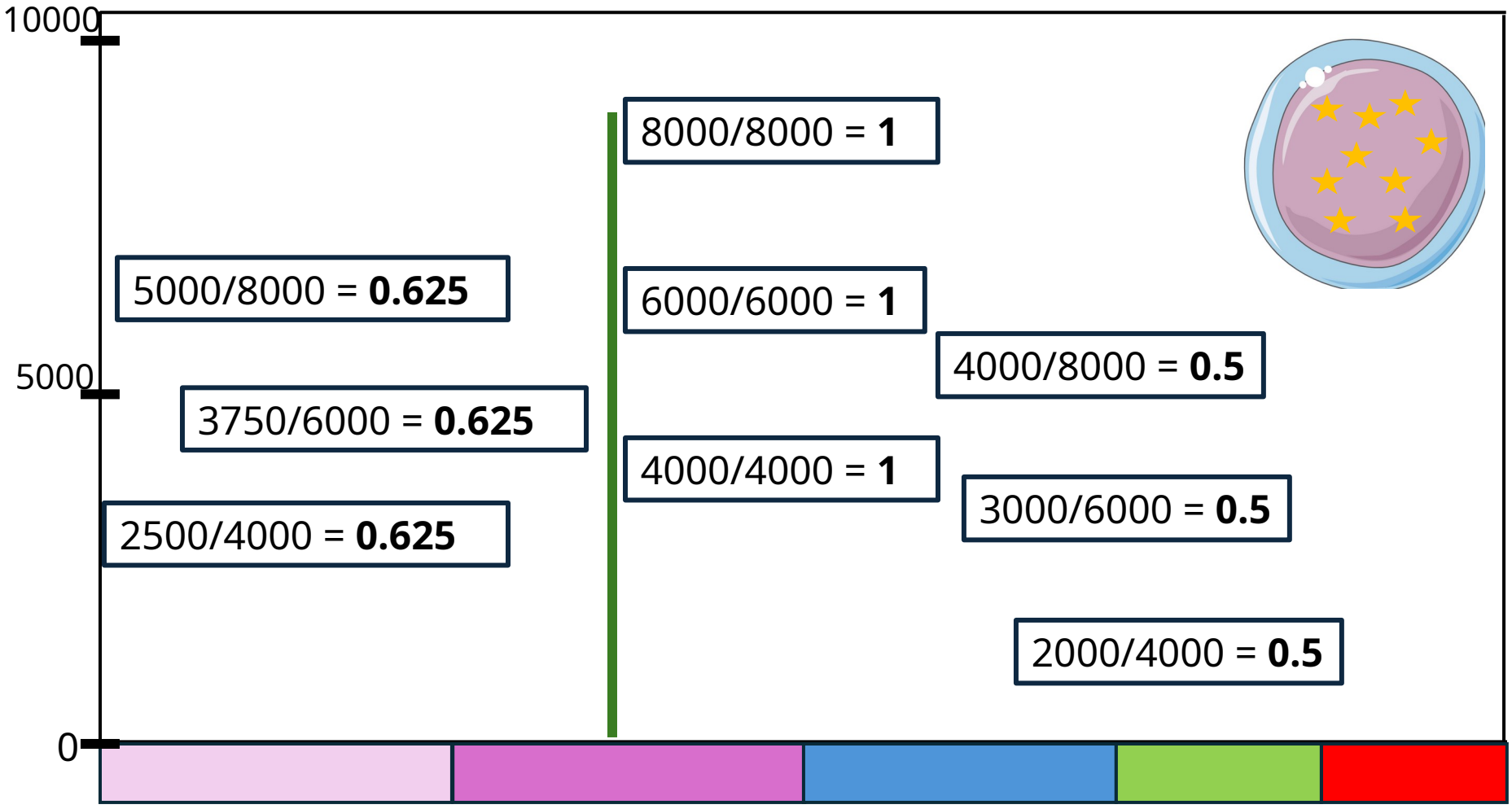


# Return the same constants



# Second essential idea: Signature



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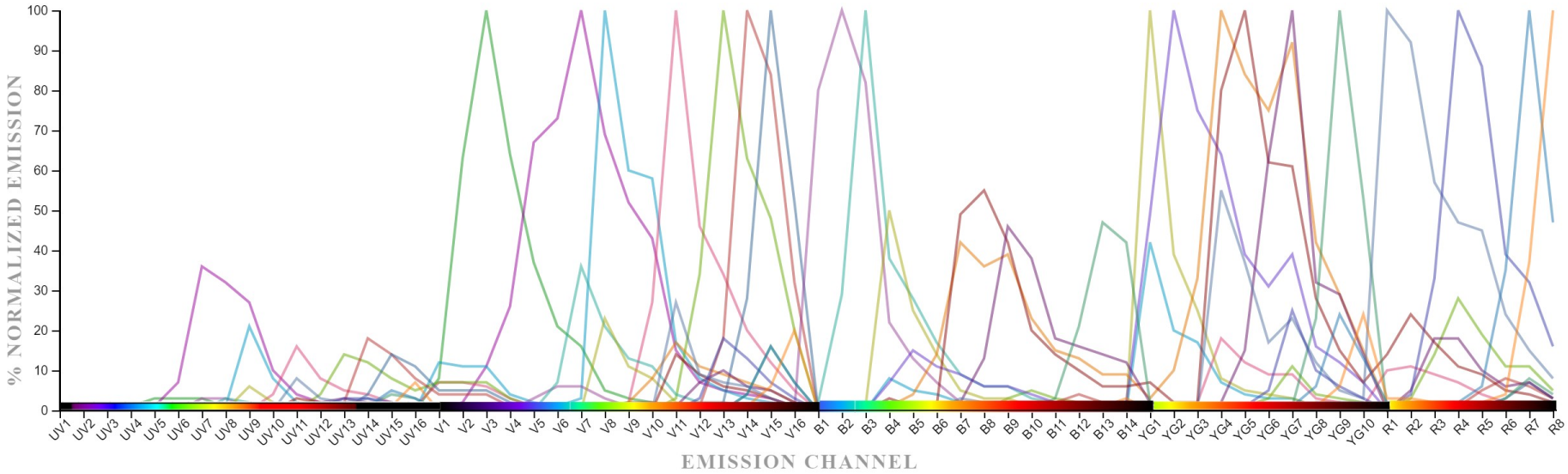
# Similar with Fluorophores (APC)



# Similar with Fluorophores (APC)

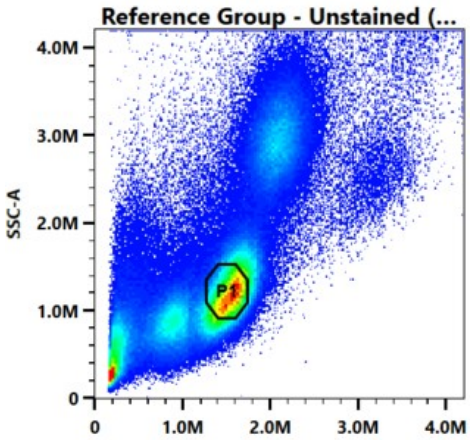


# Each Fluorophore has its own characteristic signature

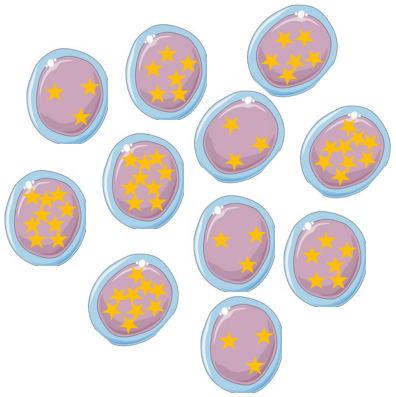



| | | | | | | |
|---|---|---|---|---|---|---|
| Pacific Blue | BV510 | BV570 | BV650 | BV711 | BV750 | BV785 |
| FITC | Spark Blue™ 550 | PE | Spark YG™ 593 | 7-AAD | PE-Cy™5 | PE-Fire 700 |
| PE-Cy™7 | APC | Alexa Fluor® 700 | APC-Cy™7 | APC-Fire 810 | | |

# *When we gate for Unstained Reference Control*




The gate we draw



Selects some cells

**Unstained Reference Controls**

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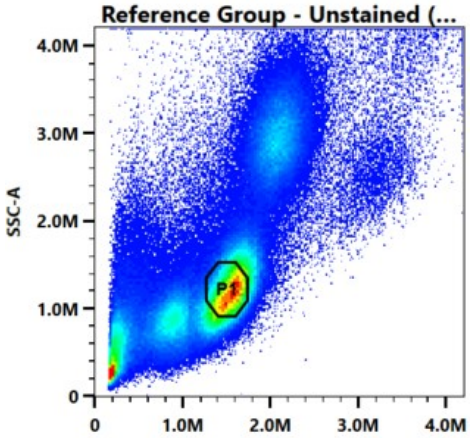


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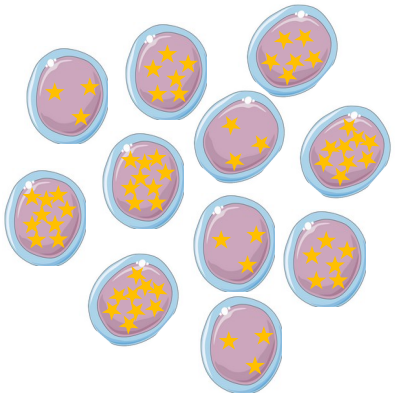
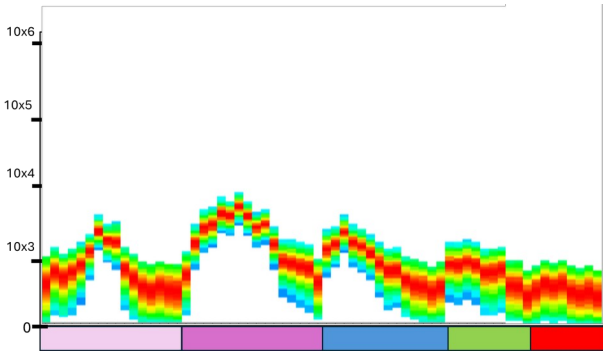


Selects some cells




The median measurement per detector is taken

**Unstained Reference Controls**

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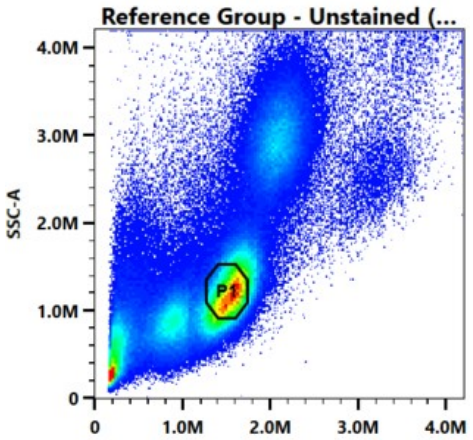


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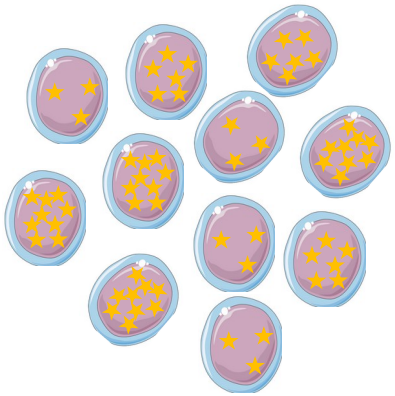

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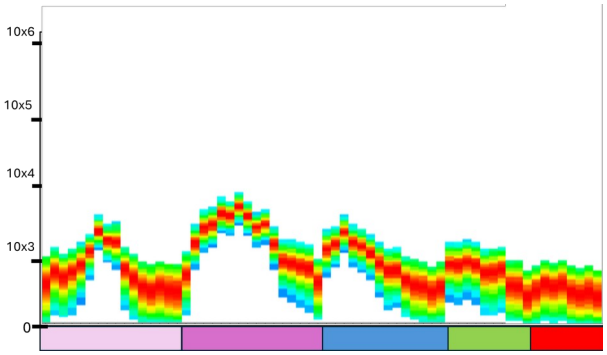


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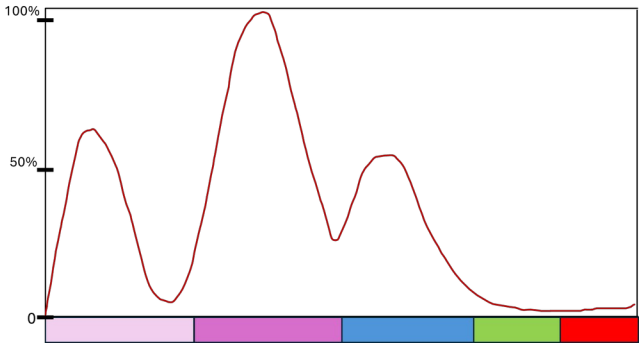


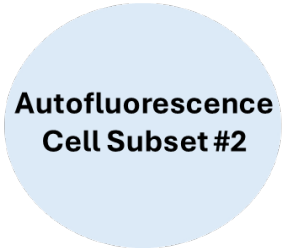

And used to generate the reference autofluorescent signature

**Unstained Reference Controls**

# A spectral cytometer needs three things for successful unmixing:

**Unstained Reference Controls**



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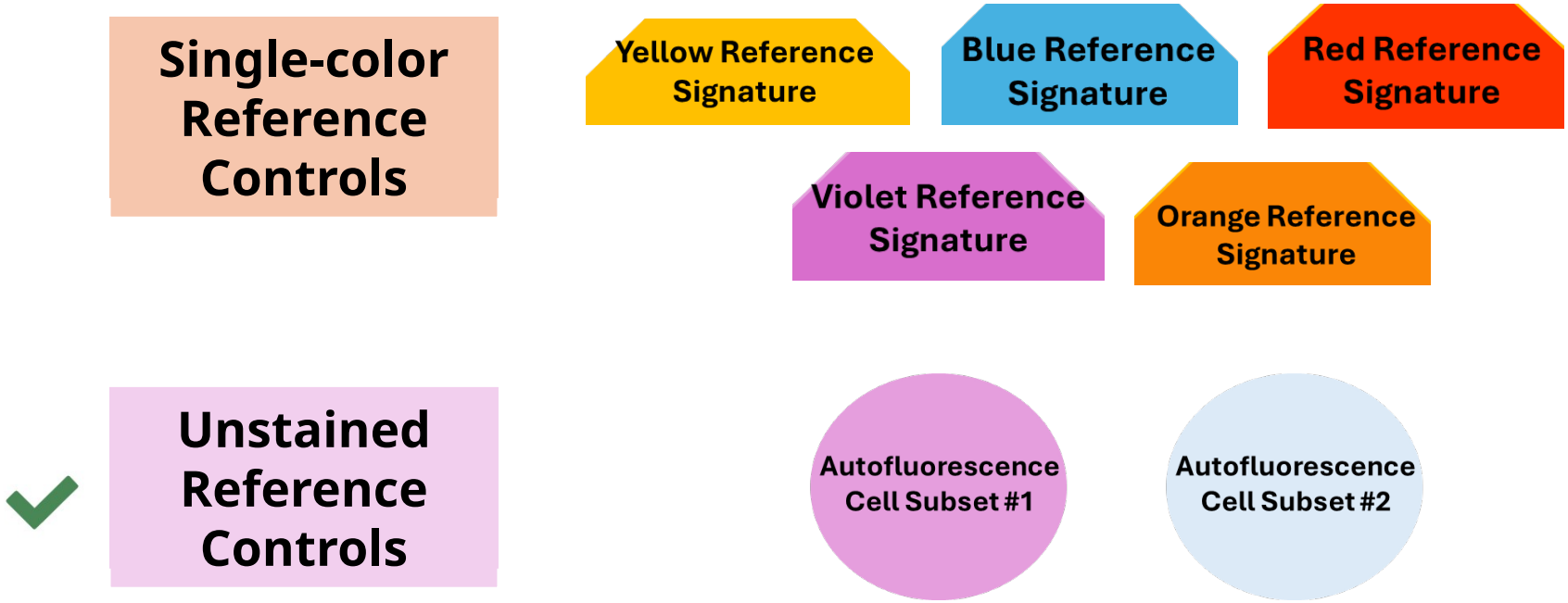
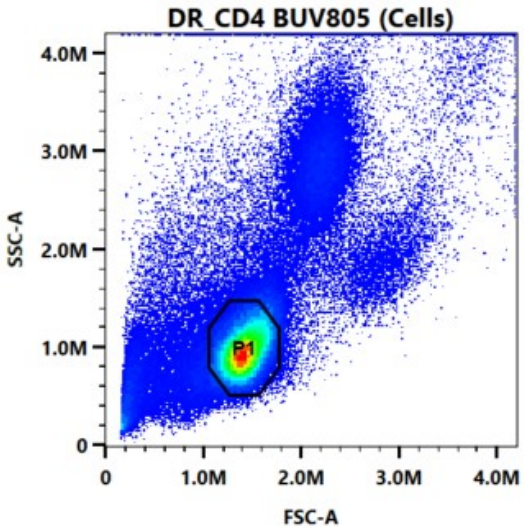
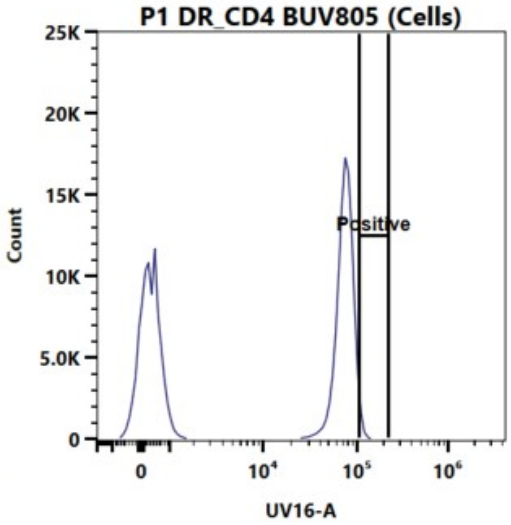
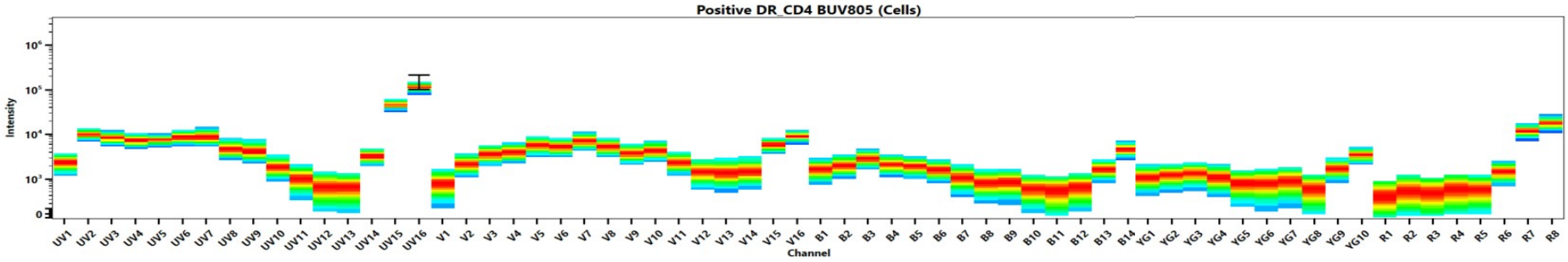


# *When we gate for Single-color Controls*



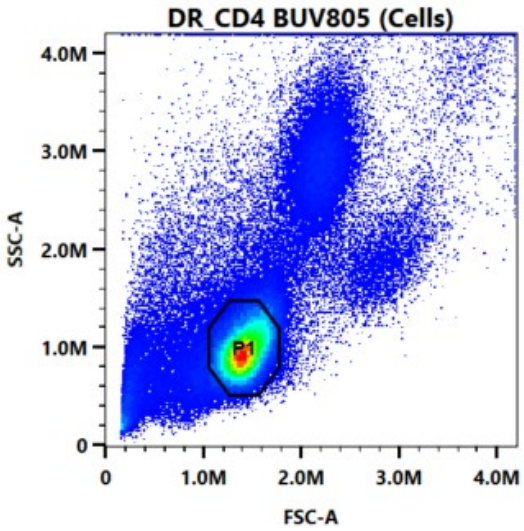



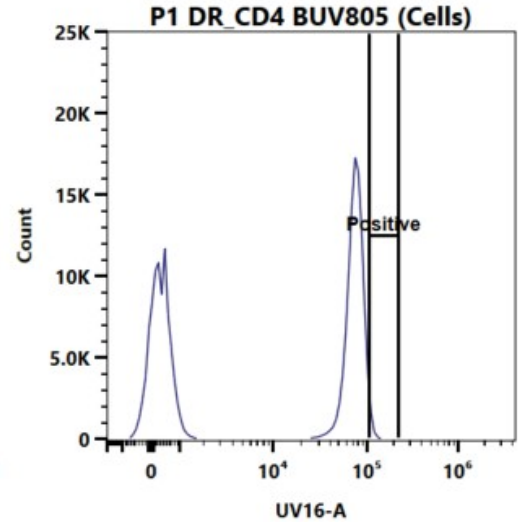



# *When we gate for Single-color Controls*

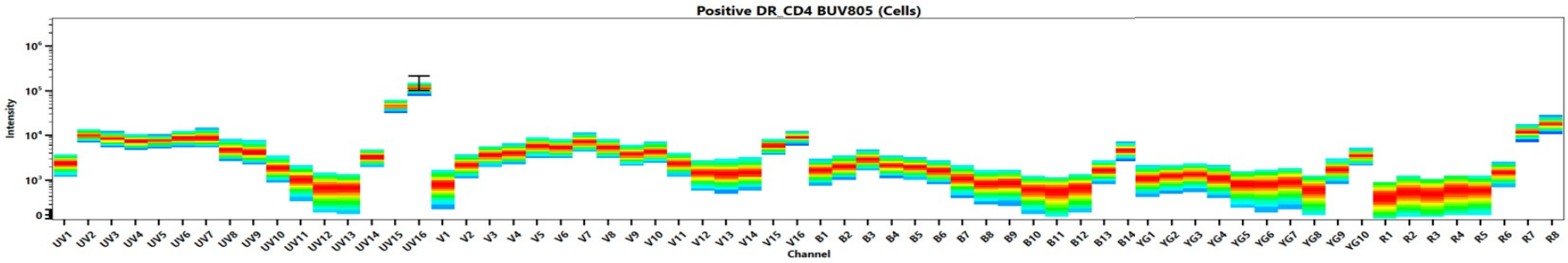
These

gated cells




Make up both the positive and negative peaks



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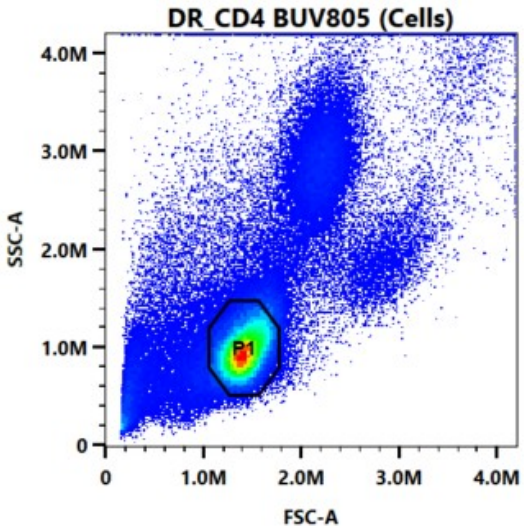
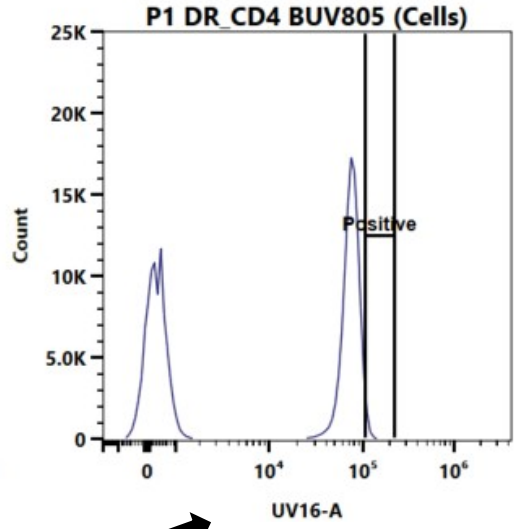
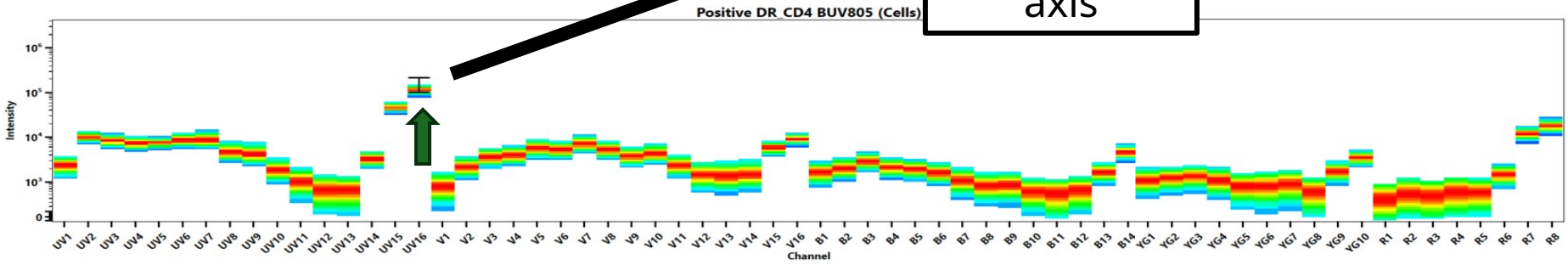



Make up both the positive and negative peaks

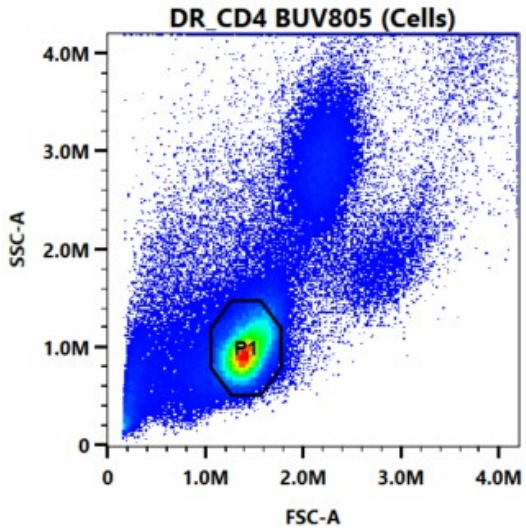
Is your x-axis

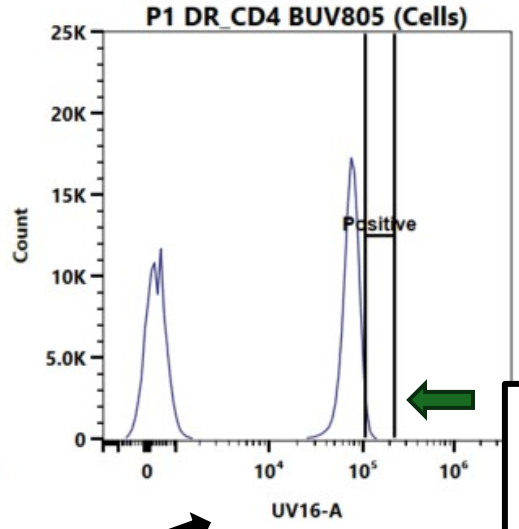



The selected detector

# *When we gate for Single-color Controls*

These gated cells

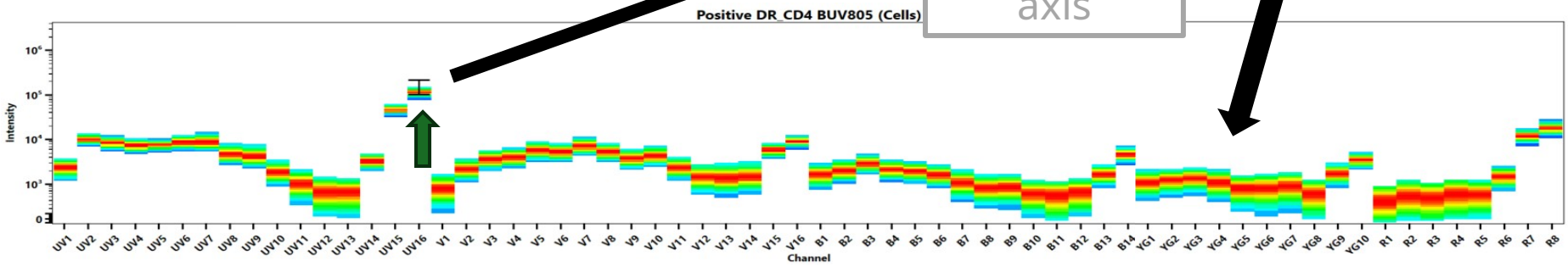



Make up both the positive and negative peaks

The cells within the positive gate

Is your x-axis



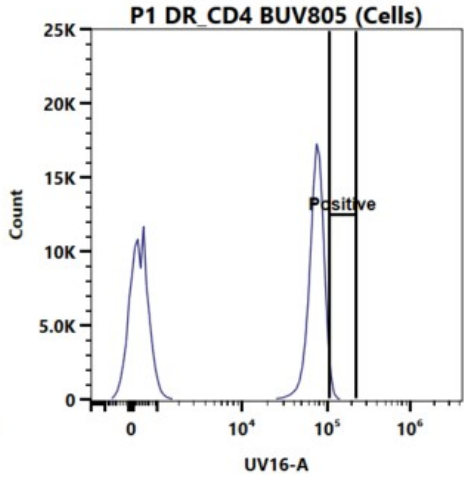

The selected detector

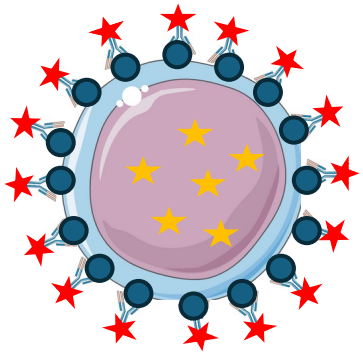
Make up the displayed signature

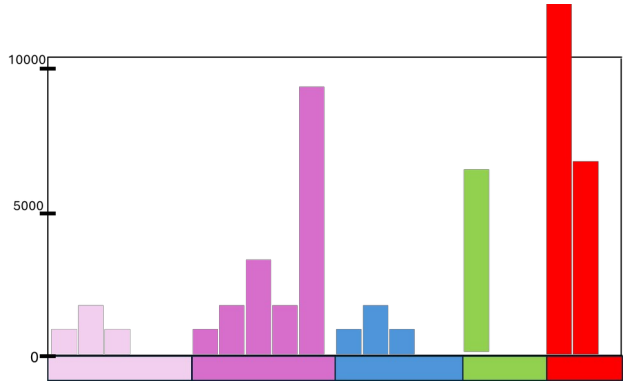
# *And if that wasn't enough…*



# The signal coming from the positive gate in our single-color controls is a mixture

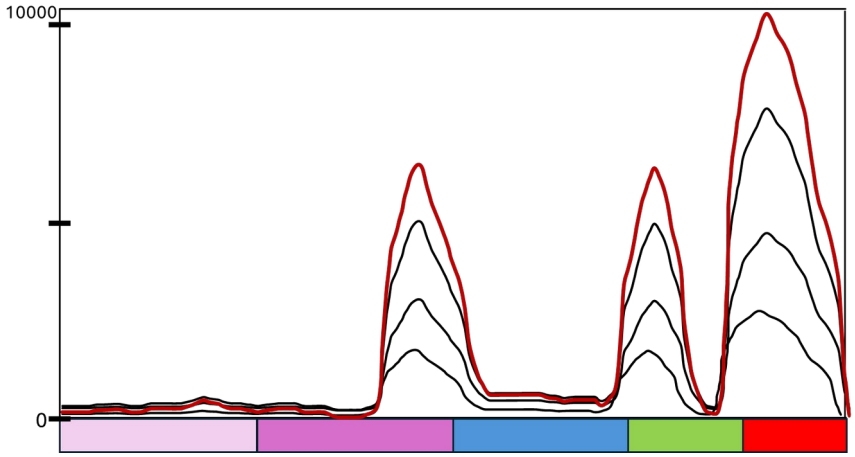


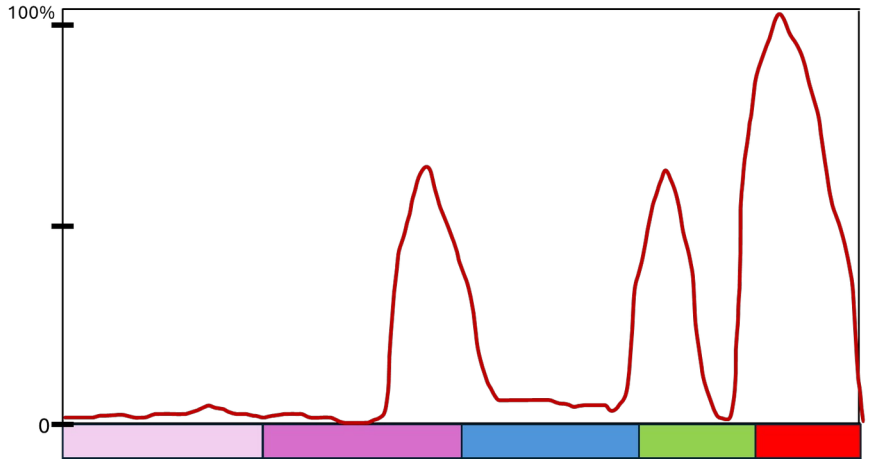



Total Signal = Positive Signal (from Fluorophore) + Negative Signal (from Autofluorescence)

# Essential concepts:

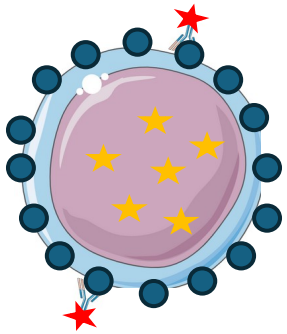


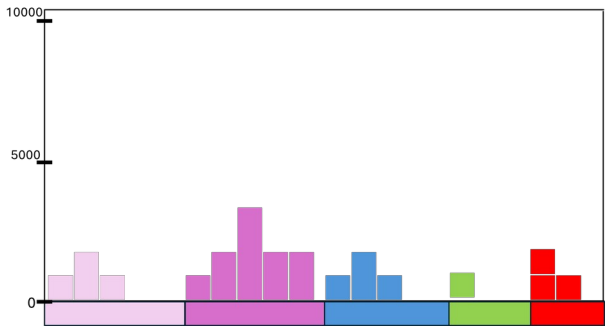

**Brightness**

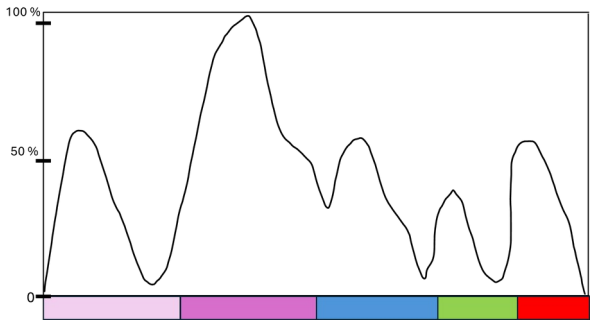



**Signature**

# How do we isolate the Fluorophore signature?

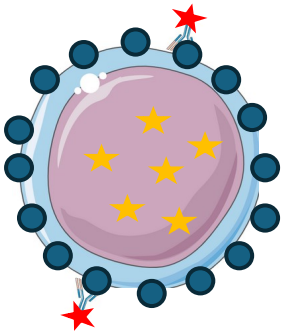


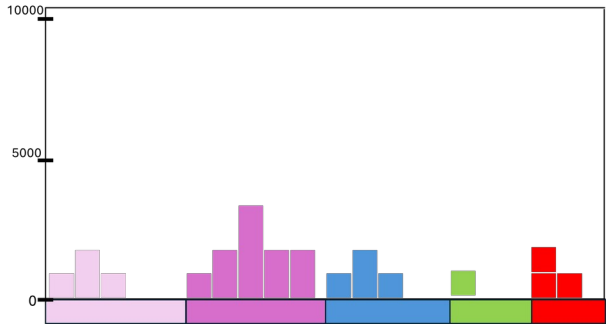


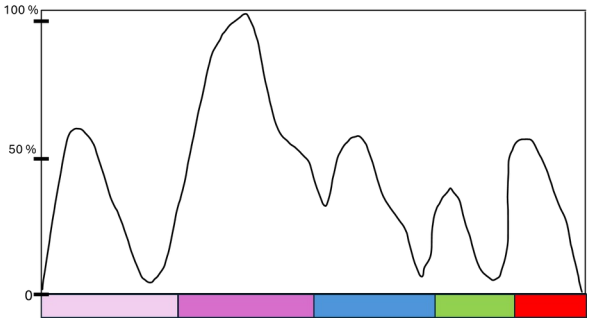




Total Signal

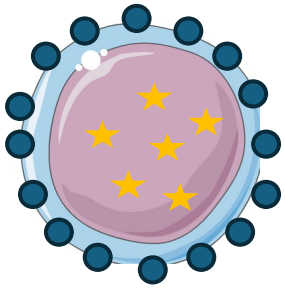
# How do we isolate the Fluorophore signature?

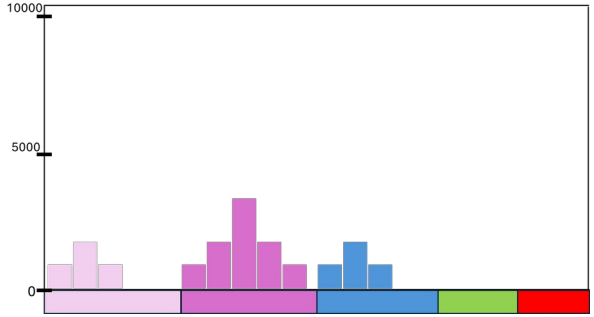


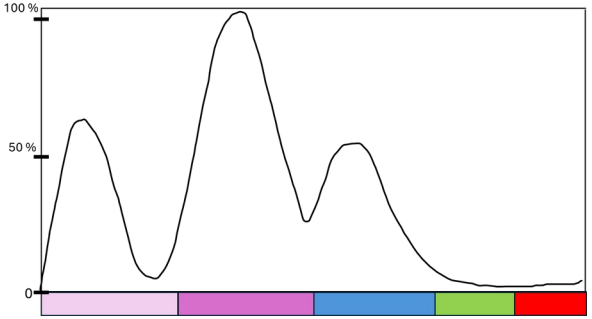



Total Signal

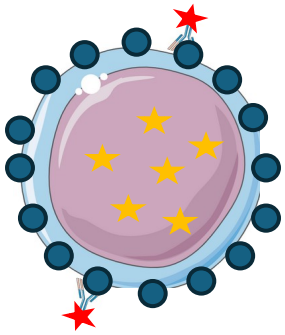


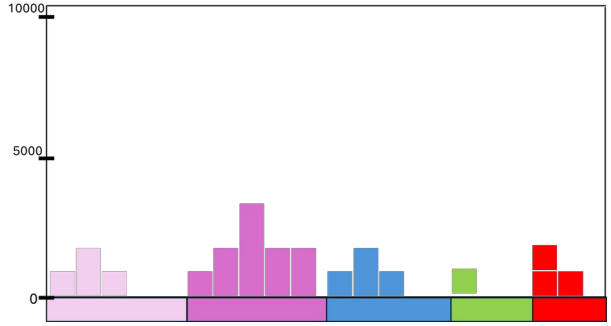


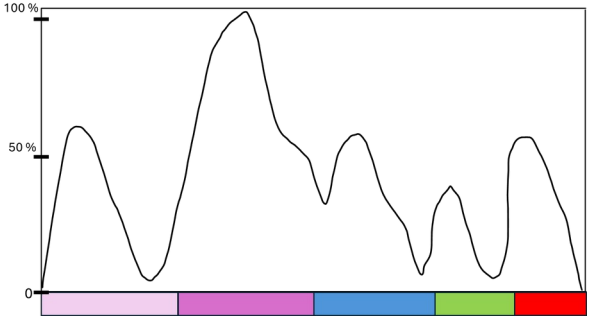




Negative Signal (from Autofluorescence)

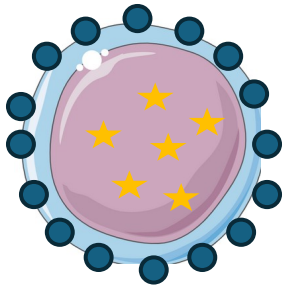
# How do we isolate the Fluorophore signature?

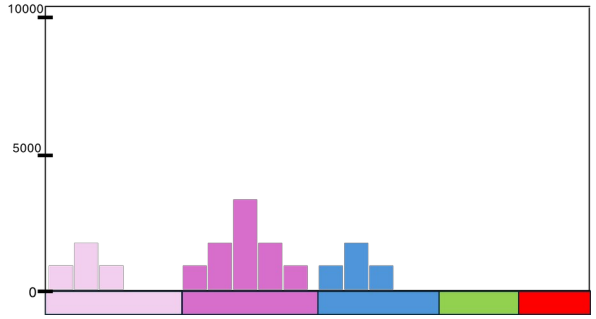


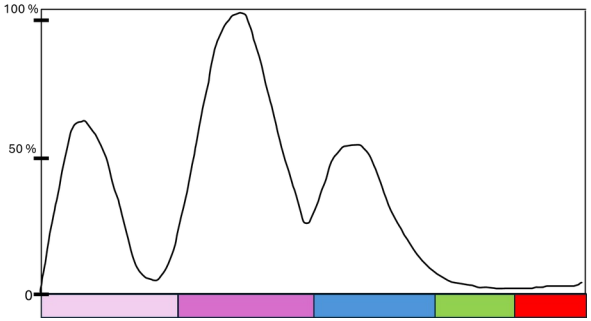



Total Signal






Negative Signal (from Autofluorescence)

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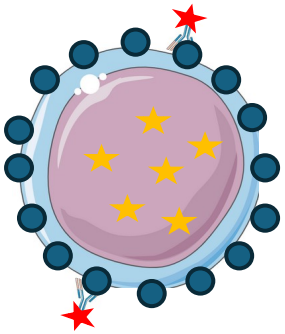
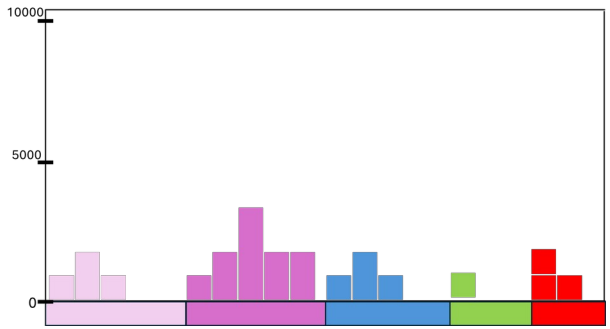
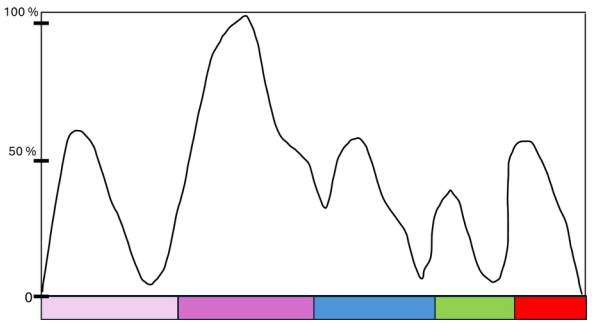
Total Signal - Negative Signal (from Autofluorescence) = ?

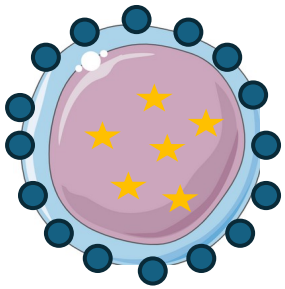
# How do we isolate the Fluorophore signature?

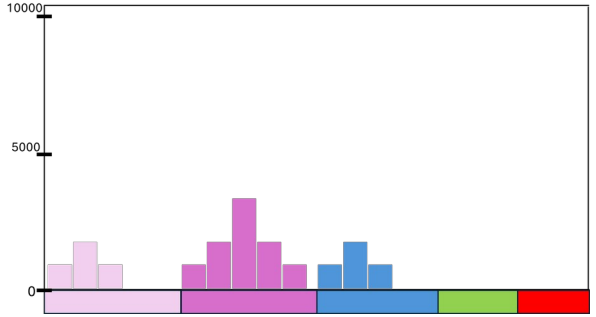


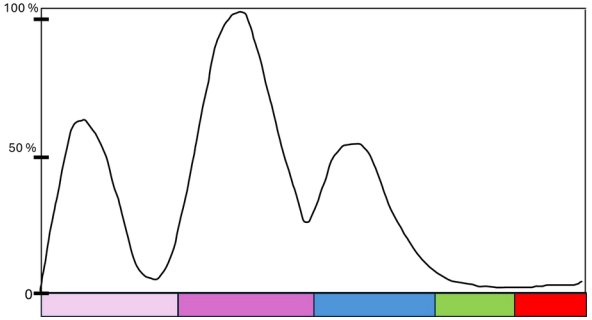



Total Signal

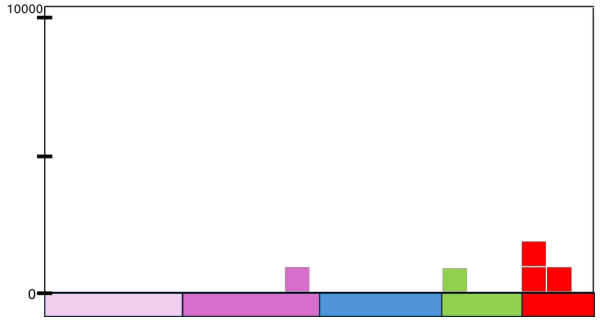


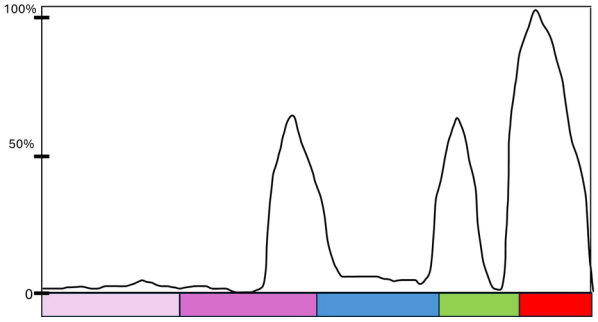



Negative Signal (from Autofluorescence)





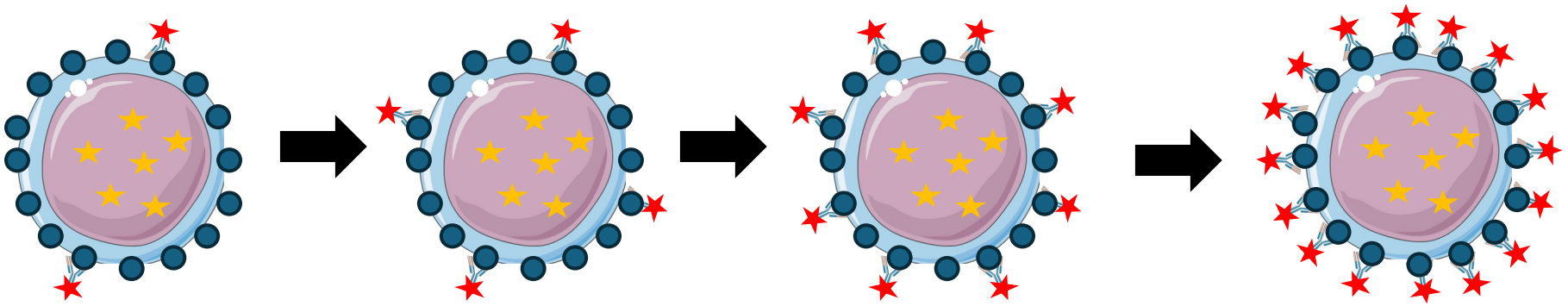




Positive Signal (from Fluorophore)

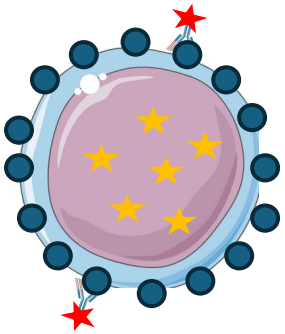
# *What happens with increased autofluorescence?*

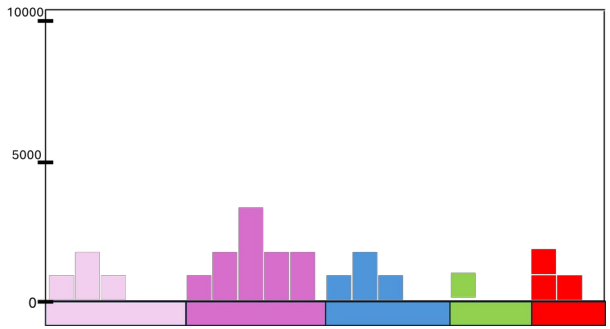


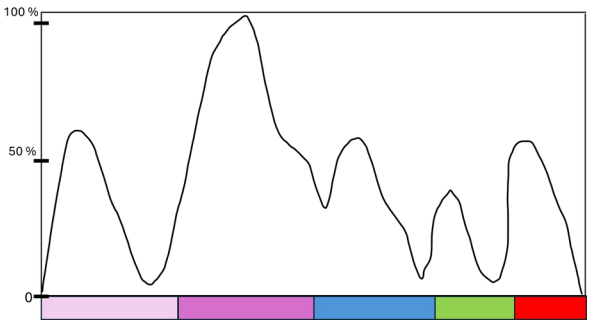
***What happens if we increase fluorophore staining?!?***



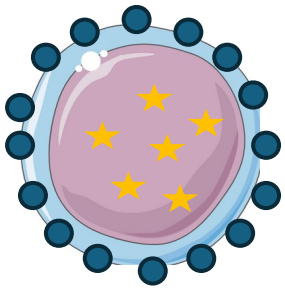
# Very Low

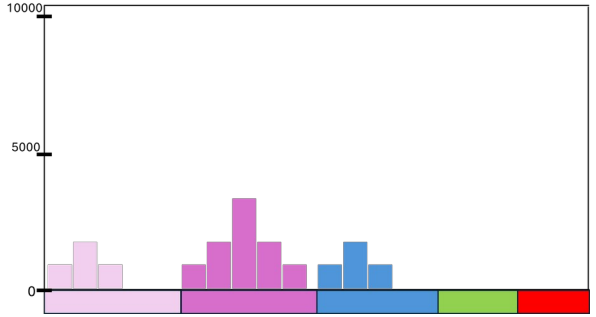


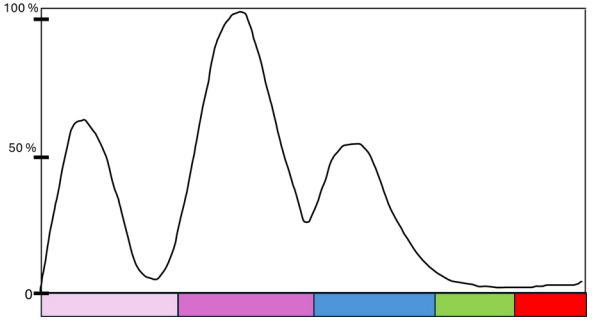



Total Signal

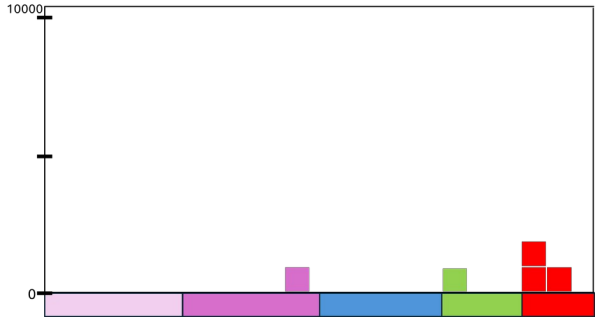


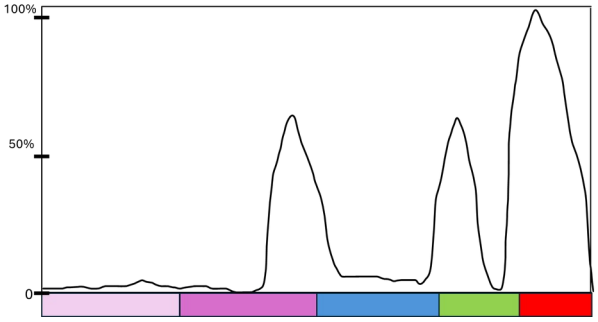



Negative Signal (from Autofluorescence)

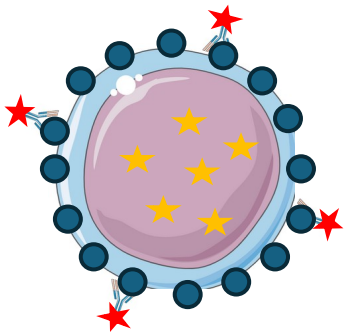


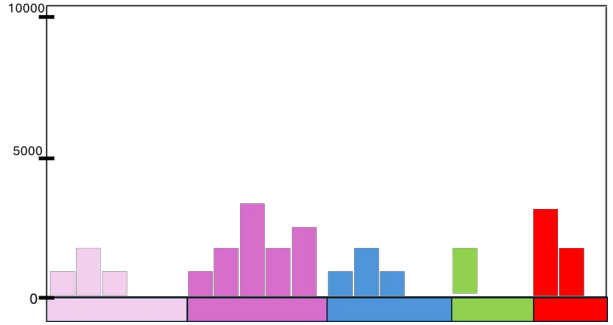


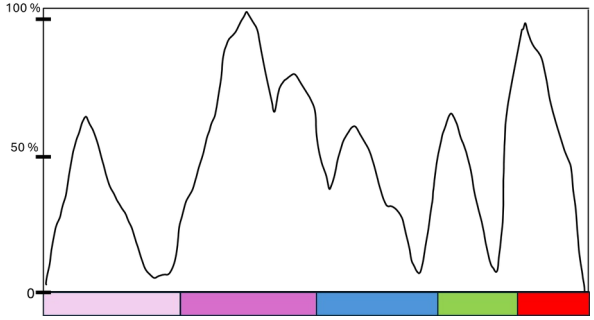




Positive Signal (from Fluorophore)

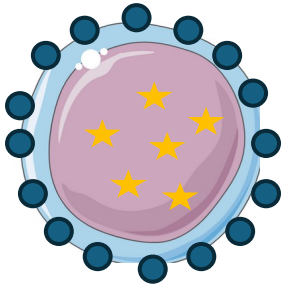
# Low

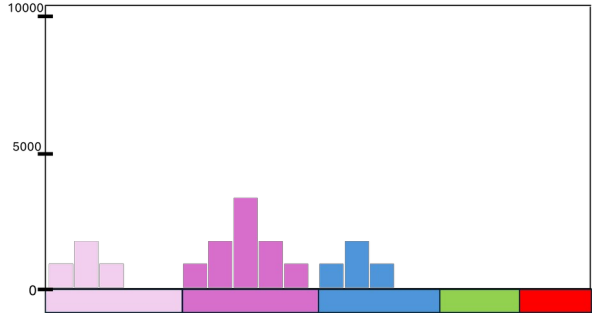


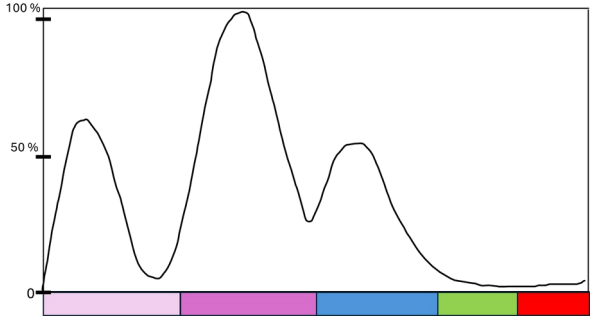



Total Signal

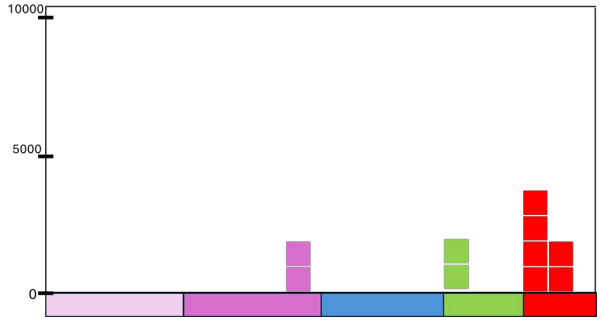


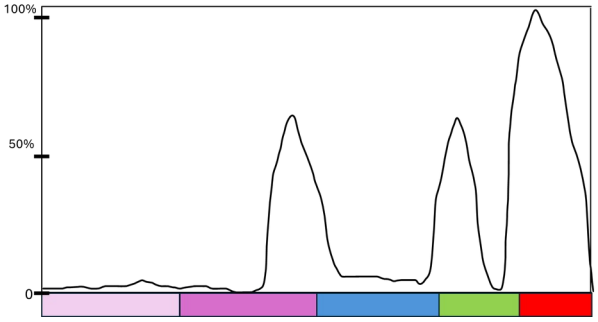



Negative Signal (from Autofluorescence)

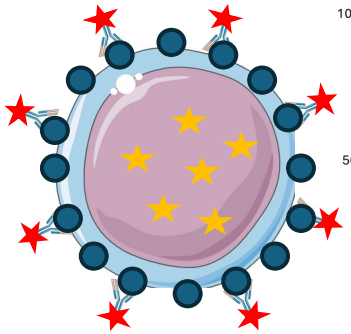


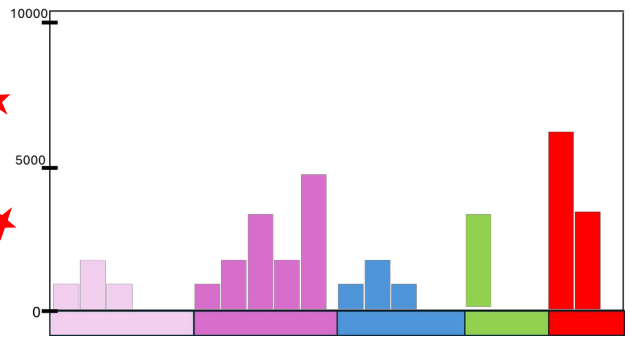


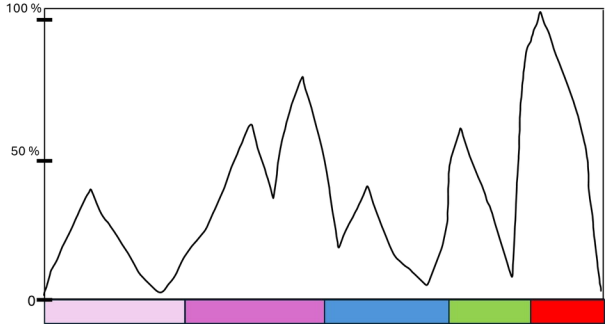




Positive Signal (from Fluorophore)

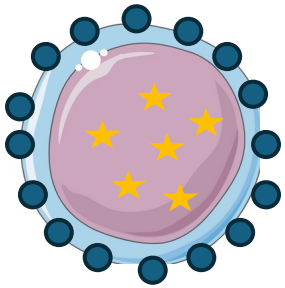
# Medium

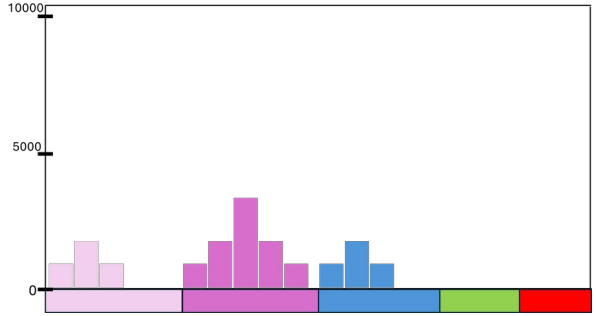


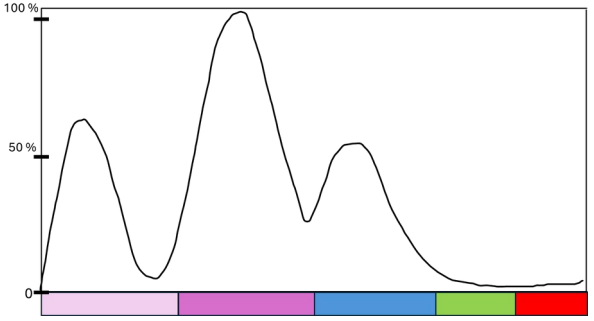



Total Signal

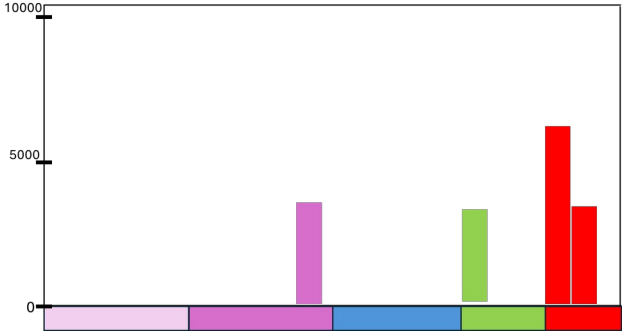


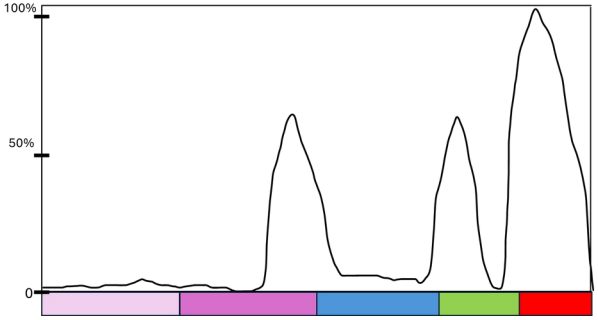



Negative Signal (from Autofluorescence)

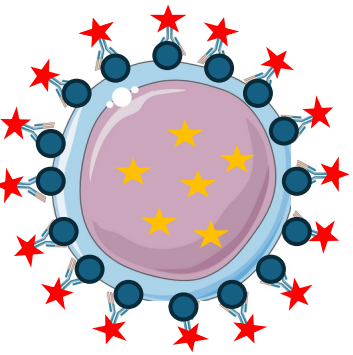


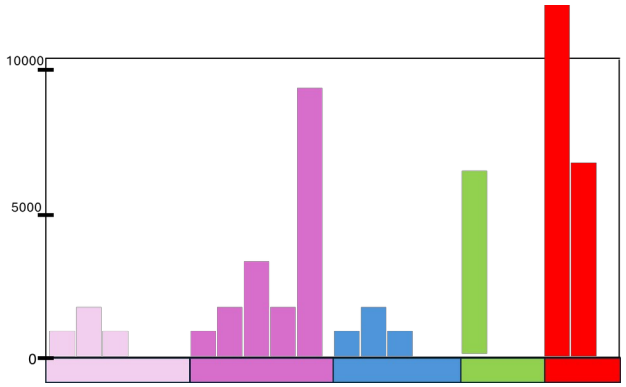


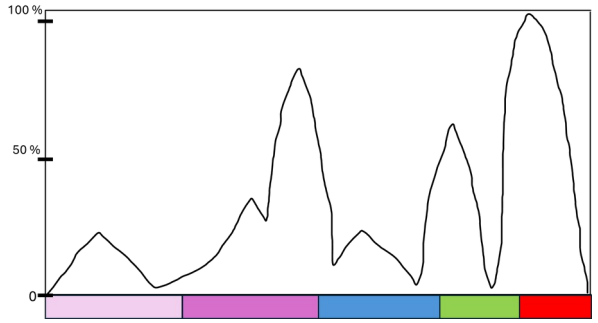




Positive Signal (from Fluorophore)

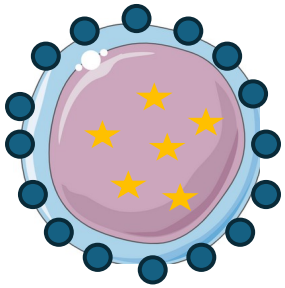
# High

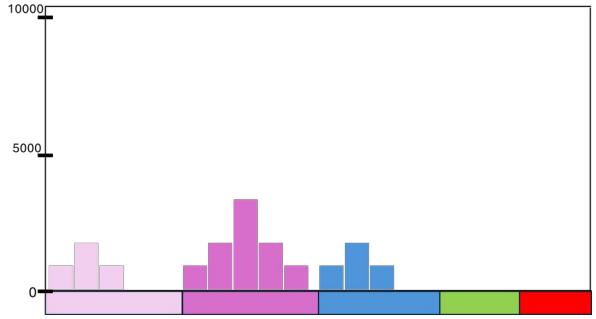


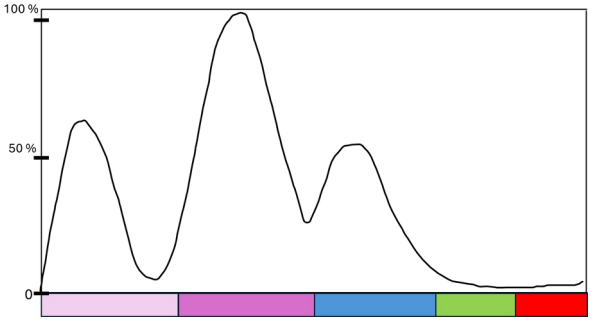



Total Signal

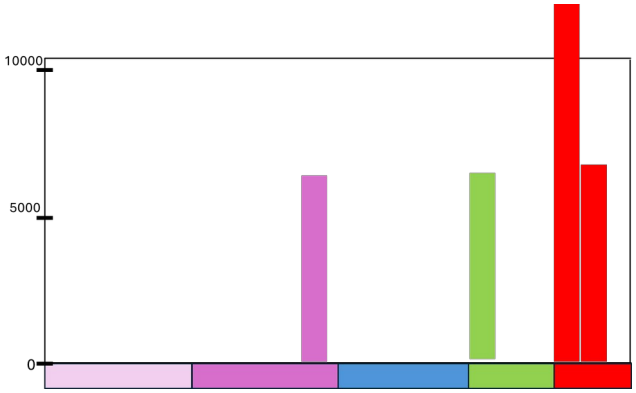


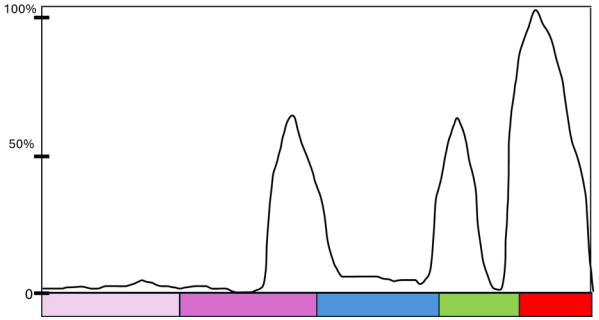



Negative Signal (from Autofluorescence)

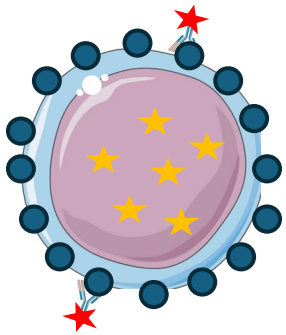


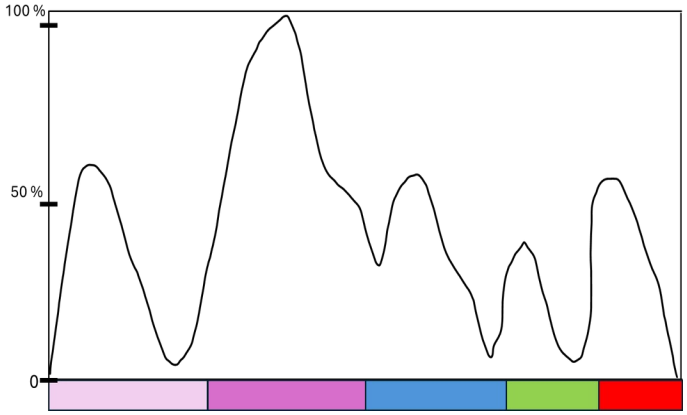


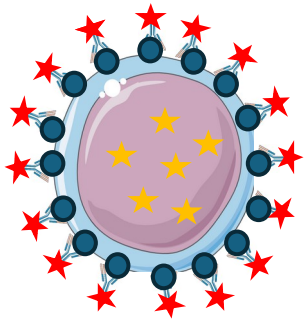



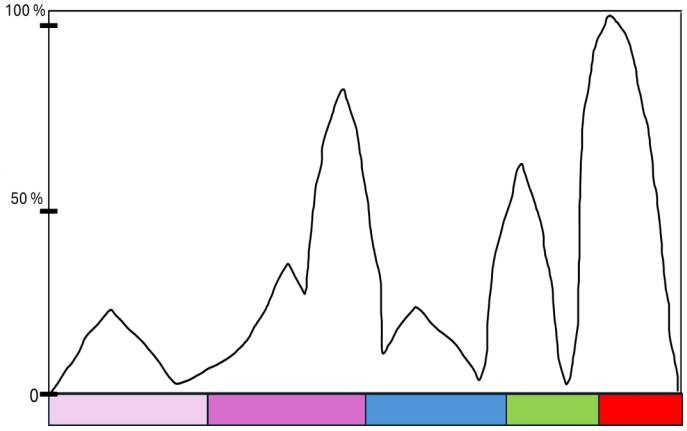

Positive Signal (from Fluorophore)

# When autofluorescence is correctly subtracted



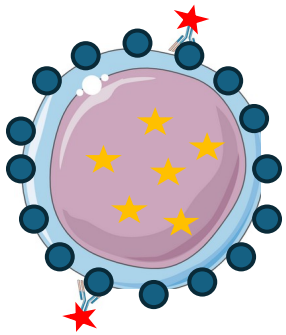


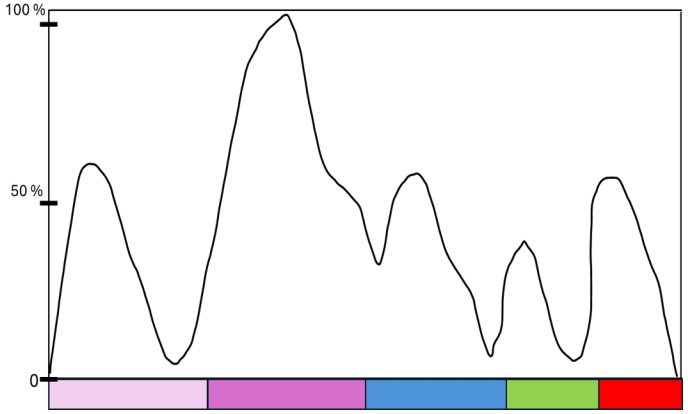



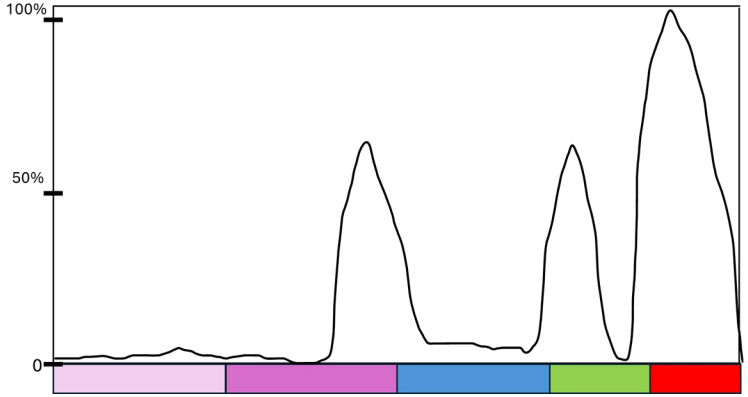


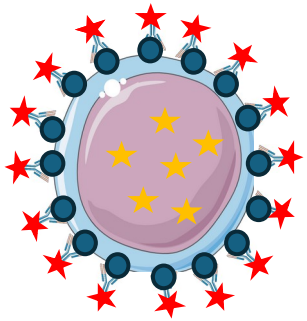
# When autofluorescence is correctly subtracted

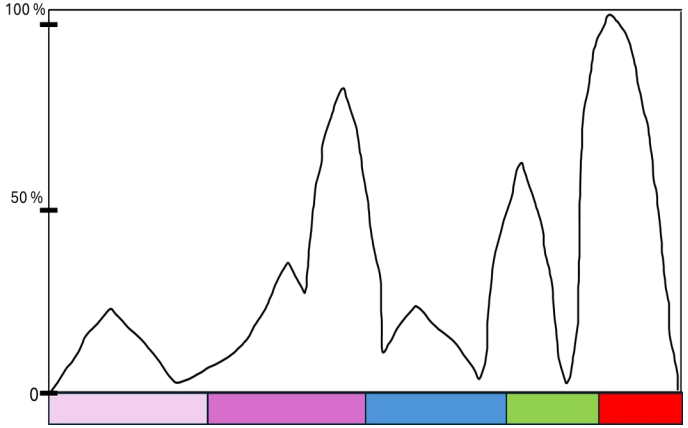
## The Fluorophores signature is the same regardless of brightness

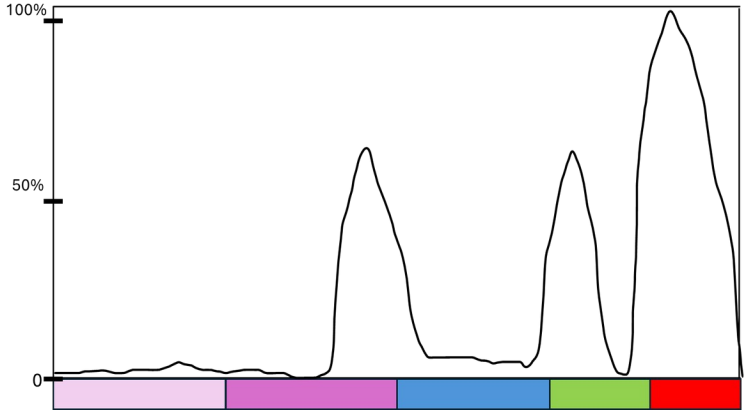




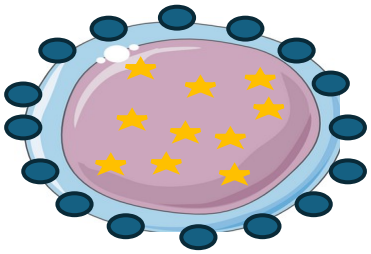


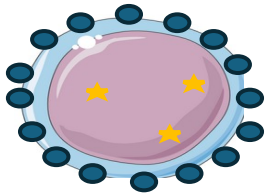
# The issue: Variation in autofluorescence

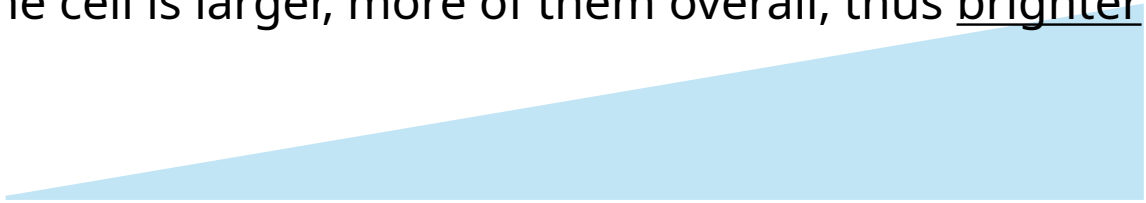


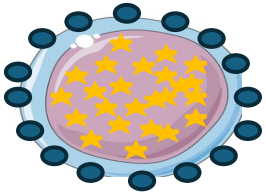




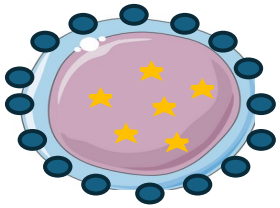
**Difference in cell size:**
Same proportion of autofluorescent molecules, but because one cell is larger, more of them overall, thus brighter



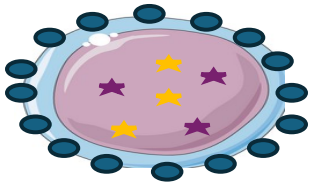




**Difference in amount of autofluorescence:**
Same cell size, just one has more autofluorescent molecules, thus brighter

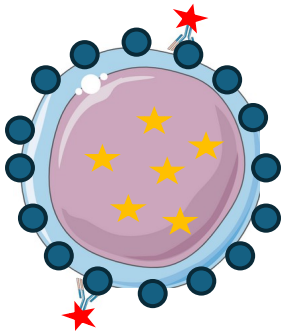


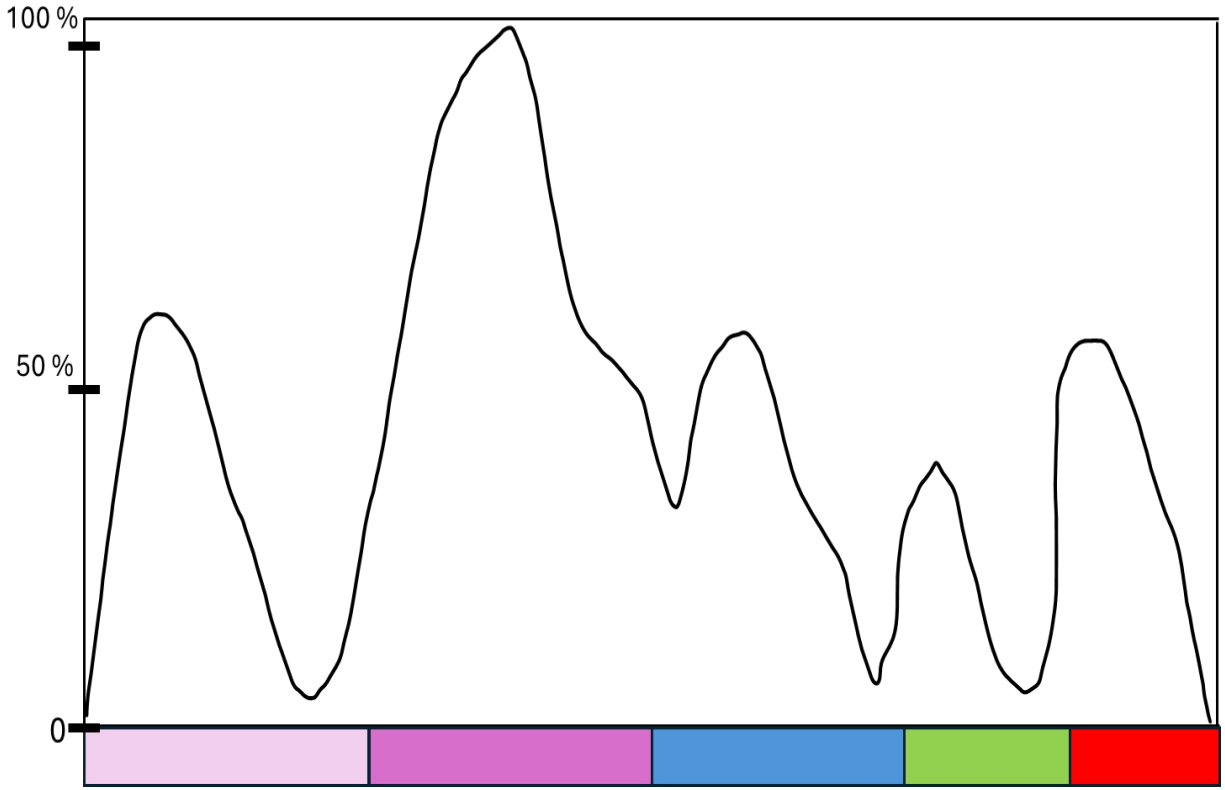




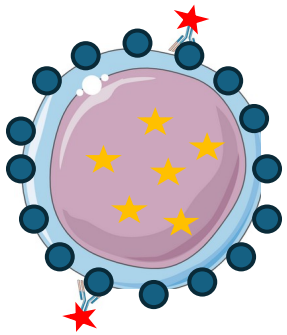
**Variation in autofluorescent molecules:**
Same cell size, but express different mixture of autofluorescent molecules, resulting in a different autofluorescent signature

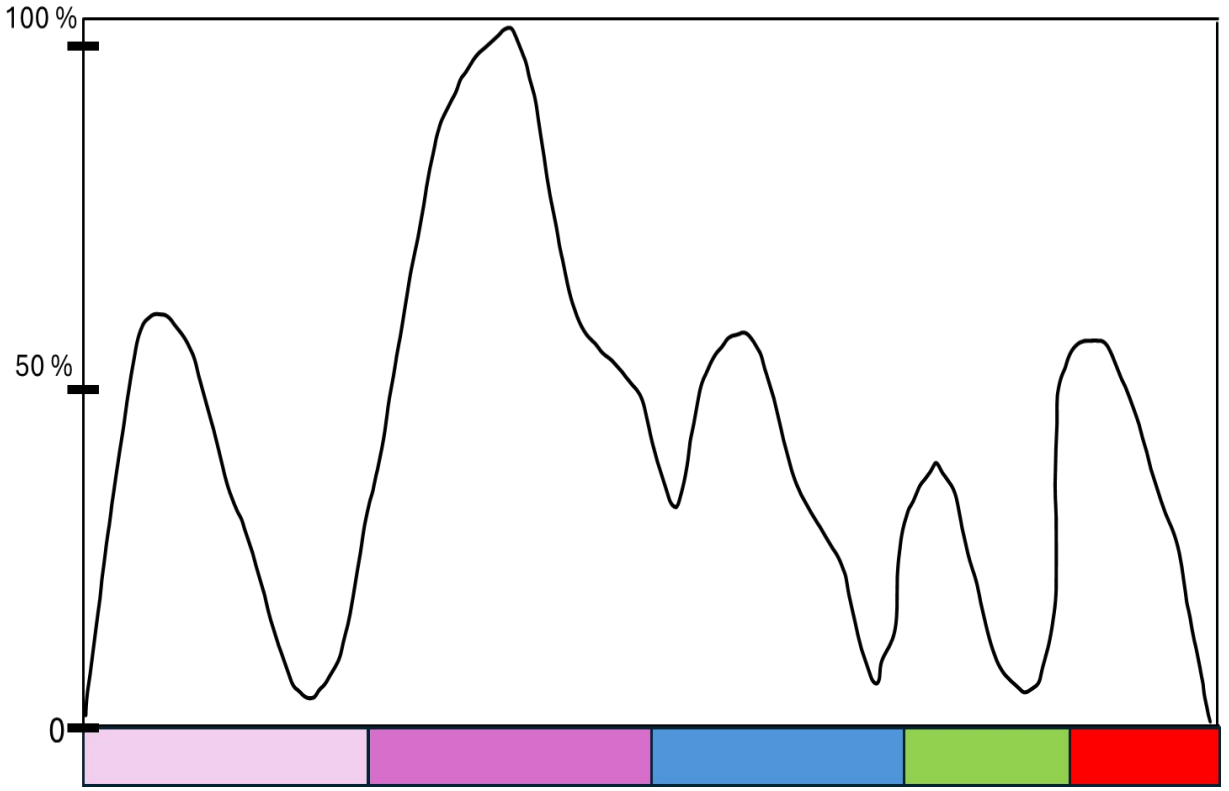
# On dim-staining cells:



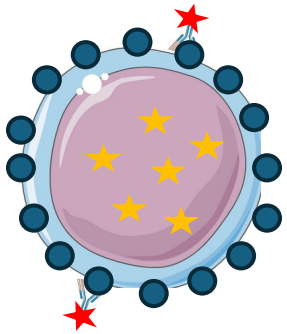


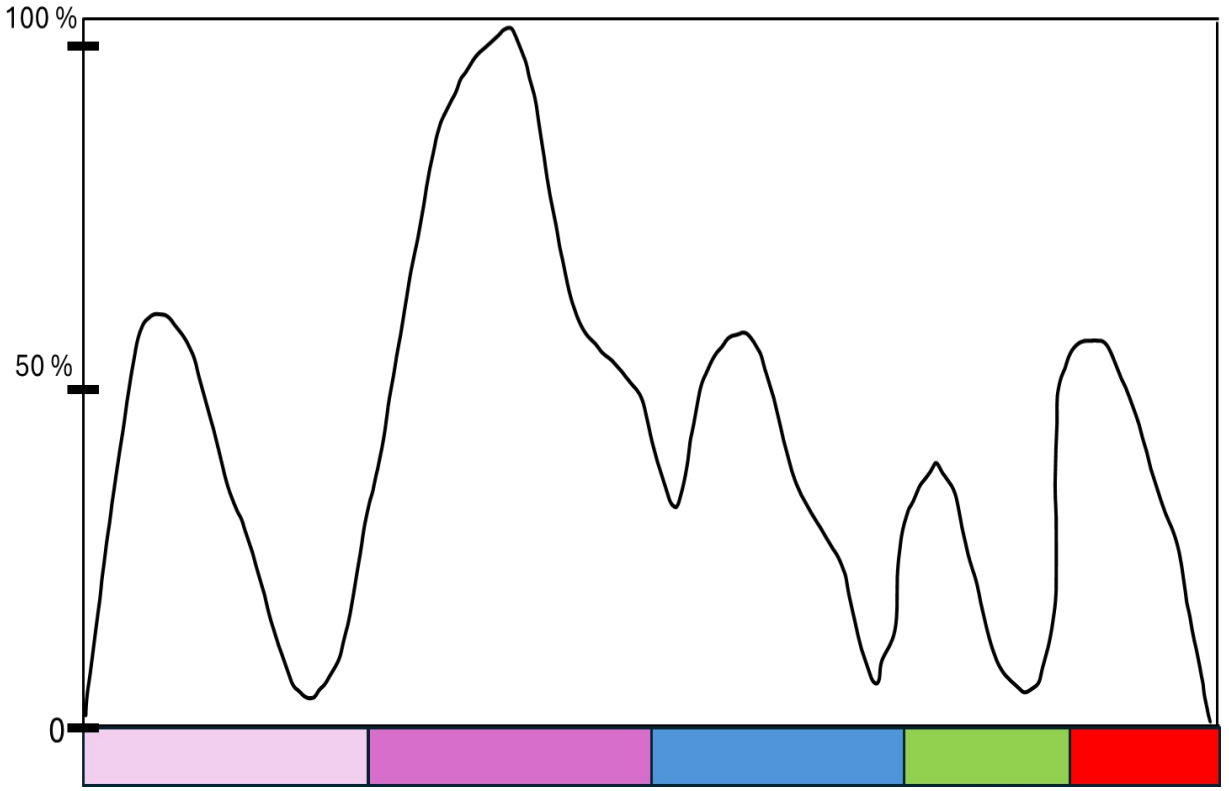
# Dim-staining cell: with correct autofluorescence subtraction



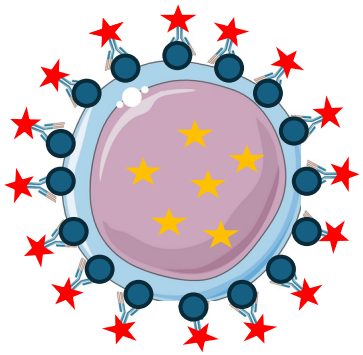


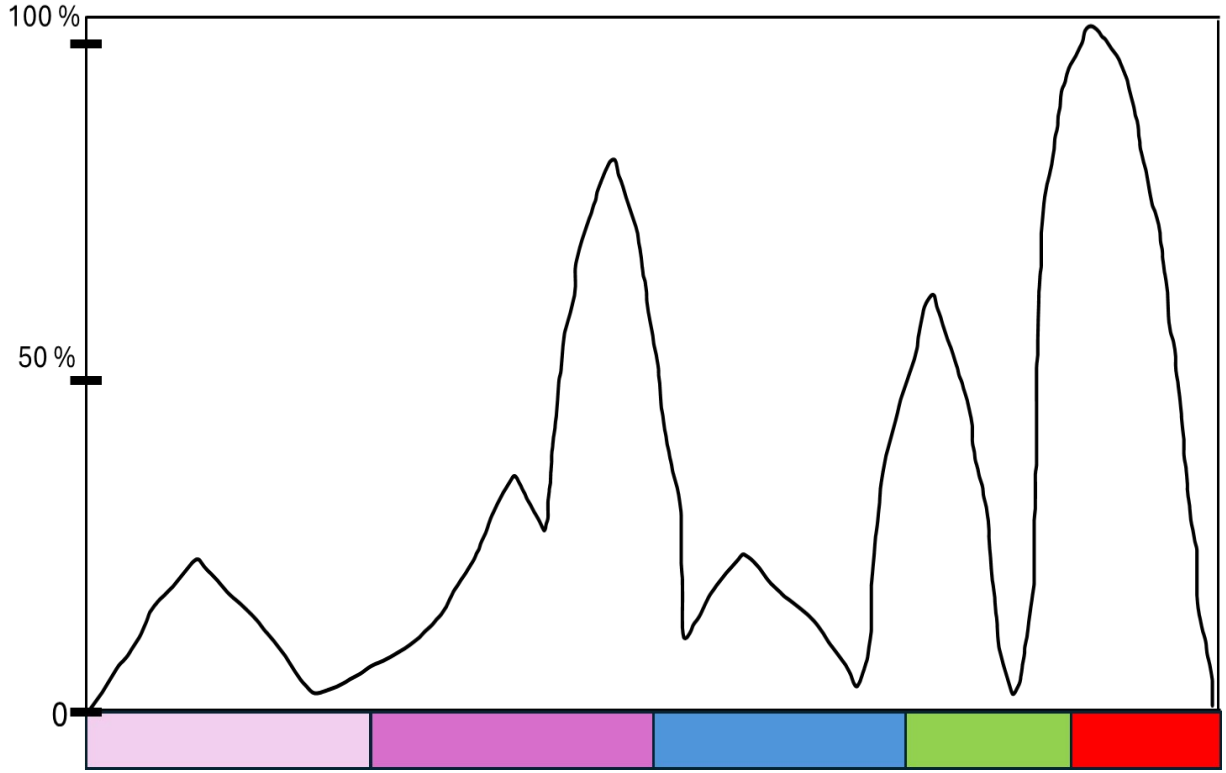
# Dim-staining cell: with missed autofluorescence subtraction



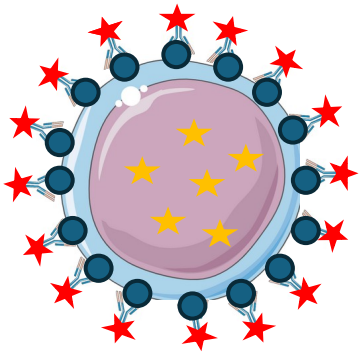


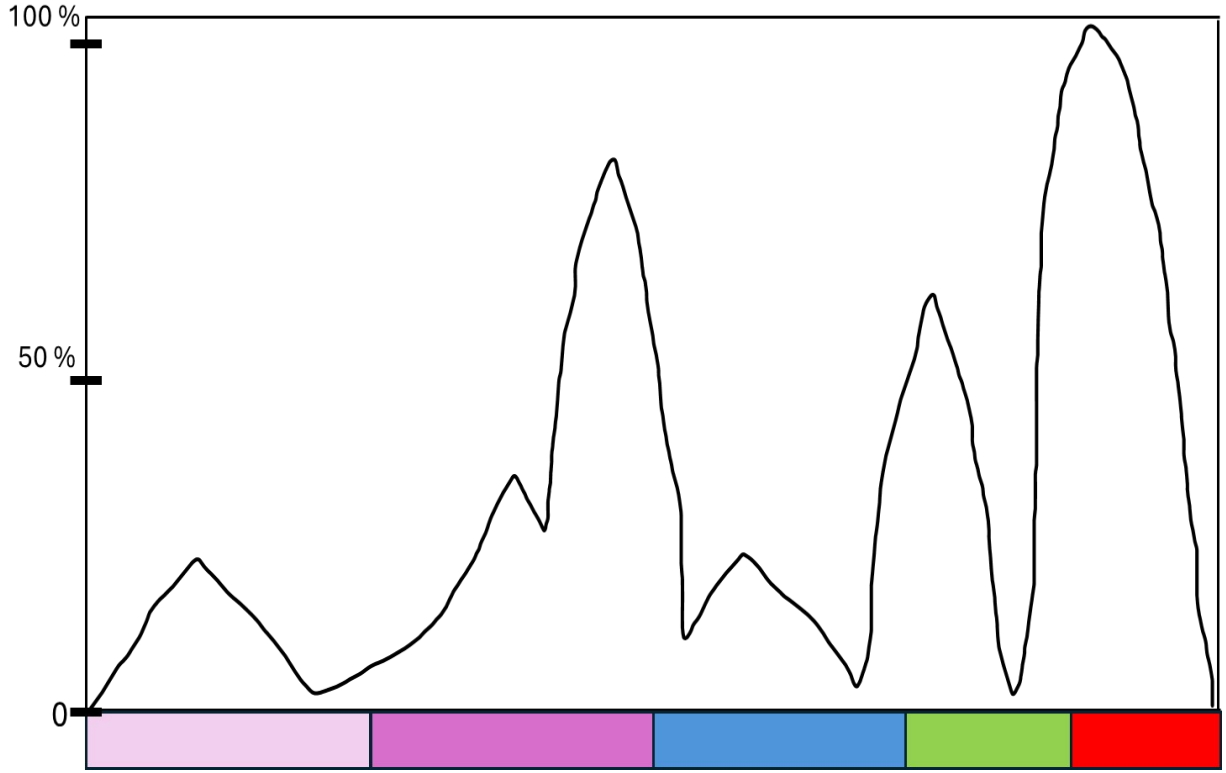
# On bright-staining cells:



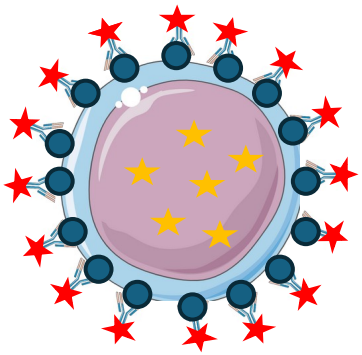


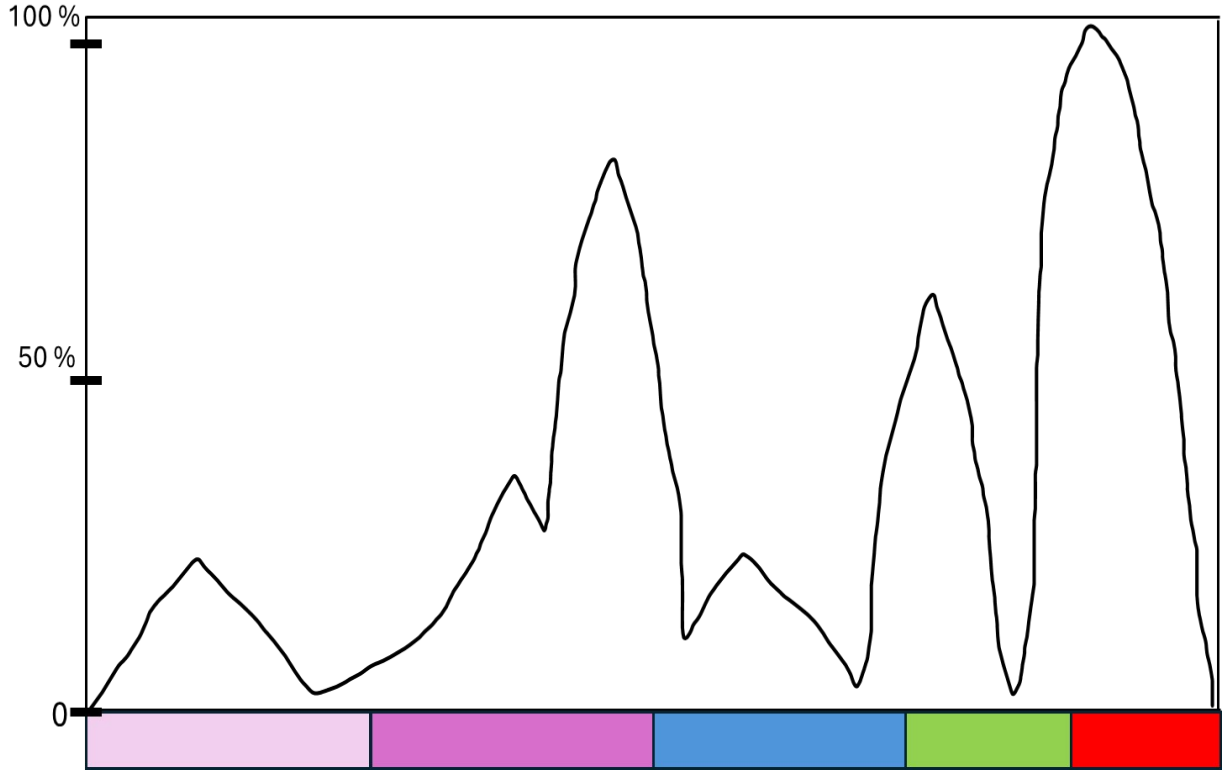
# Bright-staining cell: with correct autofluorescence subtraction



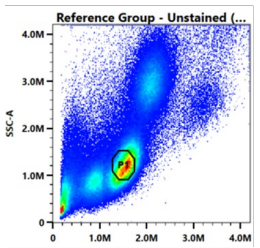


# Bright-staining cell: with missed autofluorescence subtraction

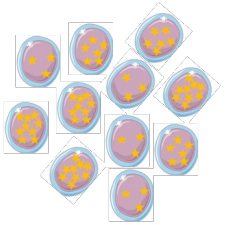




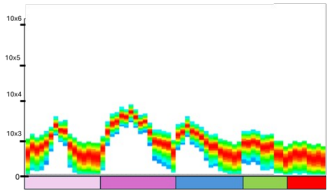
# Why would there be leftover autofluorescence signal?




The gate we draw



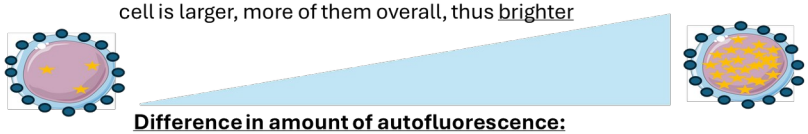
Selects some cells



The median measurement per detector is taken



**Difference in cell size:**
Same proportion of autofluorescent molecules, but because one cell is larger, more of them overall, thus brighter

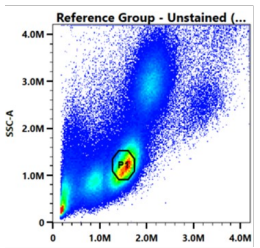


**Difference in amount of autofluorescence:**
Same cell size, just one has more autofluorescent molecules, thus brighter

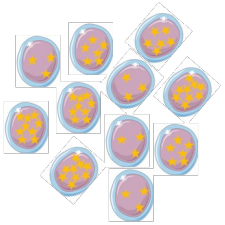


**Variation in autofluorescent molecules:**
Same cell size, but express different mixture of autofluorescent molecules, resulting in a different autofluorescent signature

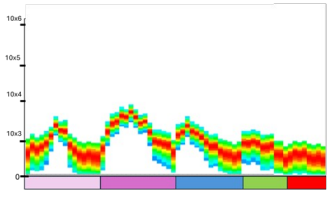
# Why would there be leftover autofluorescence signal?




The gate we draw



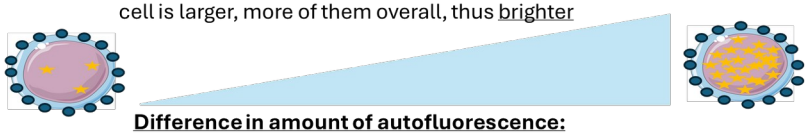
Selects some cells



The median measurement per detector is taken



**Difference in cell size:**
Same proportion of autofluorescent molecules, but because one cell is larger, more of them overall, thus brighter



**Difference in amount of autofluorescence:**
Same cell size, just one has more autofluorescent molecules, thus brighter



**Variation in autofluorescent molecules:**
Same cell size, but express different mixture of autofluorescent molecules, resulting in a different autofluorescent signature

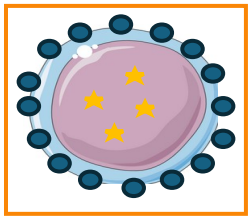
The median measurement from your unstained cells is used.

Example:
UV7 = 2000
V7 = 4000
B3 = 2000

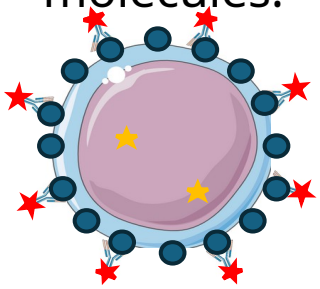
# At the population level, not everything is “average”

Our “average” autofluorescent cell for the entire population

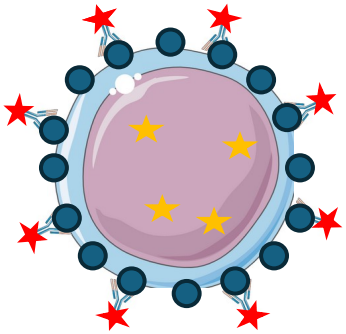
UV7 = 2000
V7 = 4000
B3 = 2000



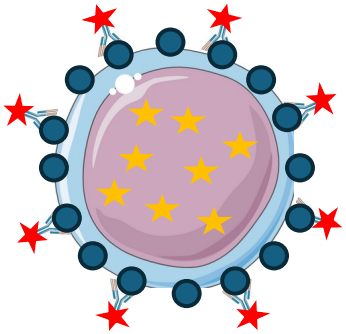
Within our single-color reference controls, individual cells may have differences in their expression of autofluorescence molecules.



UV7 = 1000
V7 = 2000
B3 = 1000

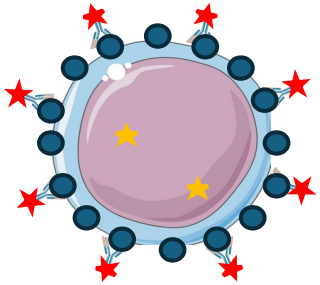


UV7 = 2000
V7 = 4000
B3 = 2000



UV7 = 4000
V7 = 8000
B3 = 4000

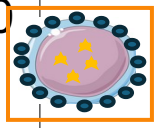
# Leftovers after autofluorescence subtraction



UV7 = 1000
V7 = 2000
B3 = 1000

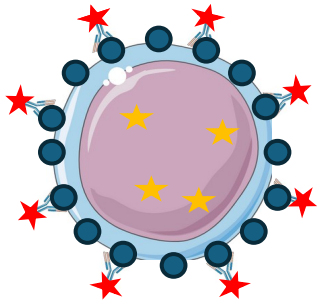
-

UV7 = 2000
V7 = 4000
B3 = 2000



=

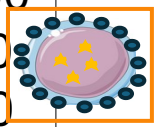
UV7 = -1000
V7 = -2000
B3 = -1000



UV7 = 2000
V7 = 4000
B3 = 2000

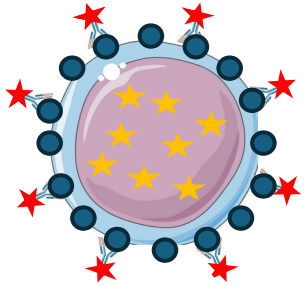
-

UV7 = 2000
V7 = 4000
B3 = 2000



=

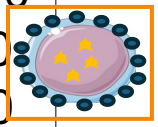
UV7 = 0
V7 = 0
B3 = 0



UV7 = 4000
V7 = 8000
B3 = 4000

-

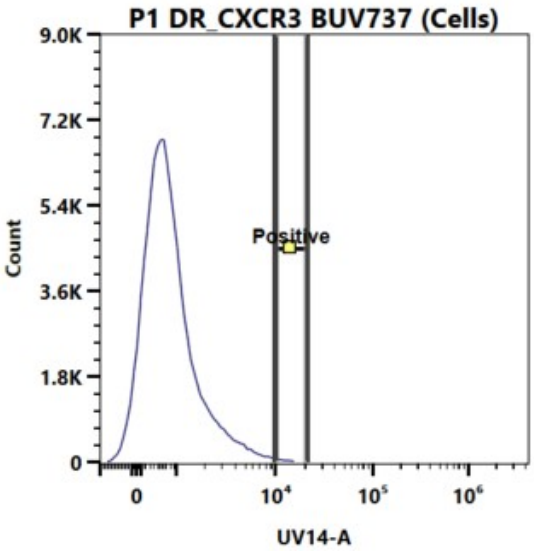
UV7 = 2000
V7 = 4000
B3 = 2000

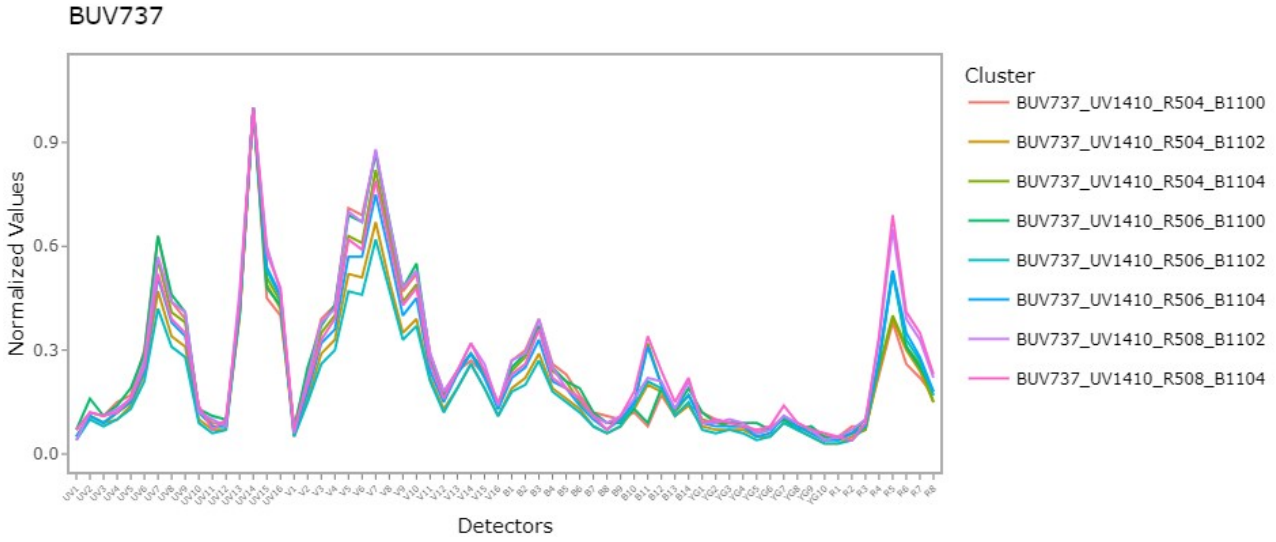


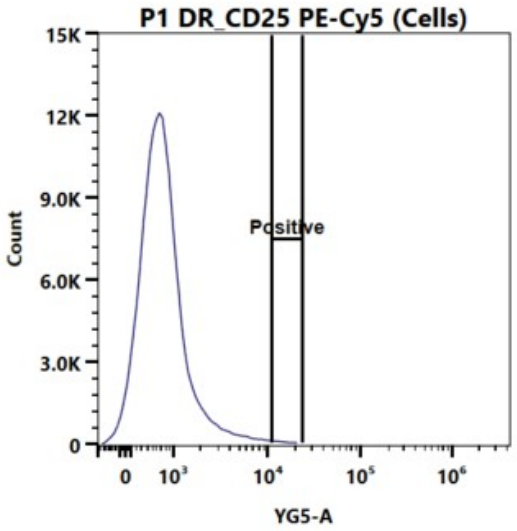
=

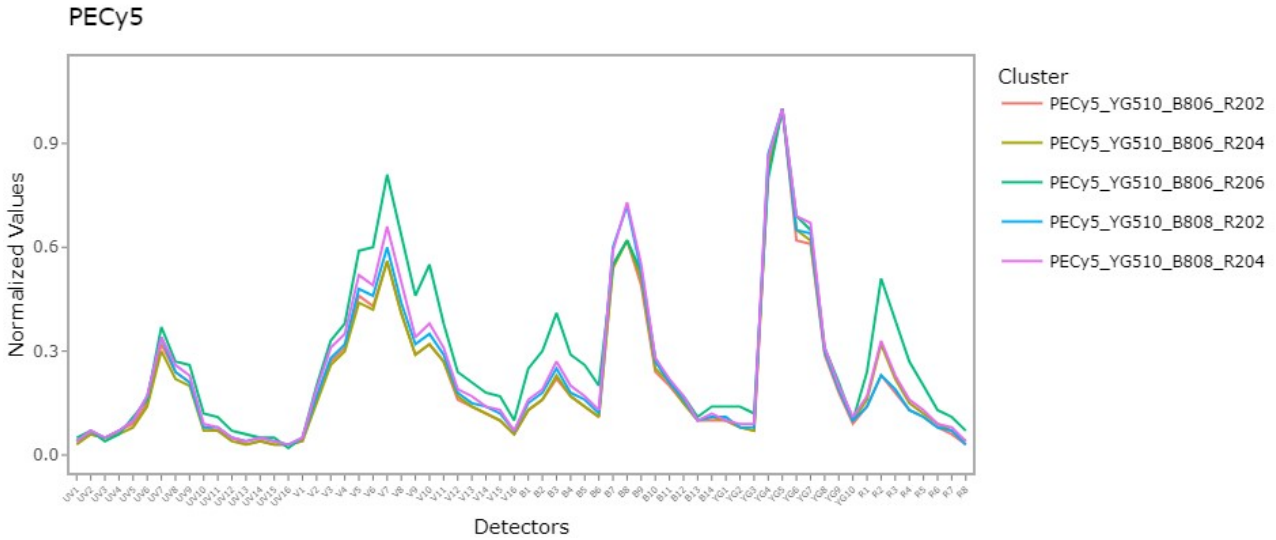
UV7 = +2000
V7 = +4000
B3 = +2000

# Adventures in Real World Data
# (from my recent experiment)

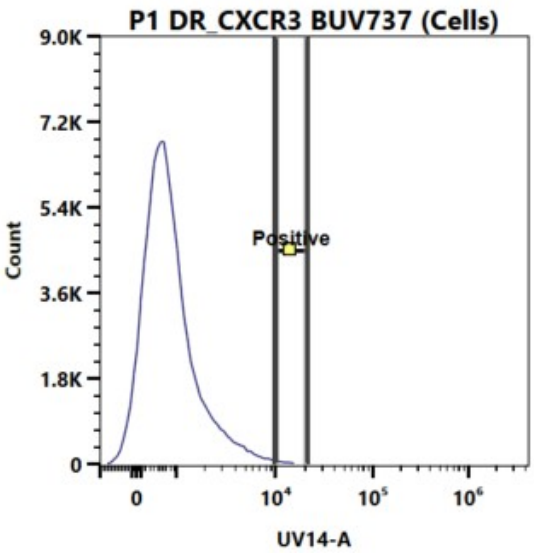


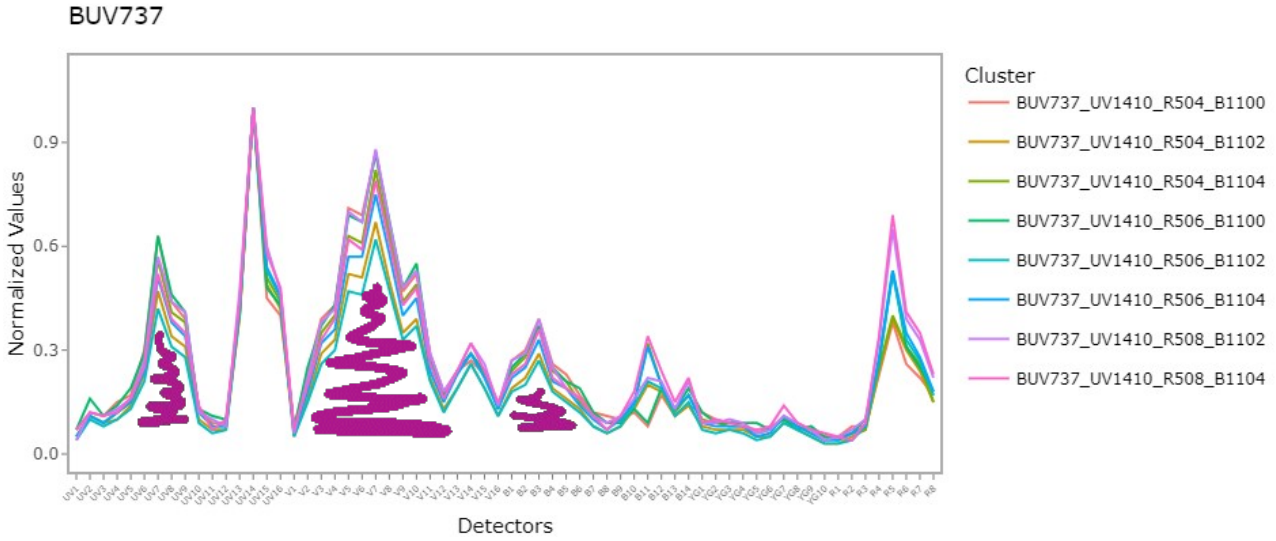



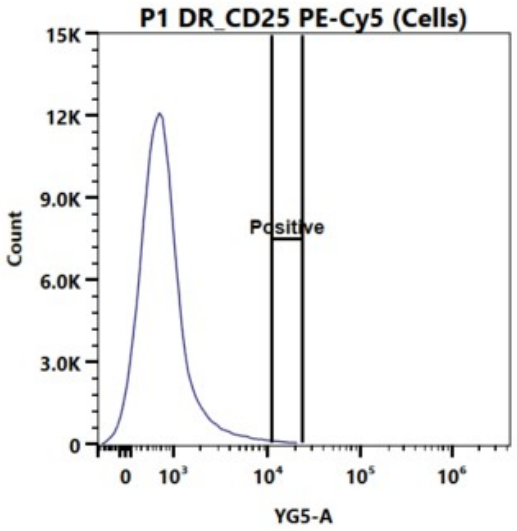



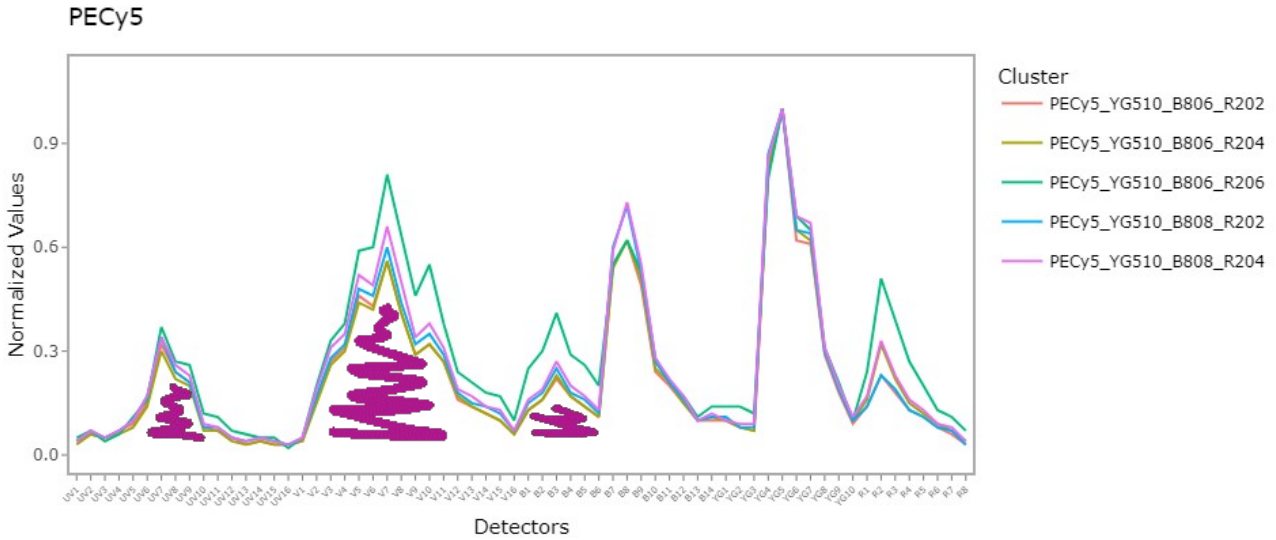



# Substantial leftover autofluorescence affecting signature for dim marker*fluorophore combinations

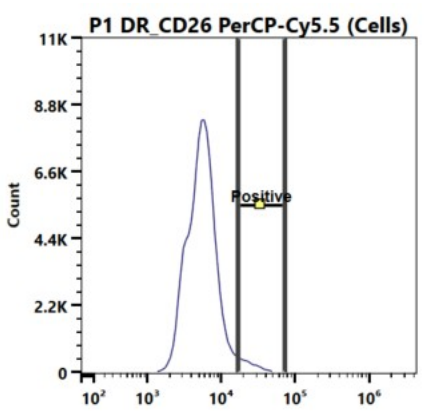


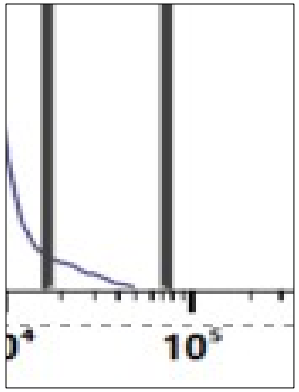



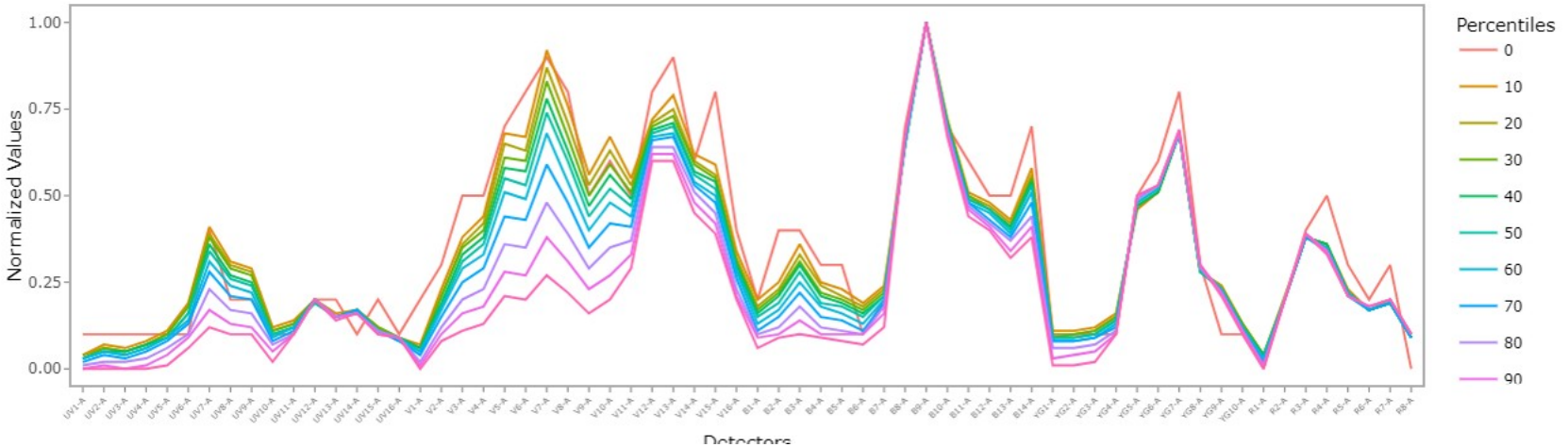



# Be cautious when using cell controls for dim fluorophores or tertiary markers



Split the gated cells into 10 groups by percentile, subtracted autofluorescence, and then normalized



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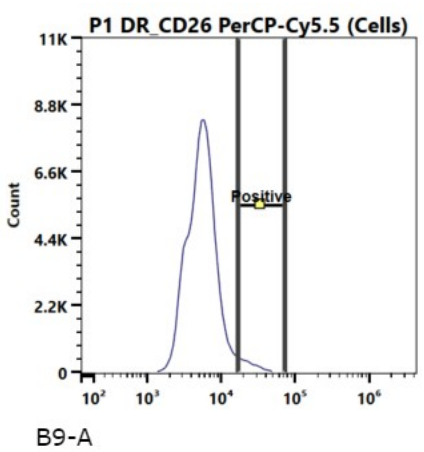


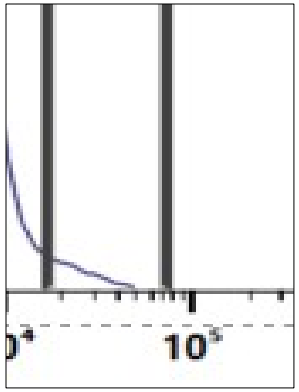

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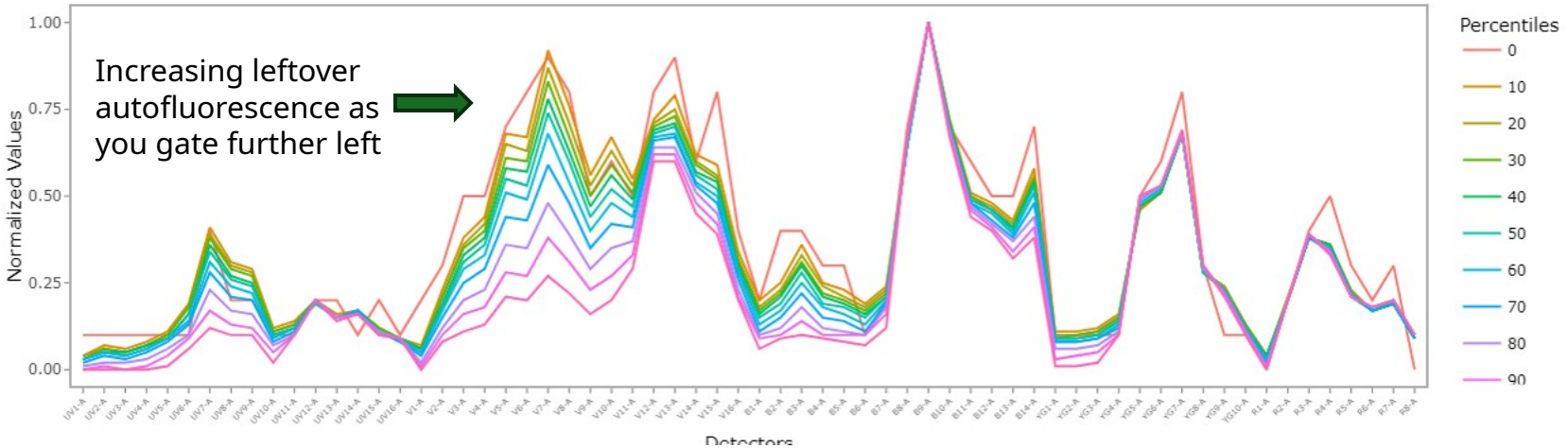
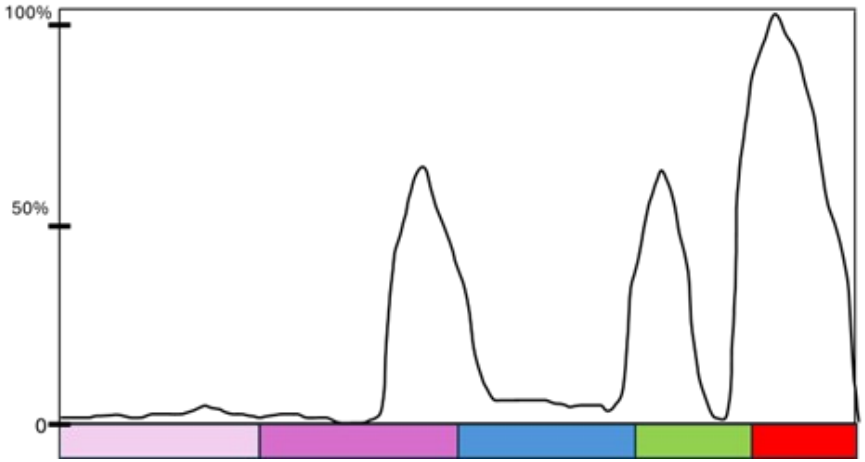
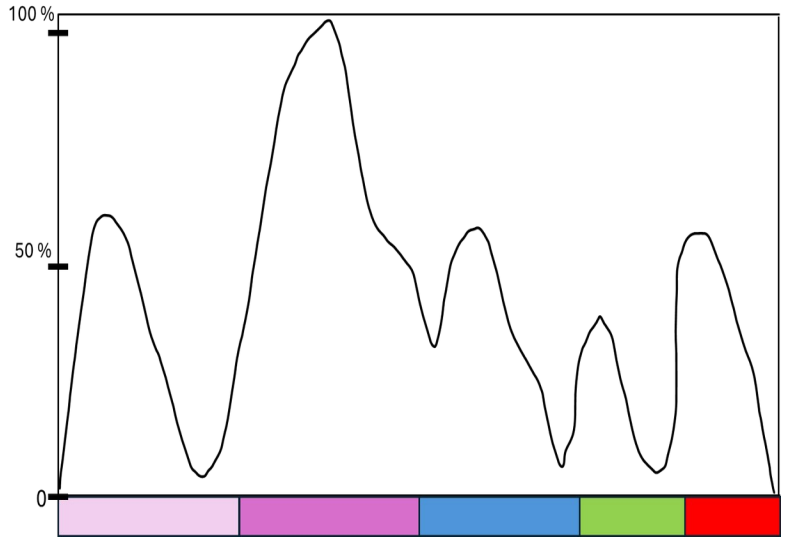
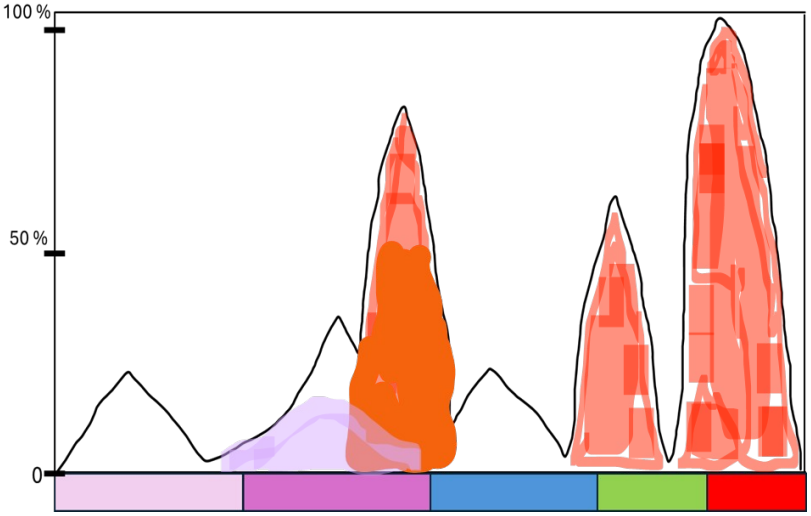


# In terms of the fluorophore signature used for unmixing



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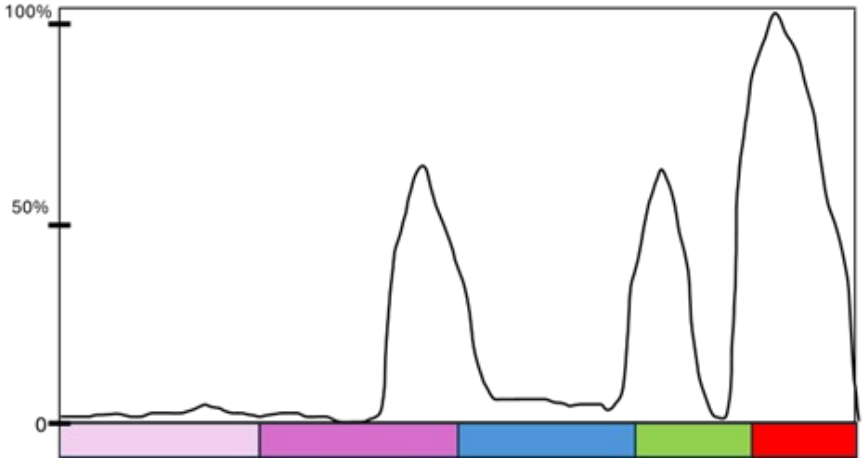


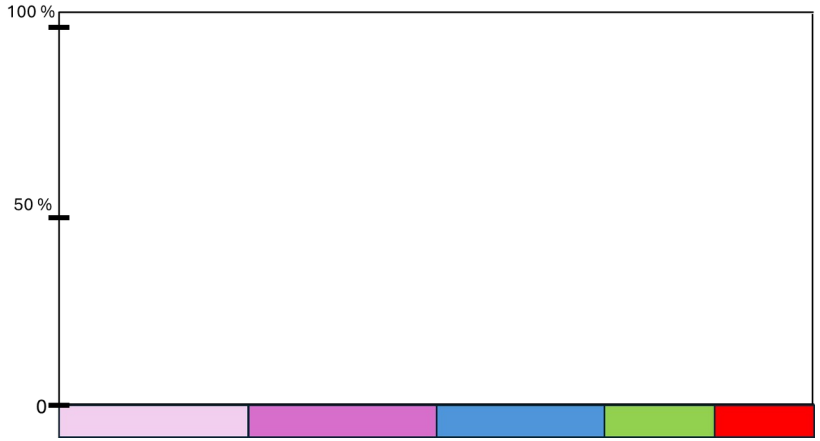


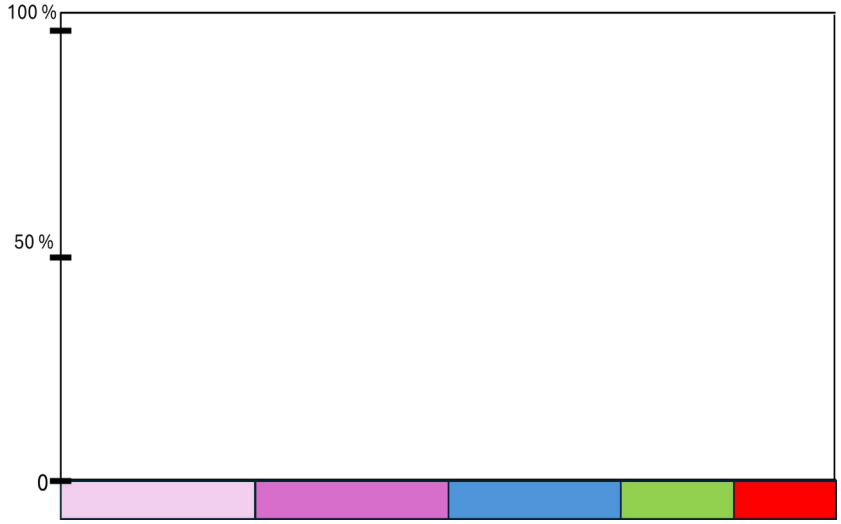


When gating single-color controls, the brightest staining cells are more resilient to signature changes due to leftovers

# One major exception: Antibody aggregates!

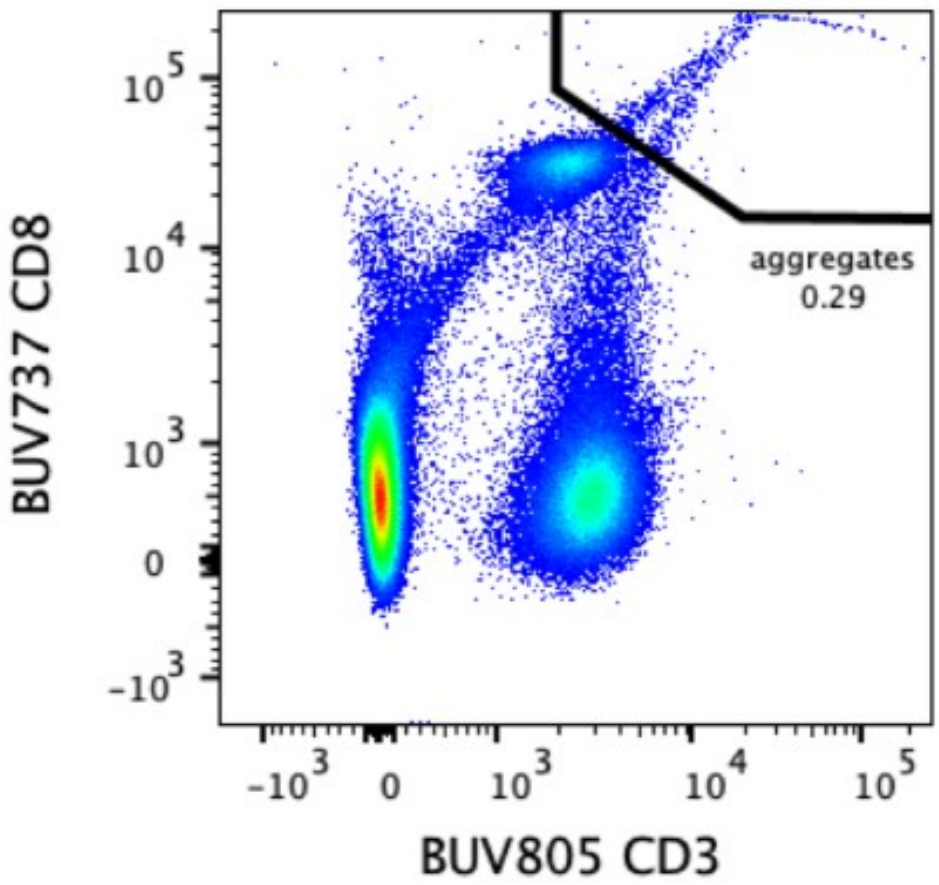


Figure from Laura Johnston (formerly University of Chicago) wonderful blogpost!
https://voices.uchicago.edu/ucflow/2020/09/30/how-to-identify-bad-flow-cytometry-data-part-1/

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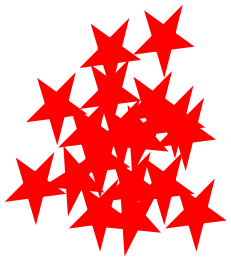
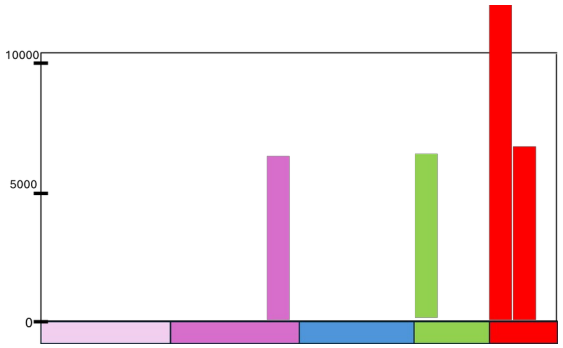
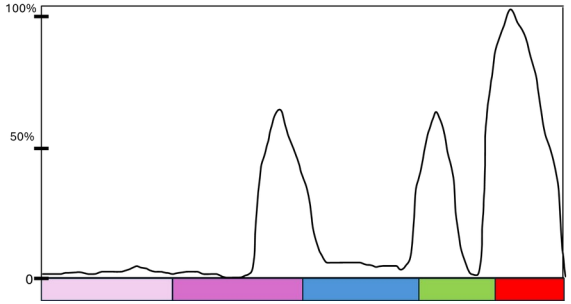
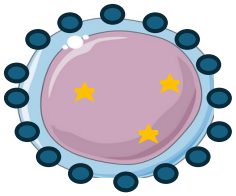
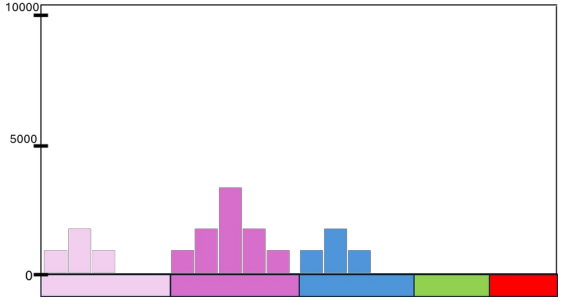


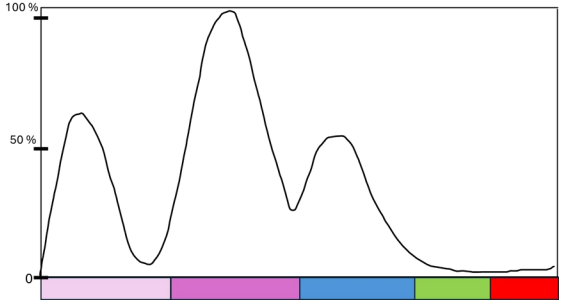



Total Signal



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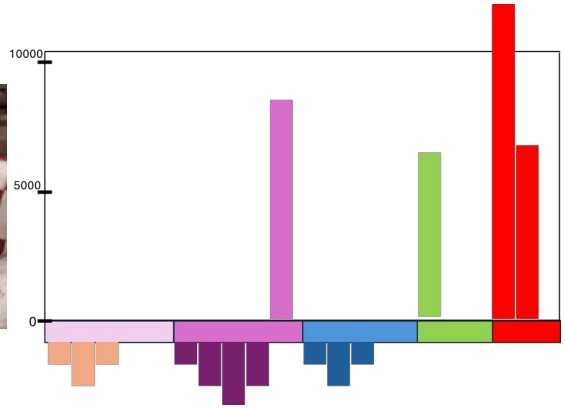
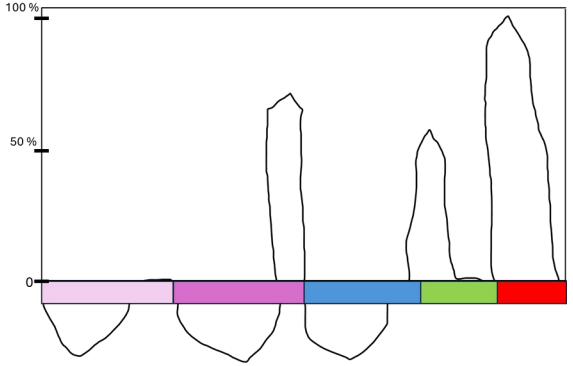
Negative Signal (from Autofluorescence)

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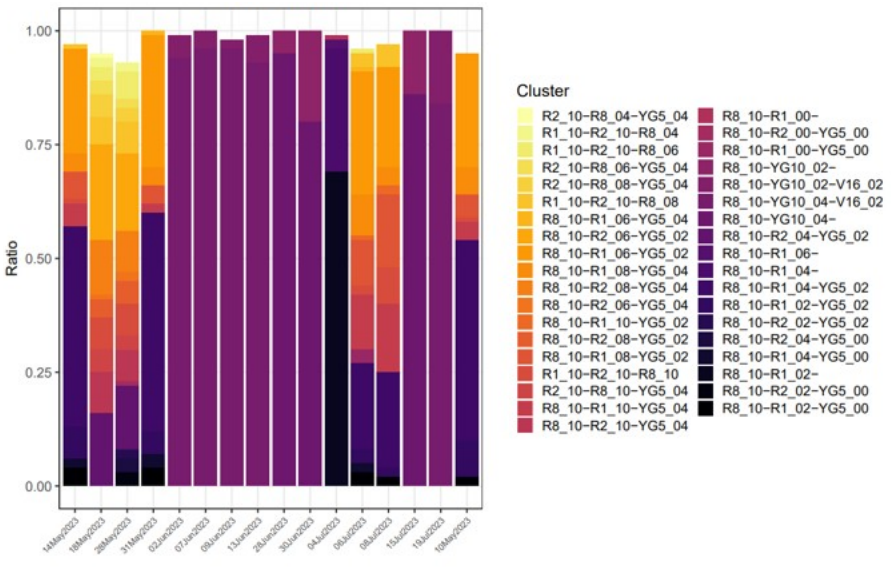
Positive Signal (from Fluorophore)

# Not all problems are due to autofluorescence



# *Your fluorophores can also vary in their signature!!!*




- ❑ Manufacturer Lot Variation
- ❑ Cross-pollinating BUV and BV fluorophores
- ❑ Tandem degradation
- ❑ FRET

**And others!**

But that’s a problem for Thursday’s Single Color and Autofluorescence Troubleshooting session.

# For now: The three rules for good unmixing controls

Previewing Thursday's session

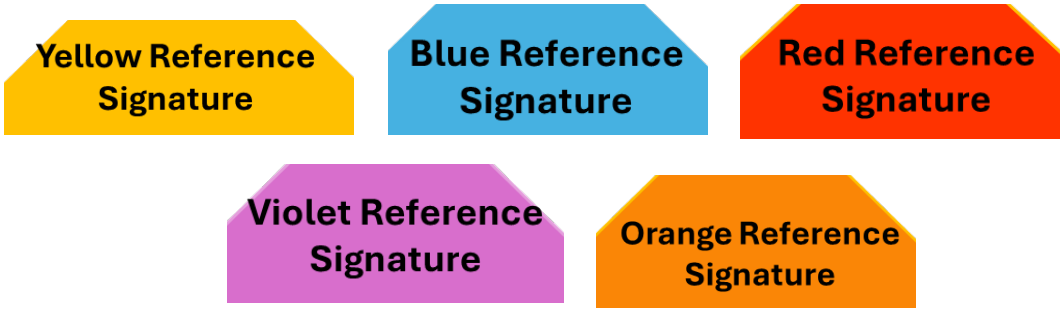
1. The single stained control must be as bright or brighter than the fully stained sample.
2. The exact same fluorophore must be used the stain the control and the fully stained sample.
3. Background autofluorescence should be the same for the positive and negative control

THE UNIVERSITY OF CHICAGO

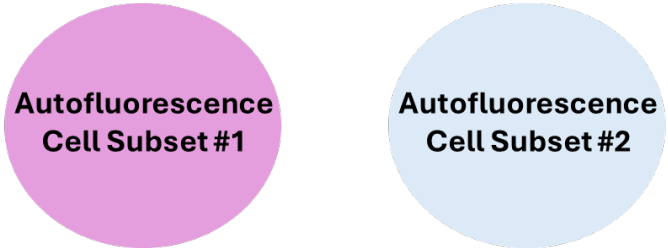
https://voices.uchicago.edu/ucflow/flow-cytometry-resources/

# A spectral cytometer needs three things for successful unmixing:

- ✔ **Single-color Reference Controls**

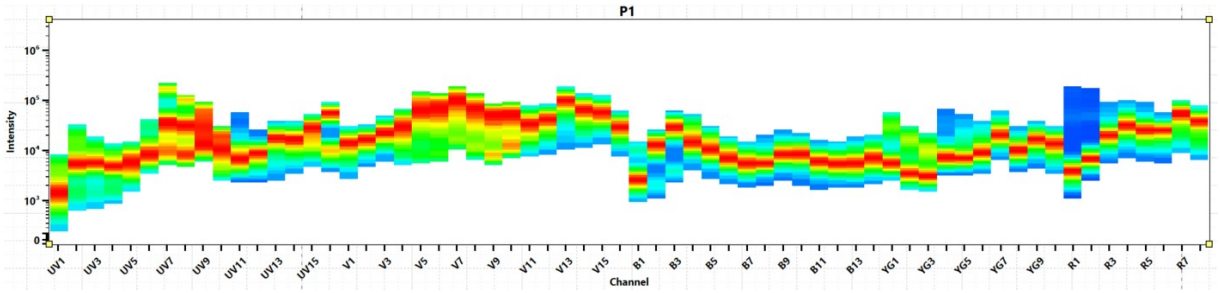



- ✔ **Unstained Reference Controls**



# A spectral cytometer needs three things for successful unmixing:

**Full-stained Samples**



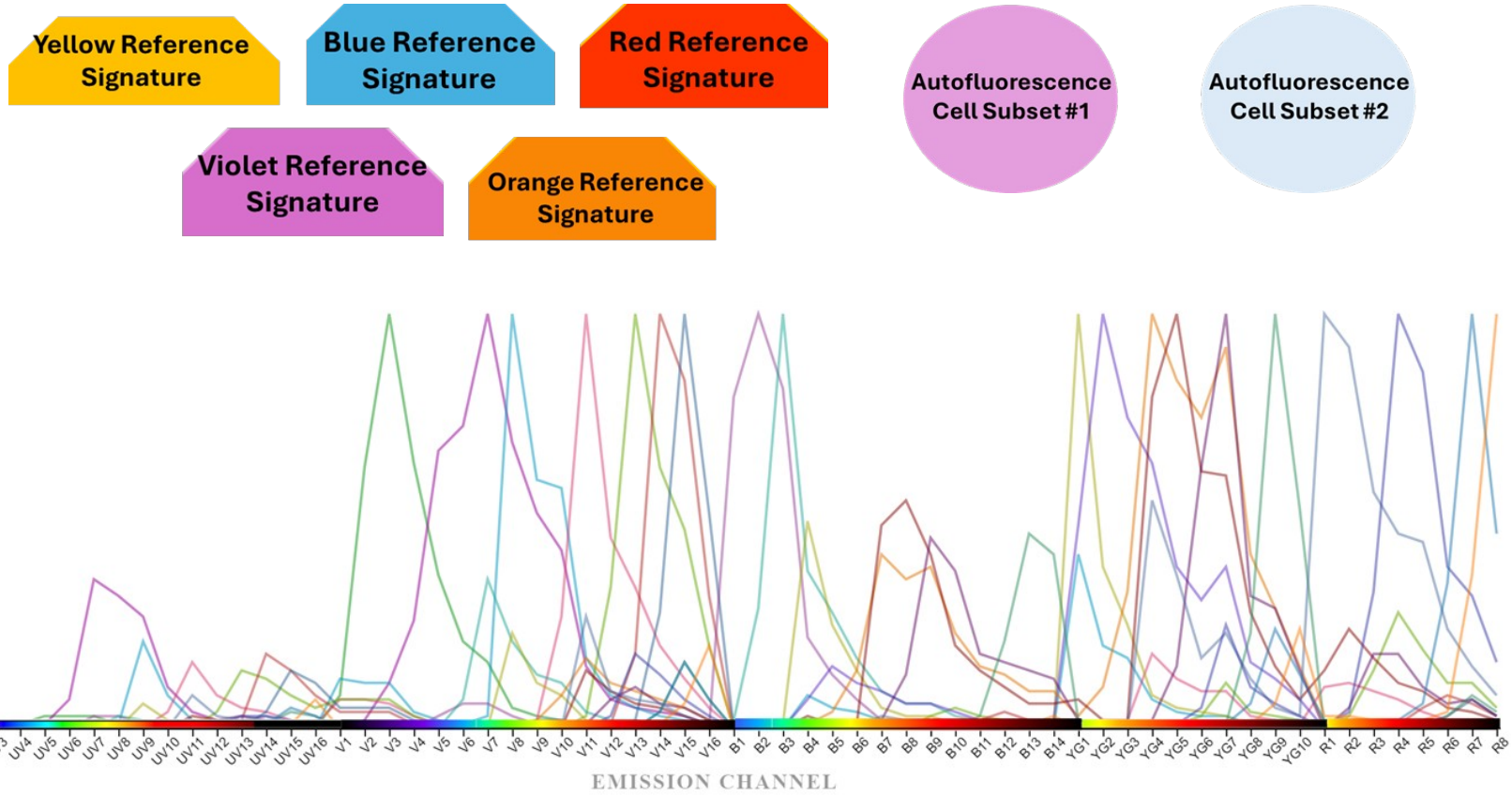

✔ **Single-color Reference Controls**

- Yellow Reference Signature
- Blue Reference Signature
- Red Reference Signature
- Violet Reference Signature
- Orange Reference Signature

✔ **Unstained Reference Controls**

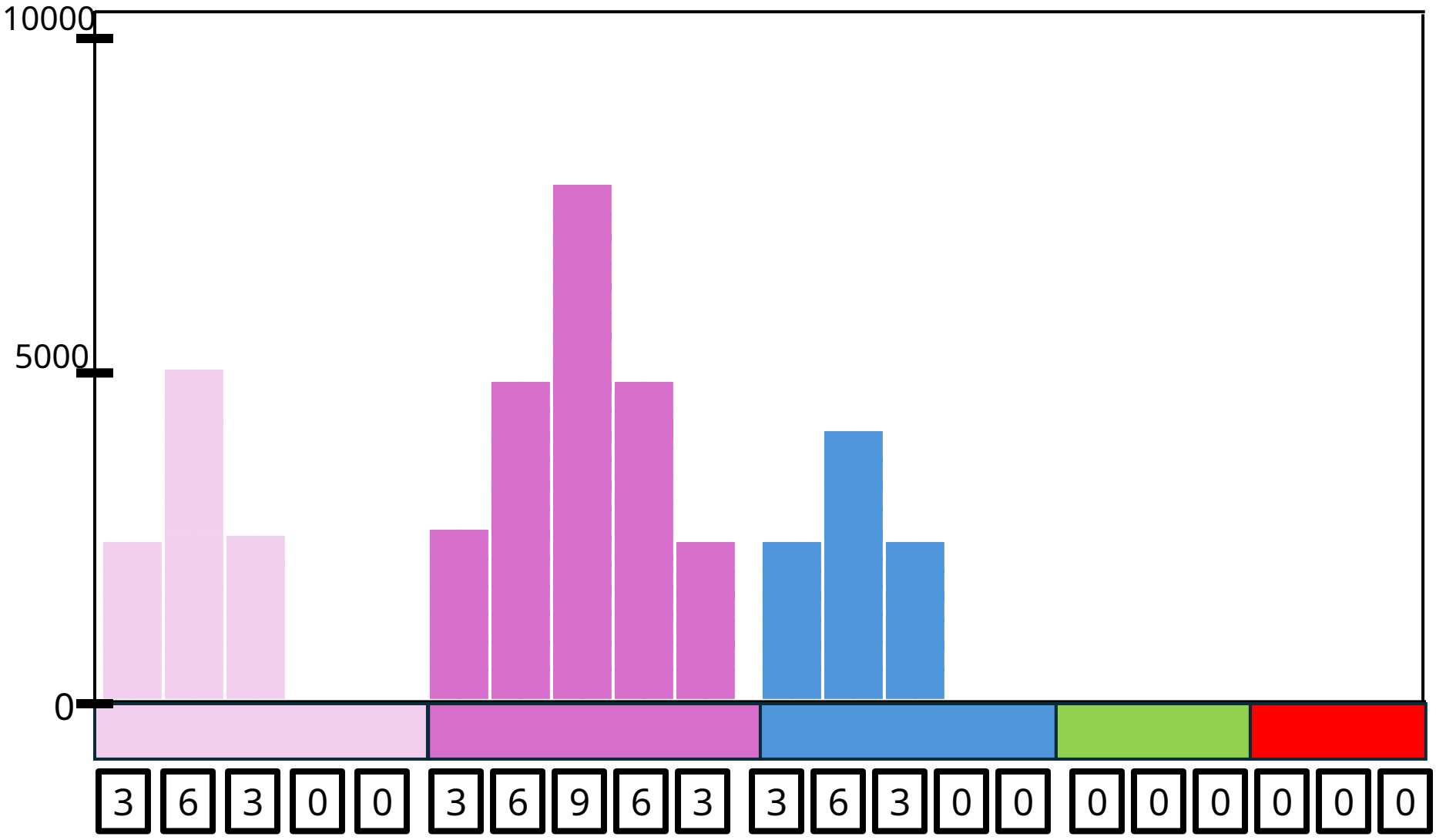
- Autofluorescence Cell Subset #1
- Autofluorescence Cell Subset #2

# Every reference control provides an unique signature




**Then what happens?**

# Each unique signature becomes a reference vector



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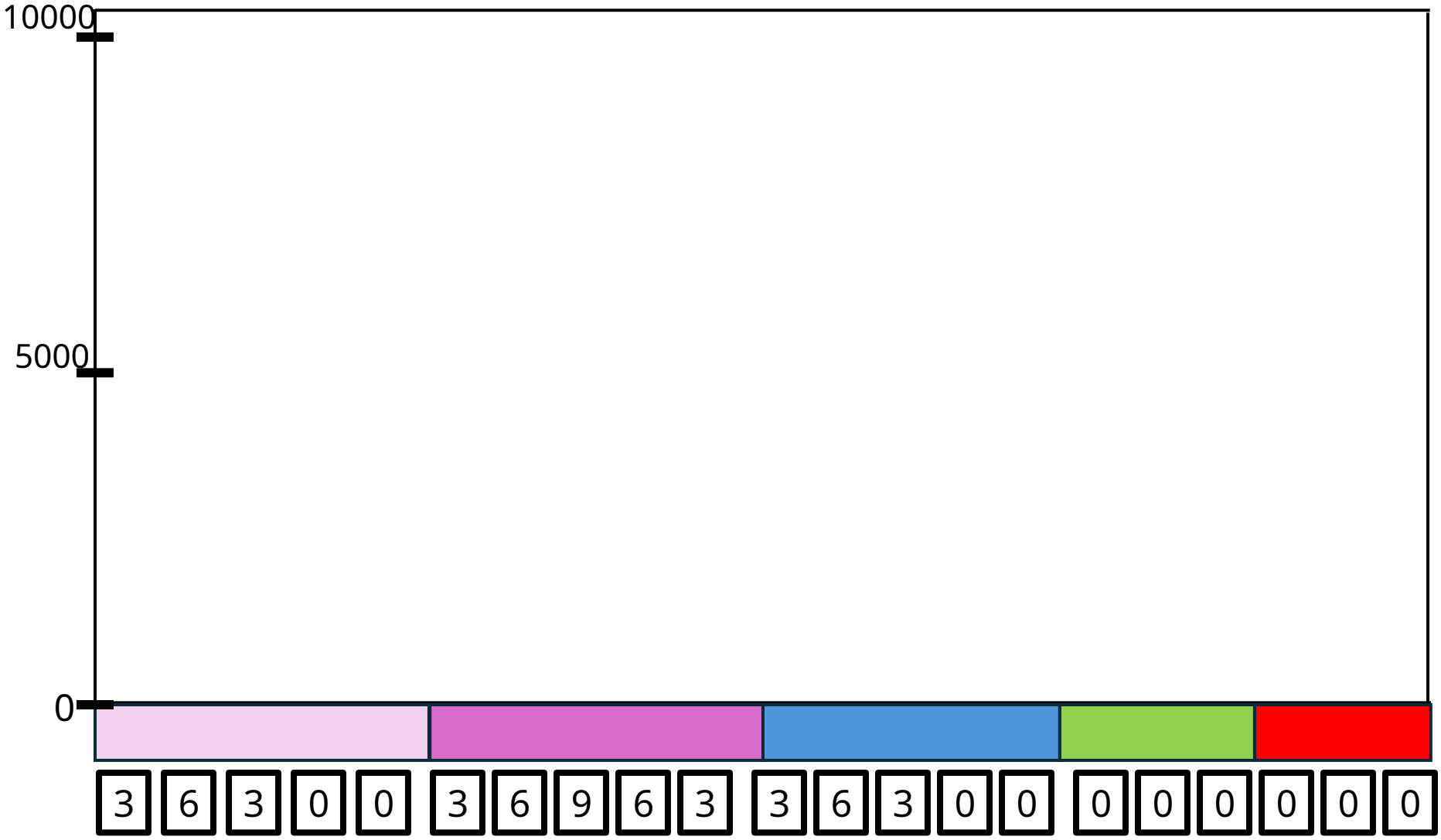

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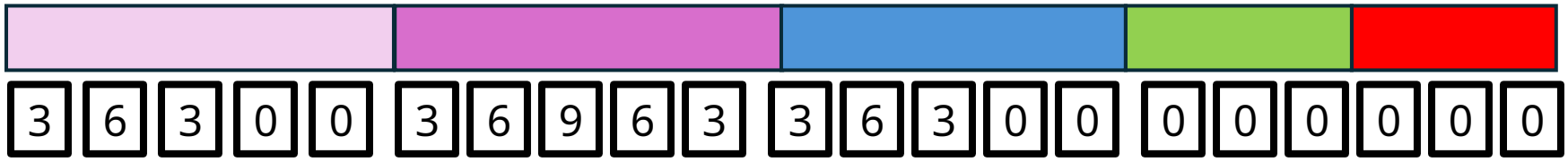
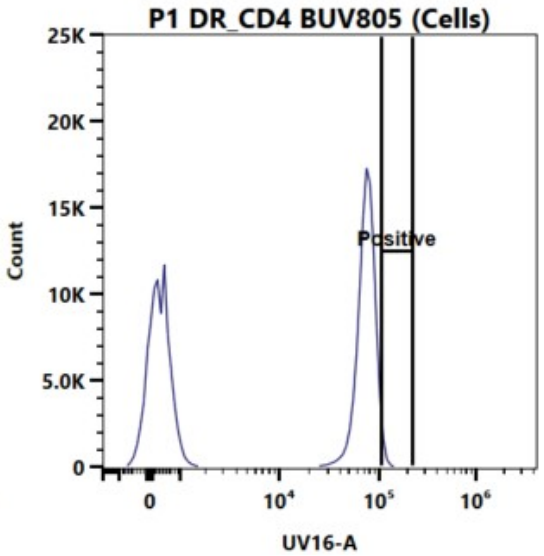


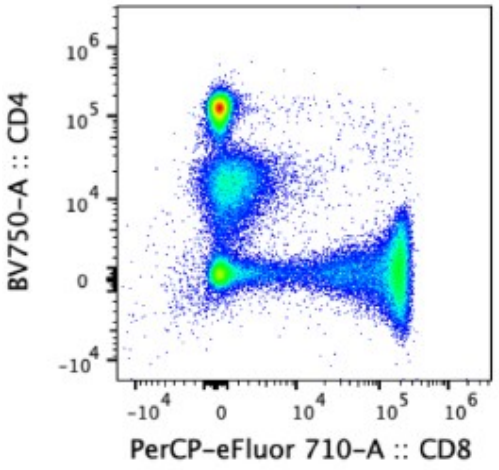
# Wait a second…. Vector…




1) Raw Data

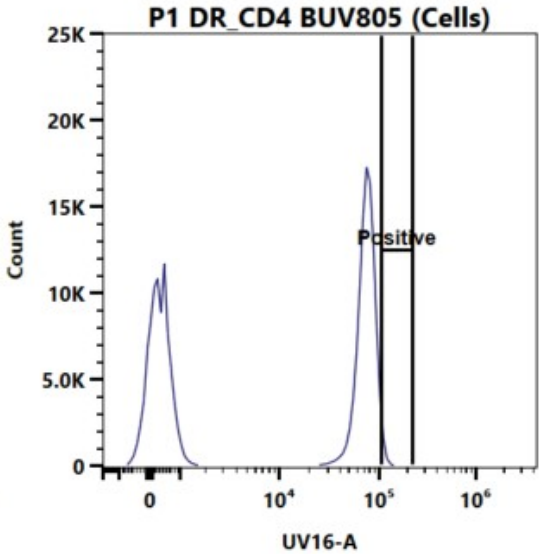


2) ???




3) Unmixed Data

# It’s not magic….

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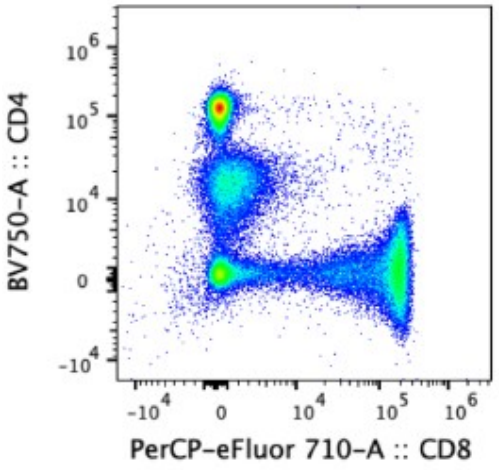
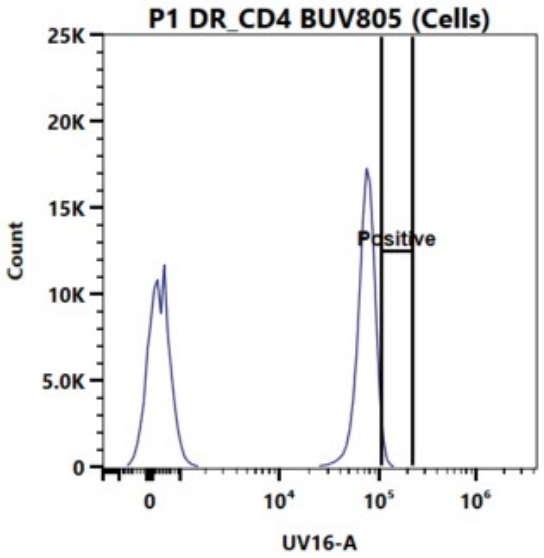
2) ???

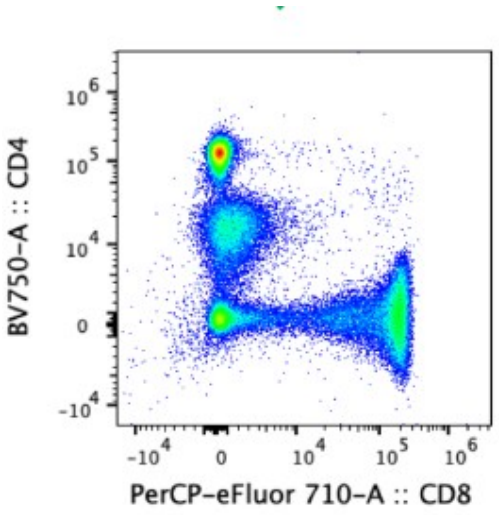



3) Unmixed Data

# It’s math!





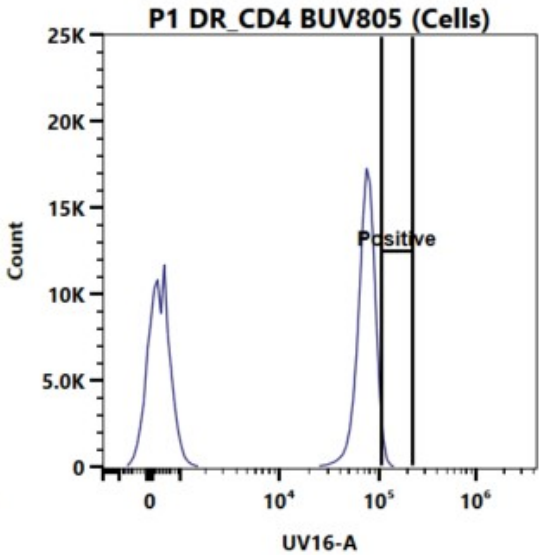




1) Raw Data

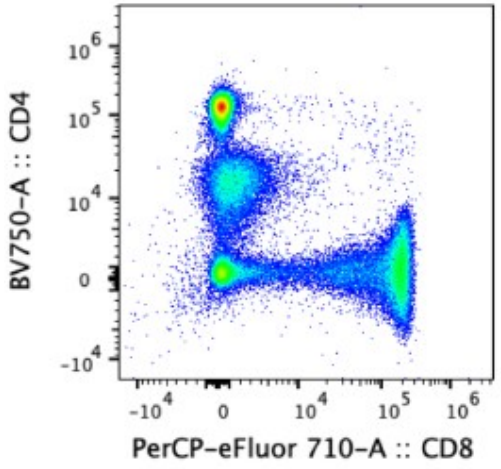
2) Linear Algebra

3) Unmixed Data

# It’s math!





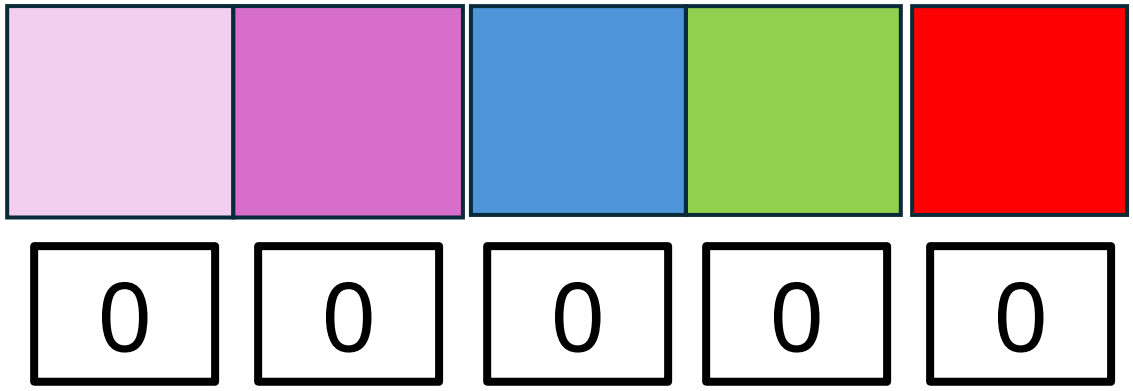

1) Raw Data

2) Linear Algebra

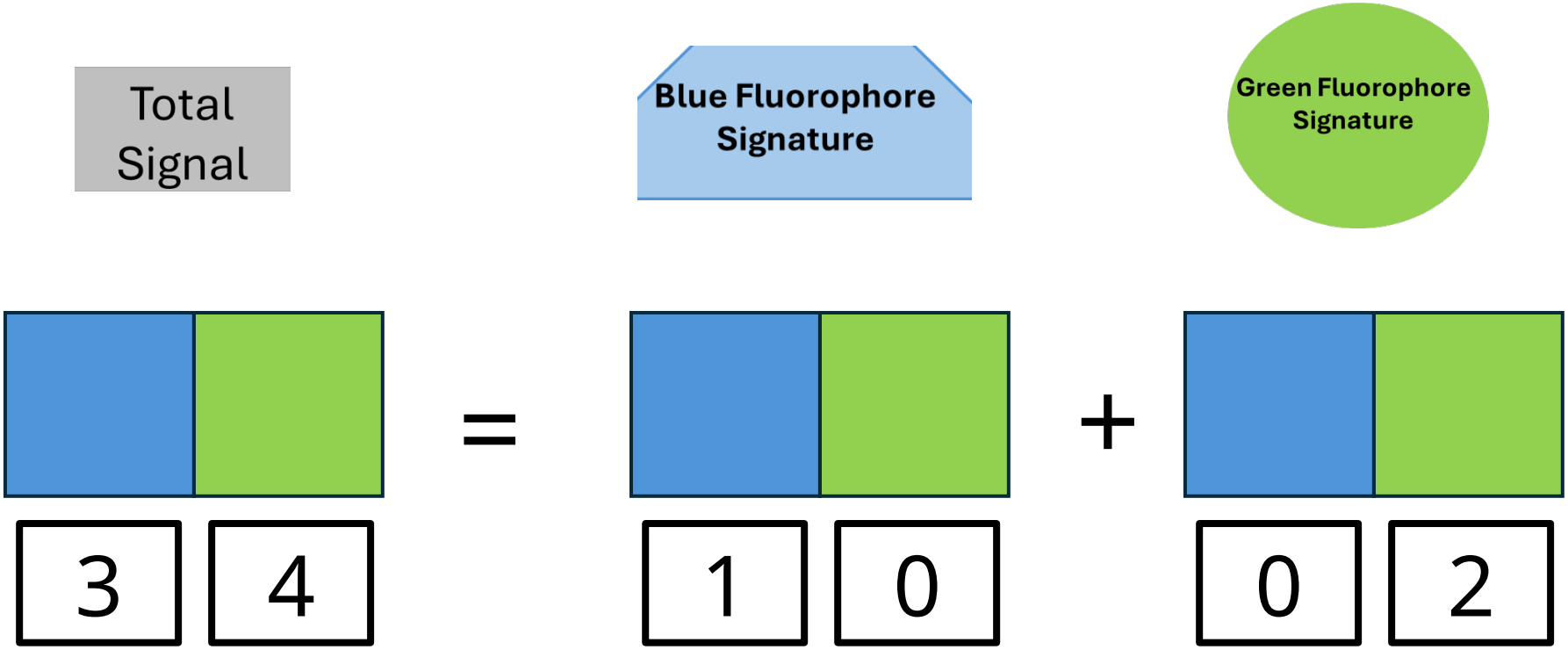
3) Unmixed Data

**Which for biologist might as well be magic**

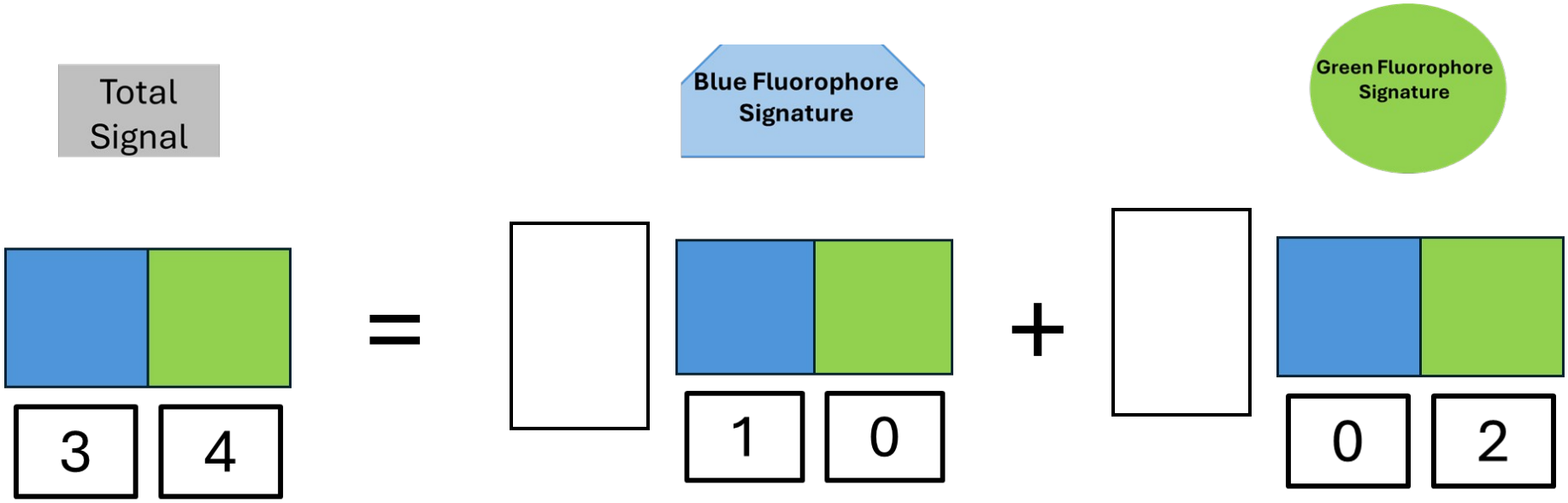
# So lets try to demystify some of underlying math



# A two-detector vector



# Finding a linear combination of Blue and Green that gives us the total



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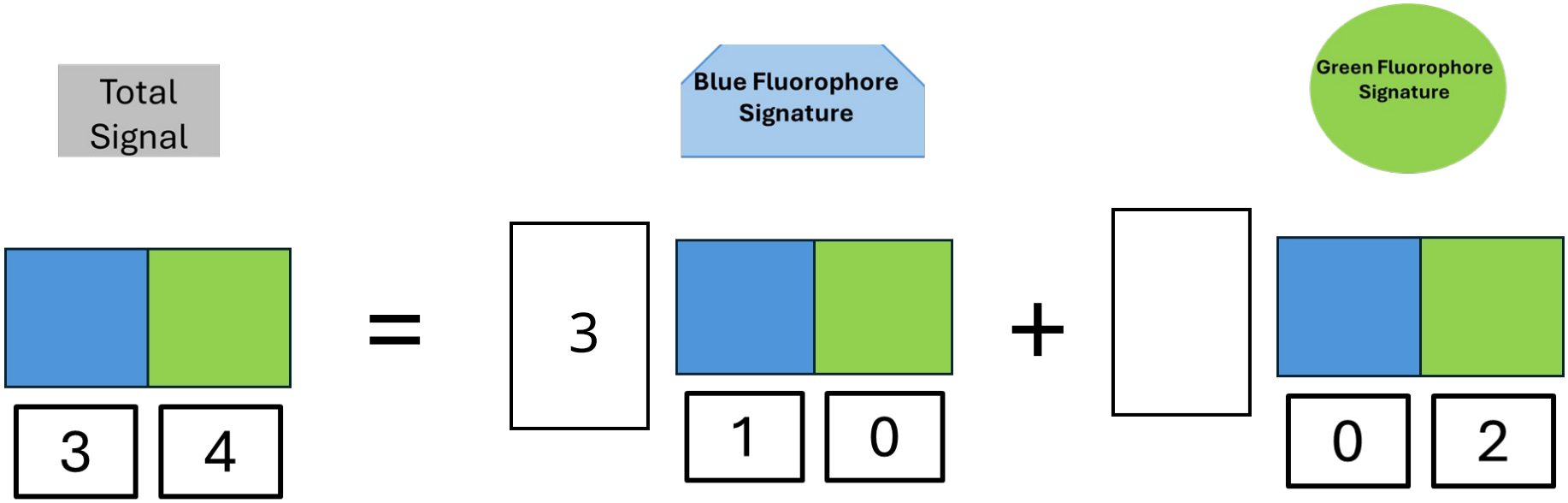

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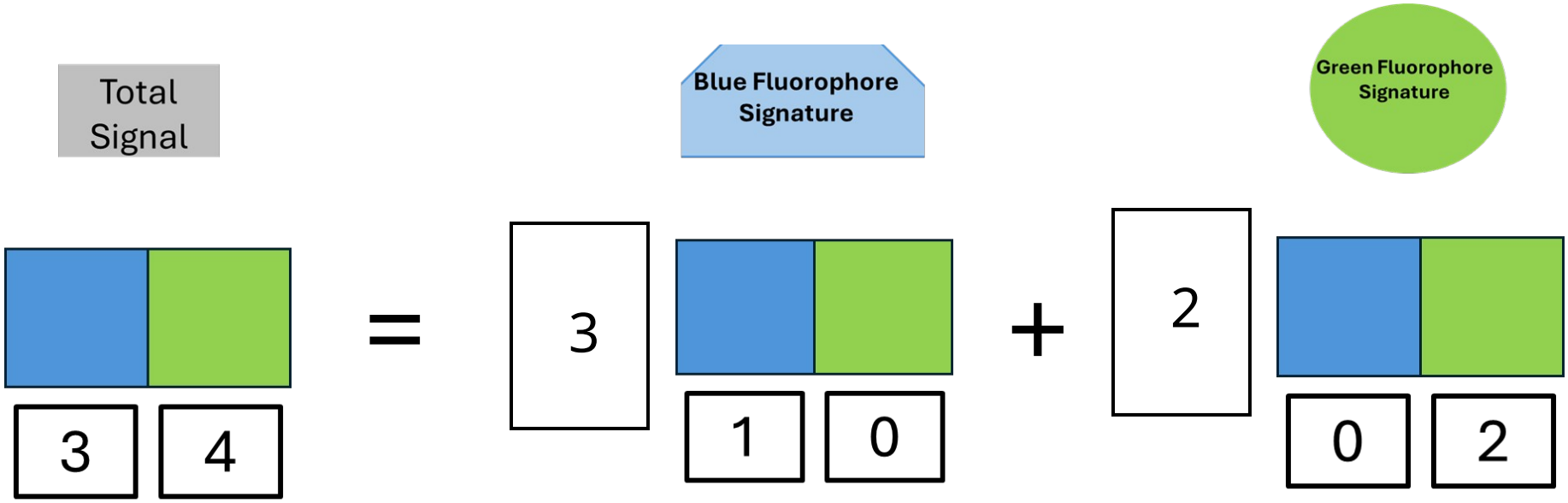
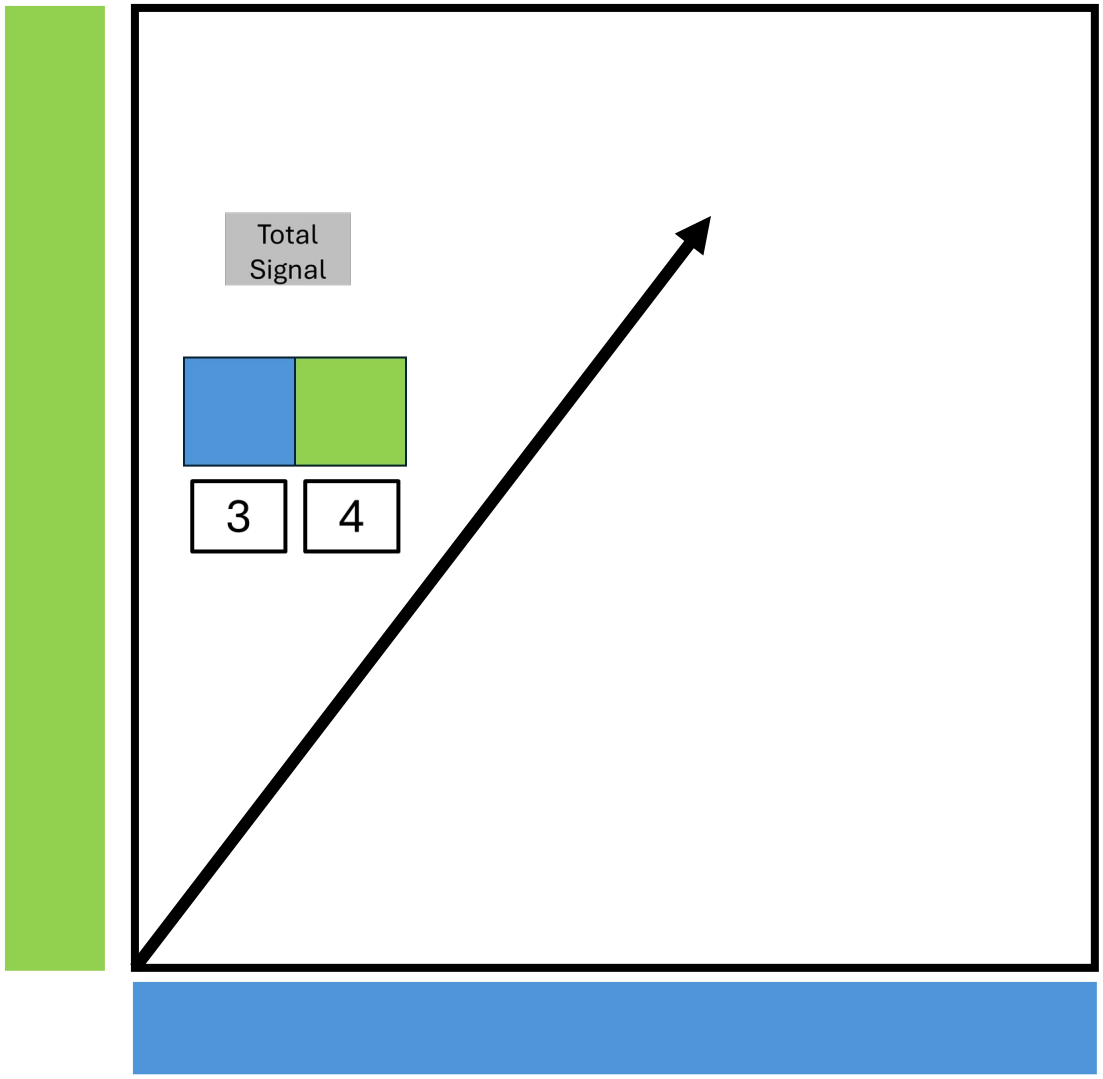


# Viewed differently:



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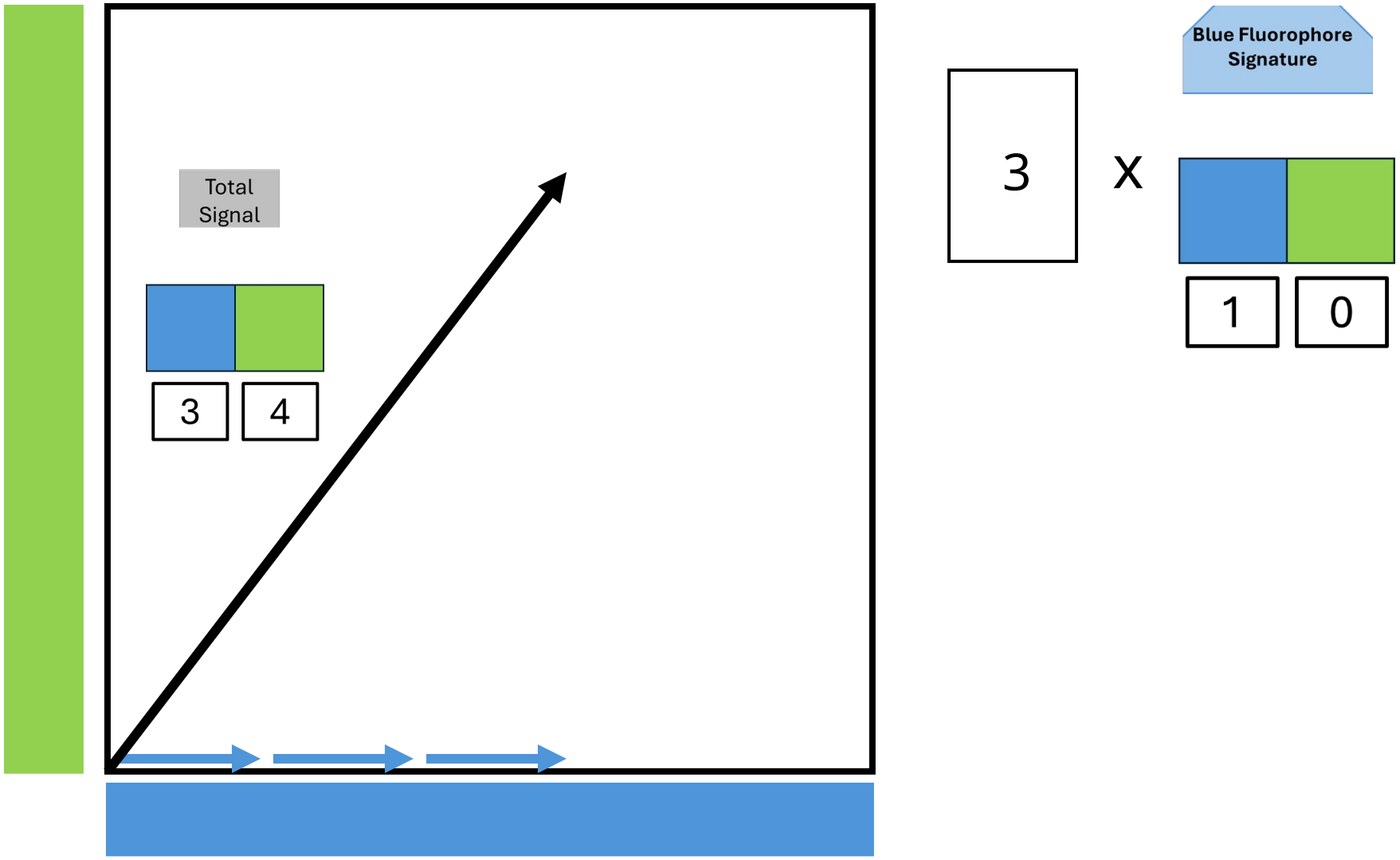

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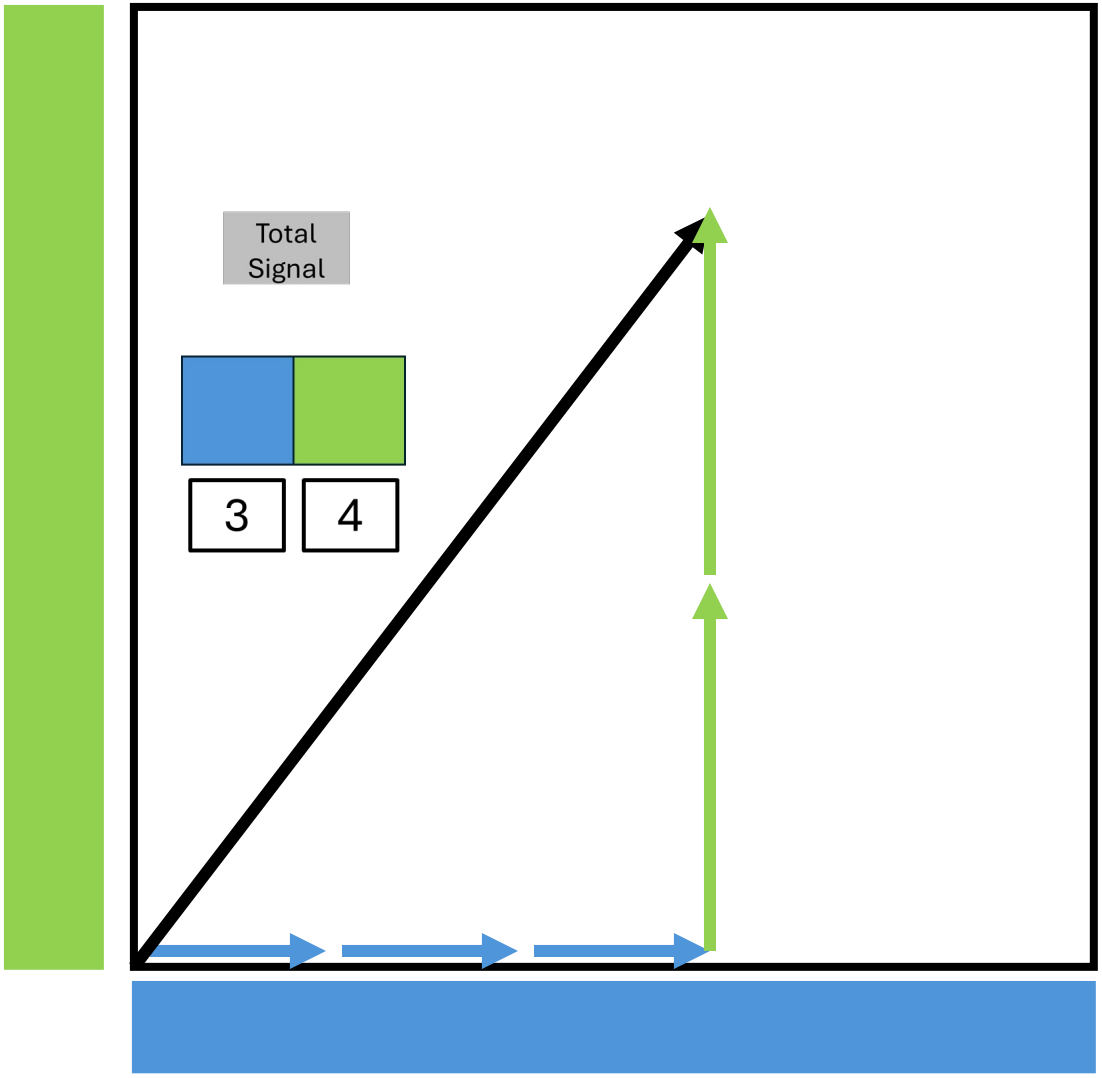
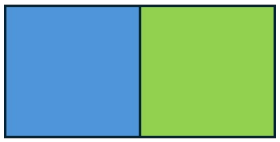



Blue Fluorophore Signature

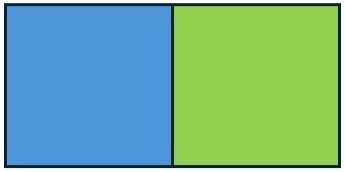
3 x



1 0

Green Fluorophore Signature

2 x



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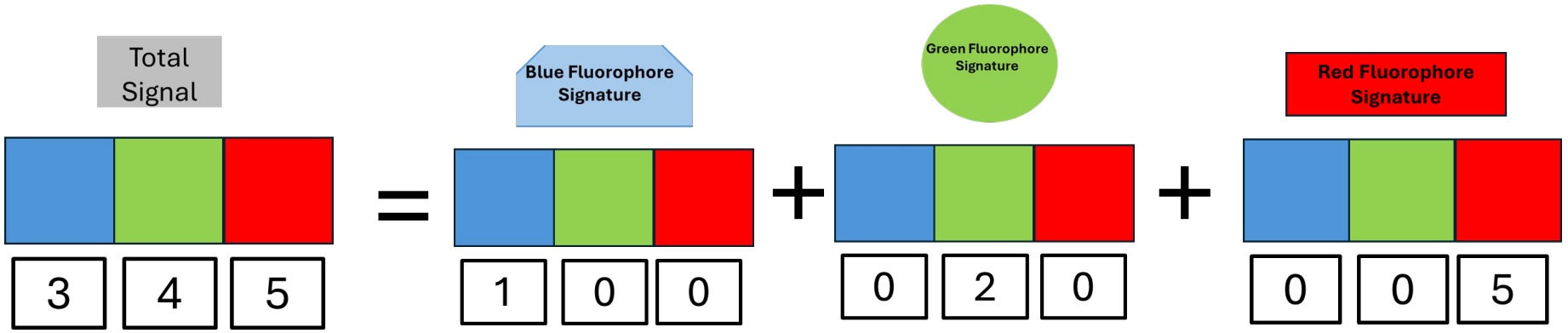
# A three-detector vector



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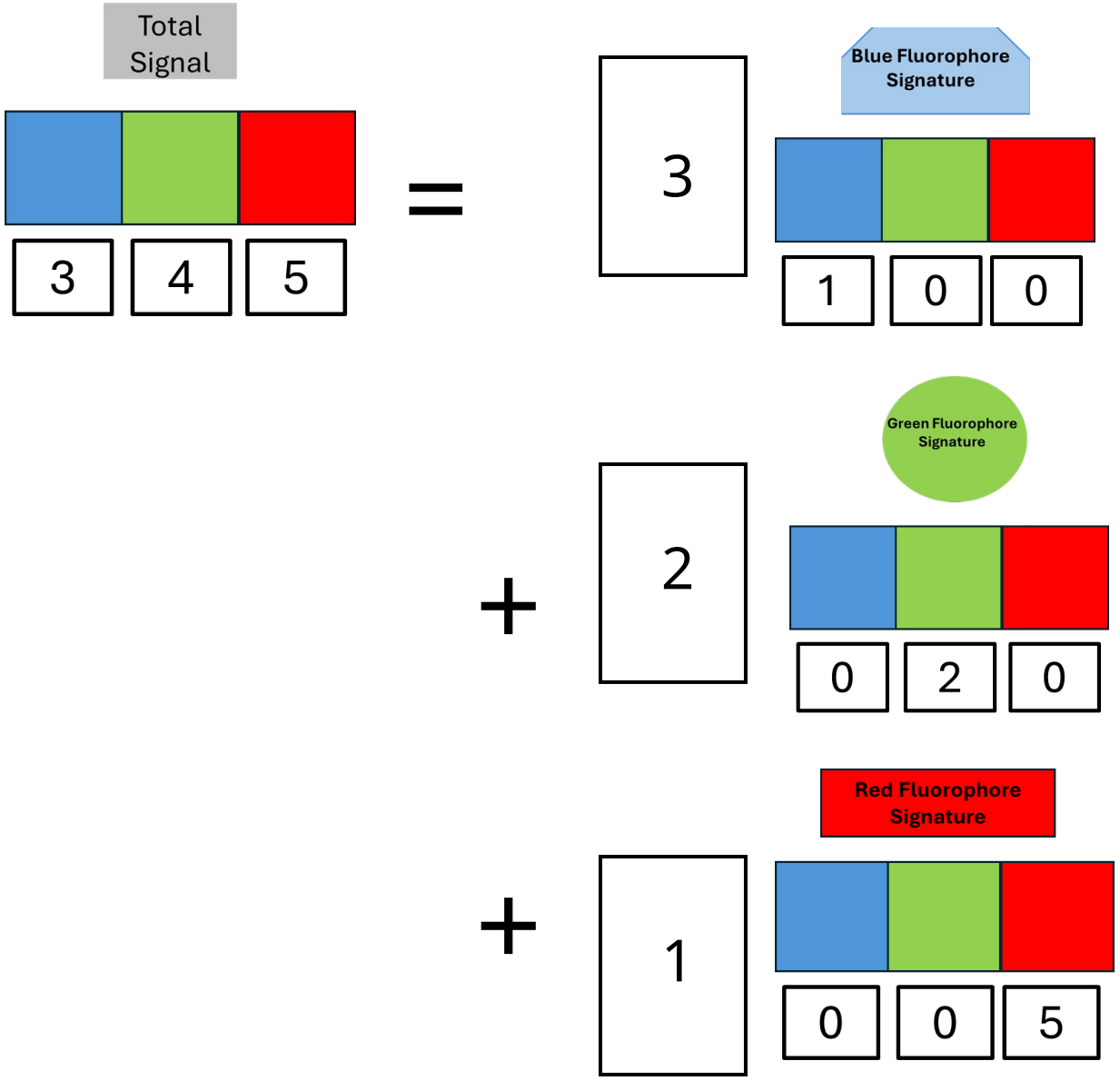
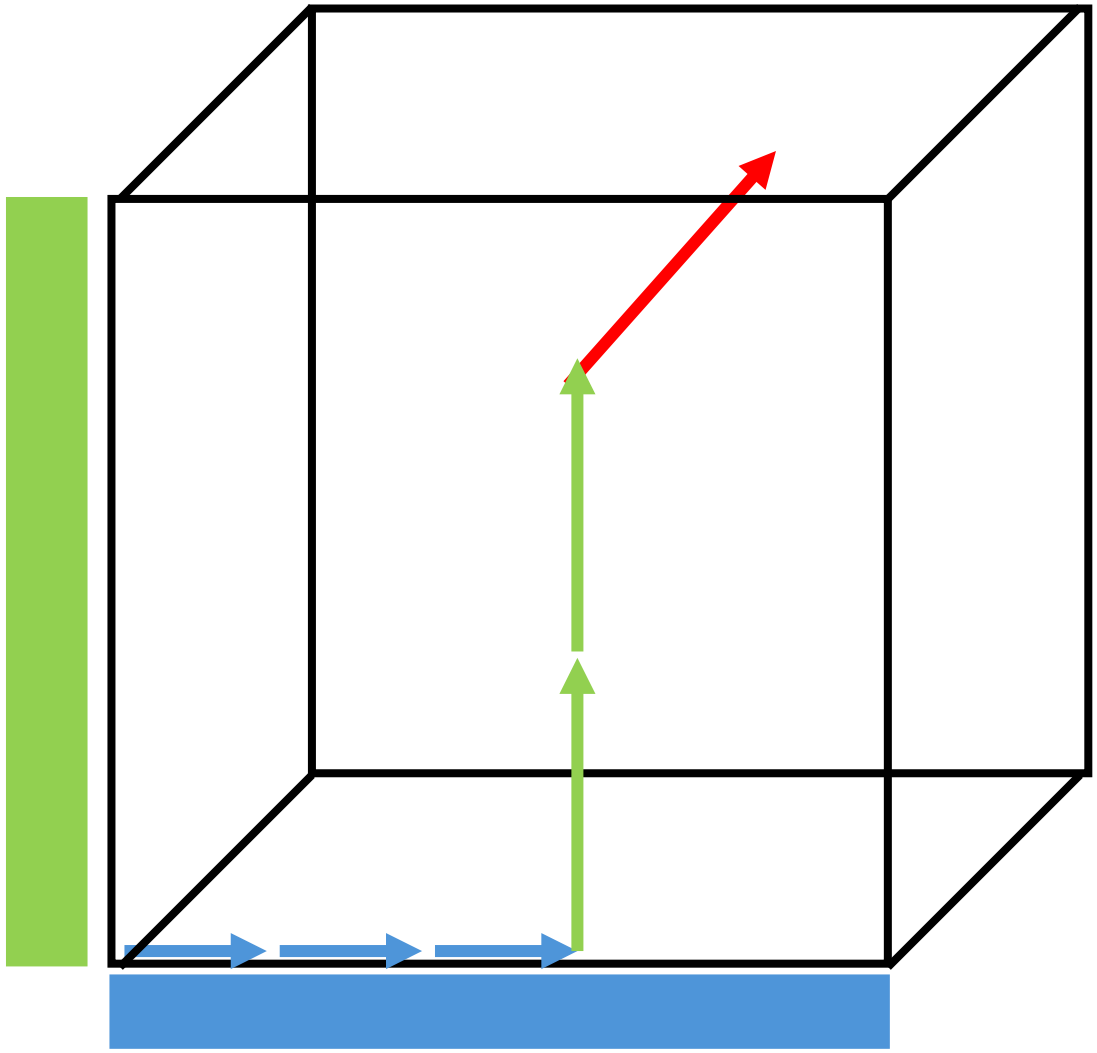
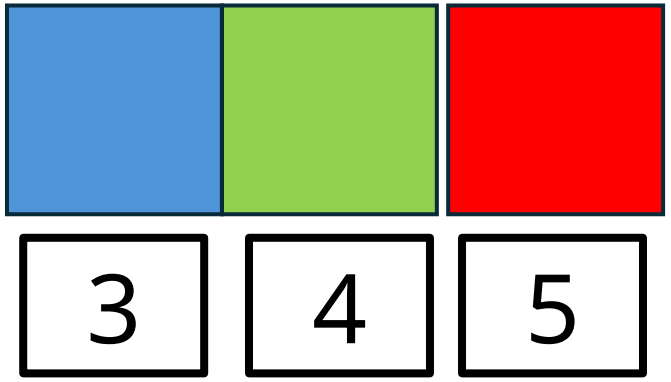
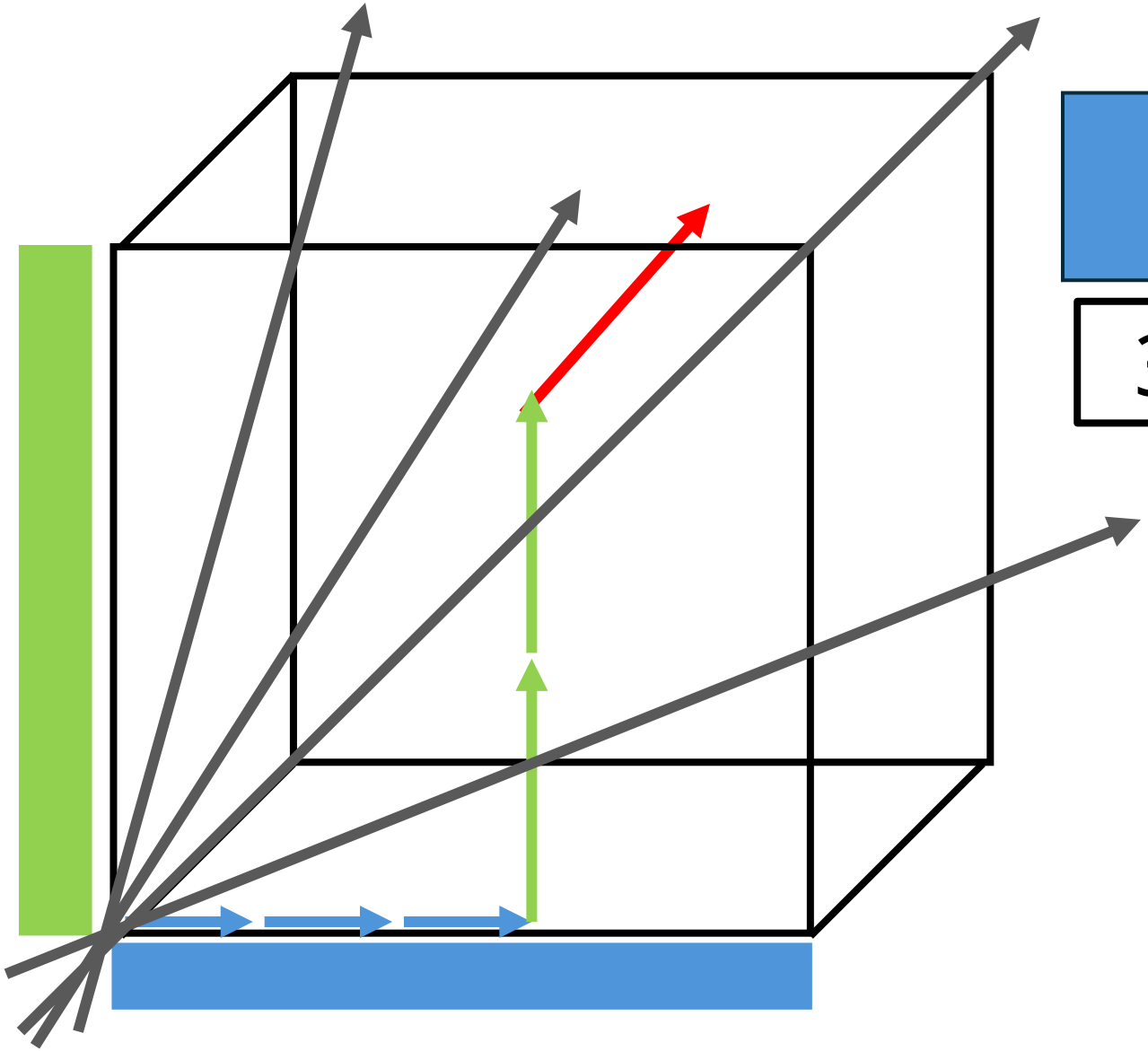


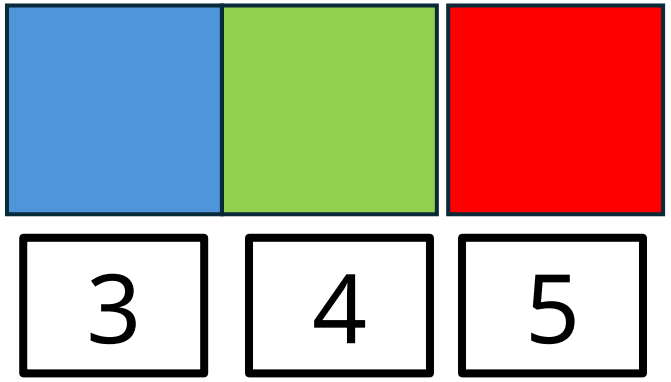
# Where David’s Art Skills End



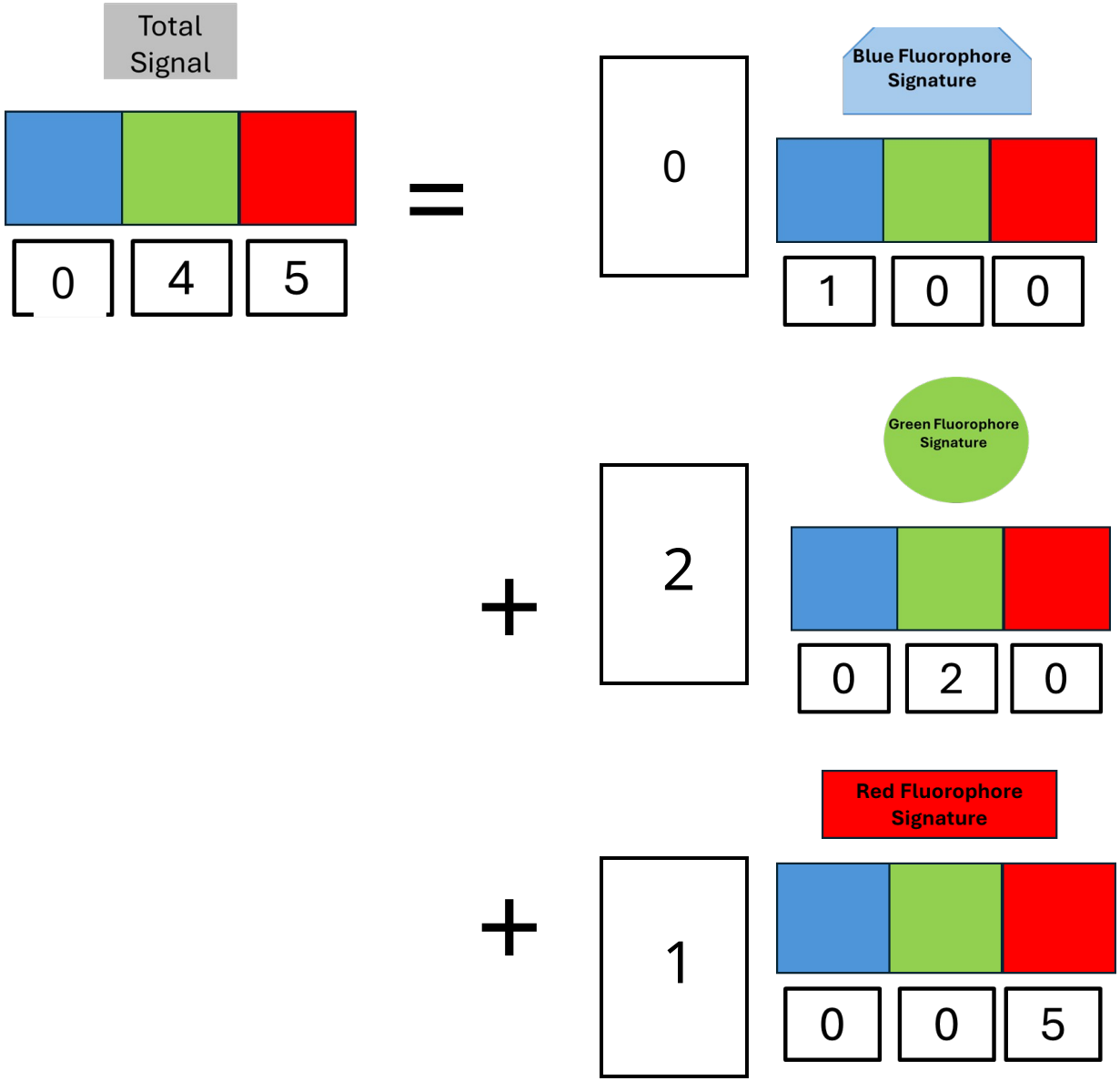


# Lines (or planes) flying through space

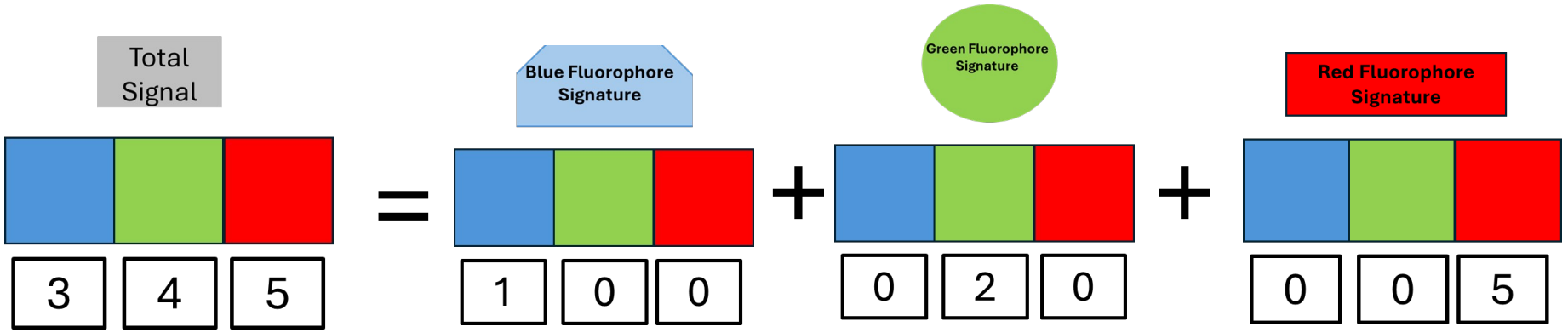


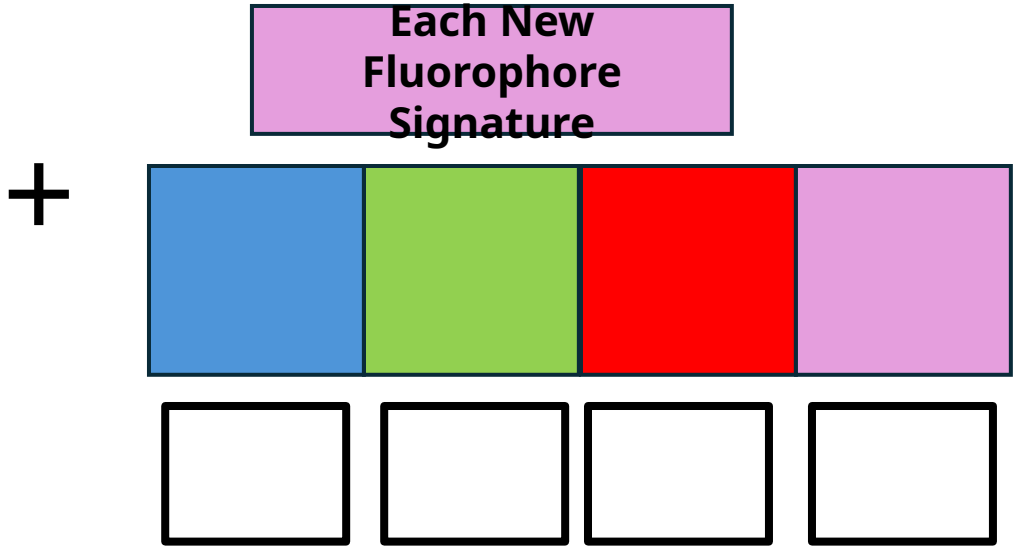


# Also explains: Presence or Absence



# Beyond three-dimensions



# Final Math Details:

- In the above examples, we had the same number of detectors as fluorophores. The matrix generated by collecting the signatures from your single-color controls is a square. This is the case with conventional Flow Cytometry.

- In Spectral Flow Cytometry, we have more detectors than Fluorophores. The matrix generated by collecting the signatures from your single-color controls is a rectangle. This is called an overdetermined system. With rare exceptions, these can’t be solved exactly.

- However, they can be approximated, which is where some variation of least-square algorithm is used (ordinary least squares in SpectroFlos case)

# If you want to learn more:

Cytometry PART A | Journal of Quantitative Cell Science | ISAC

Original Article | Free Access

## Generalized unmixing model for multispectral flow cytometry utilizing nonsquare compensation matrices

David Novo, Gérald Grégori, Bartek Rajwa

First published: 22 March 2013 | https://doi.org/10.1002/cyto.a.22272 | Citations: 54

Cytometry PART A | Journal of Quantitative Cell Science | ISAC

Review Article | Free Access

## A comparison of spectral unmixing to conventional compensation for the calculation of fluorochrome abundances from flow cytometric data

David Novo

First published: 15 July 2022 | https://doi.org/10.1002/cyto.a.24669 | Citations: 2

Cytometry PART A | Journal of Quantitative Cell Science | ISAC

Original Article | Open Access

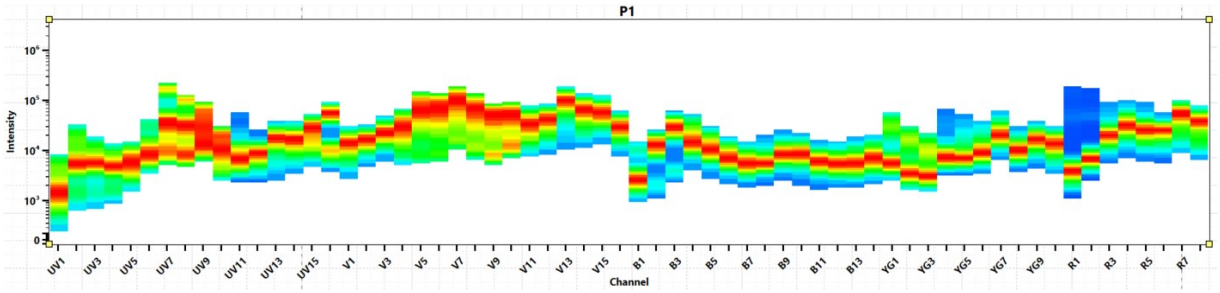
## Unlocking autofluorescence in the era of full spectrum analysis: Implications for immunophenotype discovery projects

Vanta J. Jameson, Tina Luke, Yuting Yan, Angela Hind, Maximilien Evrard, Kevin Man, Laura K. Mackay, Axel Kallies, Jose A. Villadangos, Hamish E. G. McWilliam, Alexis Perez-Gonzalez

First published: 29 March 2022 | https://doi.org/10.1002/cyto.a.24555 | Citations: 5

# A spectral cytometer needs three things for successful unmixing:

- **Full-stained Samples**




- **Single-color Reference Controls**

Yellow Reference Signature

Blue Reference Signature

Red Reference Signature

Violet Reference Signature

Orange Reference Signature

- **Unstained Reference Controls**

Autofluorescence Cell Subset #1

Autofluorescence Cell Subset #2

# Unmixing's Quandaries

Each cell in your full-stained sample is unmixed **individually**...

- Presence (or absence) of each Fluorophore
- Relative expression of each Fluorophore

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By referencing the unique signatures from the unstained & single-color reference controls…

Which are derived from taking the mean/median fluorescence measurement of a gated **population** of reference cells…

….after first subtracting out background autofluorescent signature based on a gated **population** of unstained cells.

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Variation here will impact your unmixing results

# For now: The three rules for good unmixing controls

1. The single stained control must be as bright or brighter than the fully stained sample.

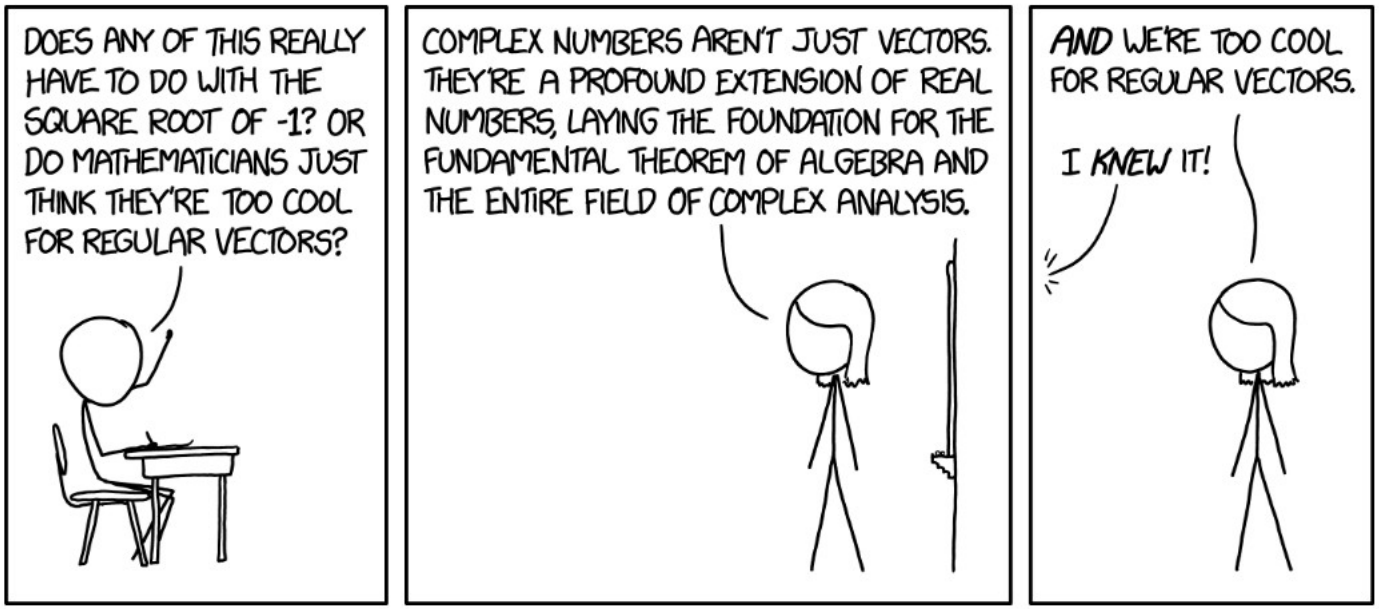
2. The exact same fluorophore must be used the stain the control and the fully stained sample.

3. Background autofluorescence should be the same for the positive and negative control

THE UNIVERSITY OF CHICAGO

https://voices.uchicago.edu/ucflow/flow-cytometry-resources/

# Questions?


DOES ANY OF THIS REALLY HAVE TO DO WITH THE SQUARE ROOT OF -1? OR DO MATHEMATICIANS JUST THINK THEY'RE TOO COOL FOR REGULAR VECTORS?
COMPLEX NUMBERS AREN'T JUST VECTORS. THEY'RE A PROFOUND EXTENSION OF REAL NUMBERS, LAYING THE FOUNDATION FOR THE FUNDAMENTAL THEOREM OF ALGEBRA AND THE ENTIRE FIELD OF COMPLEX ANALYSIS.
AND WE'RE TOO COOL FOR REGULAR VECTORS.
I KNEW IT!