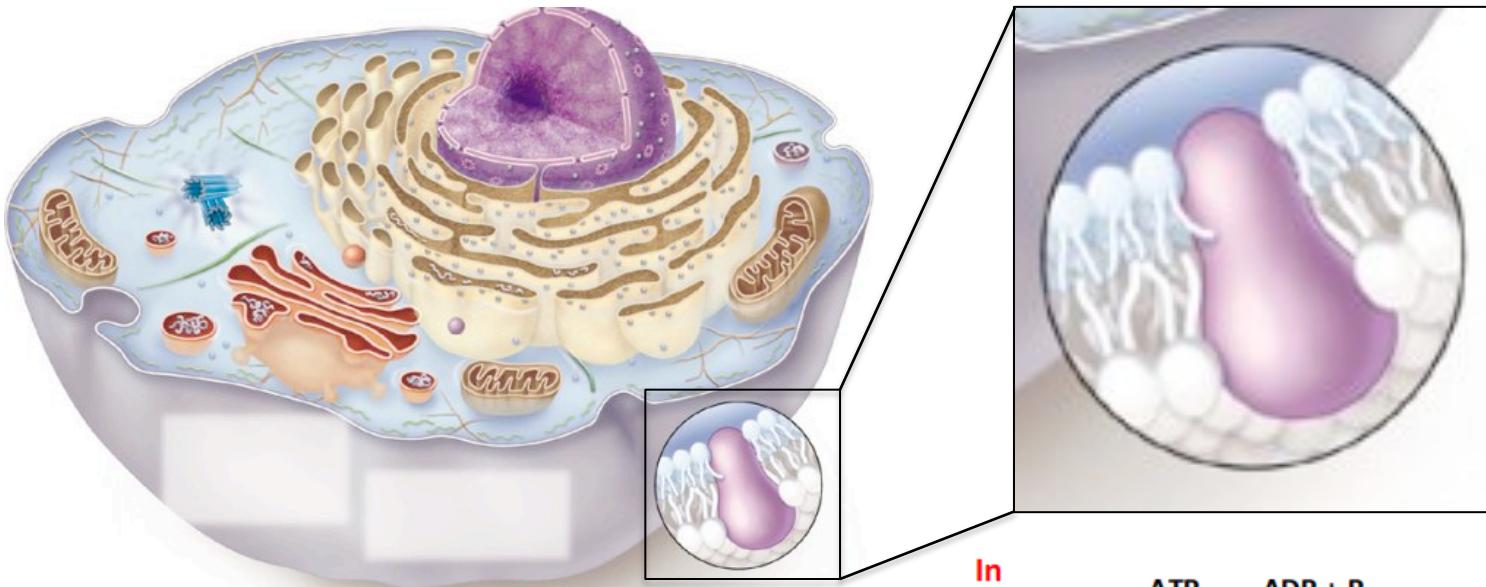


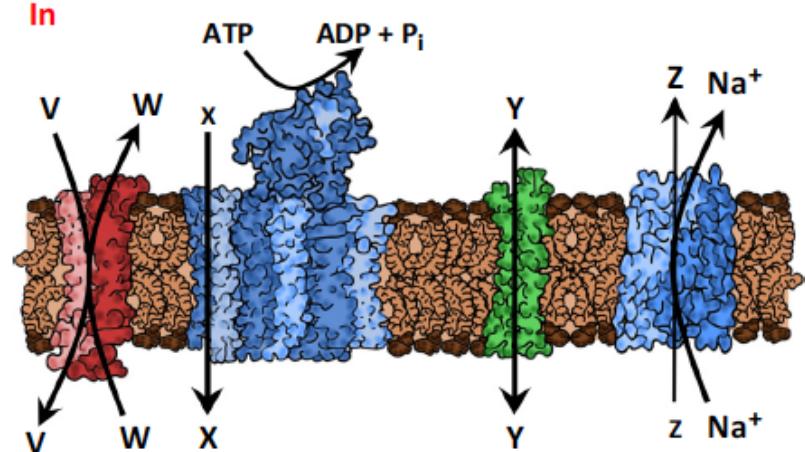
Engineering membrane protein with structure guided recombination

Claire Bedbrook

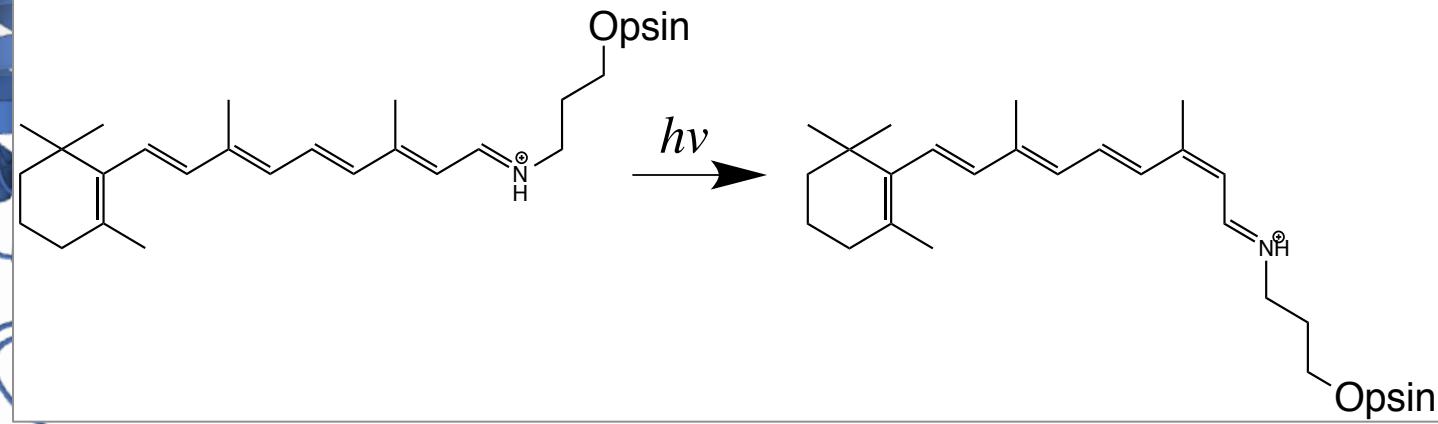
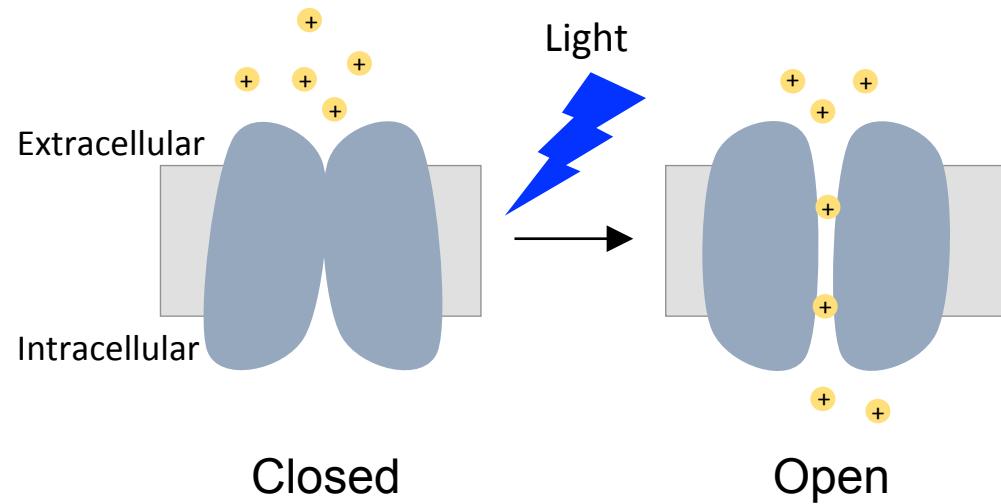
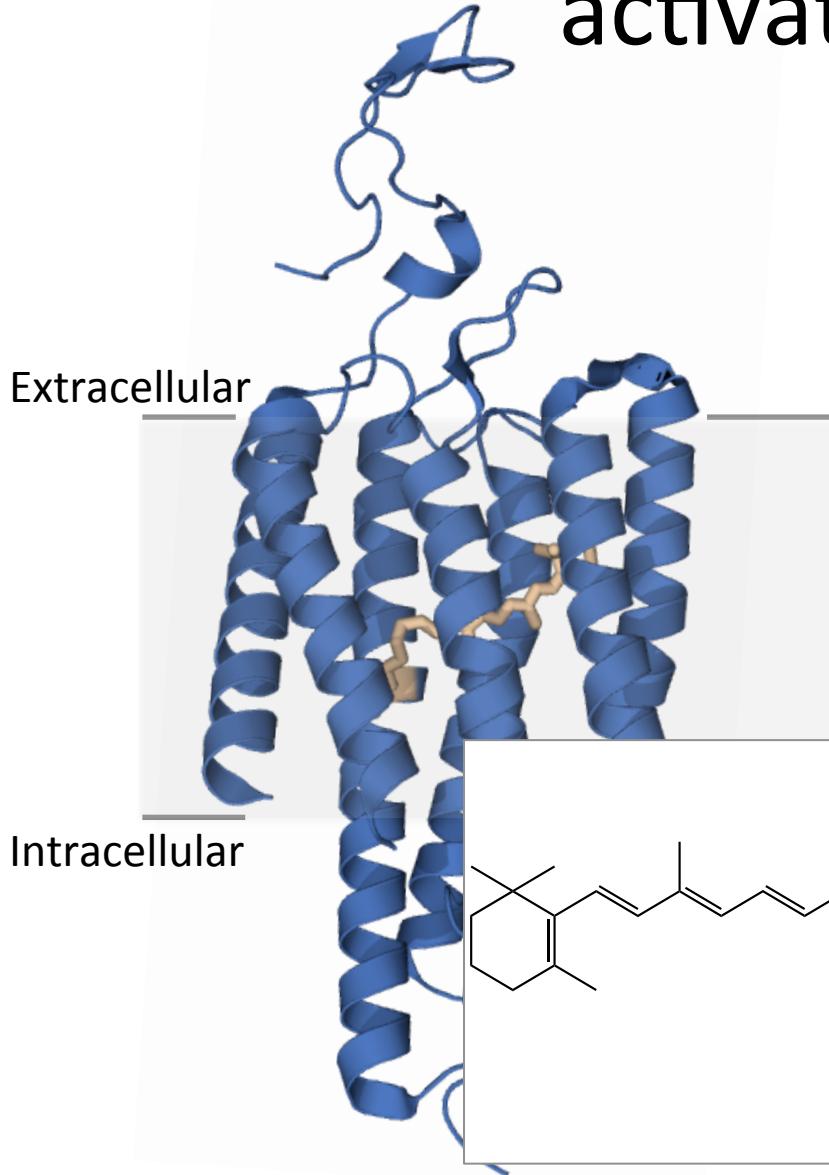
Engineering of membrane proteins



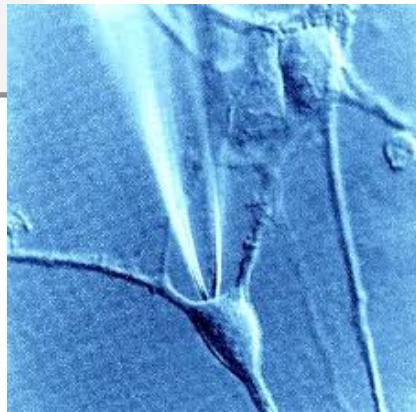
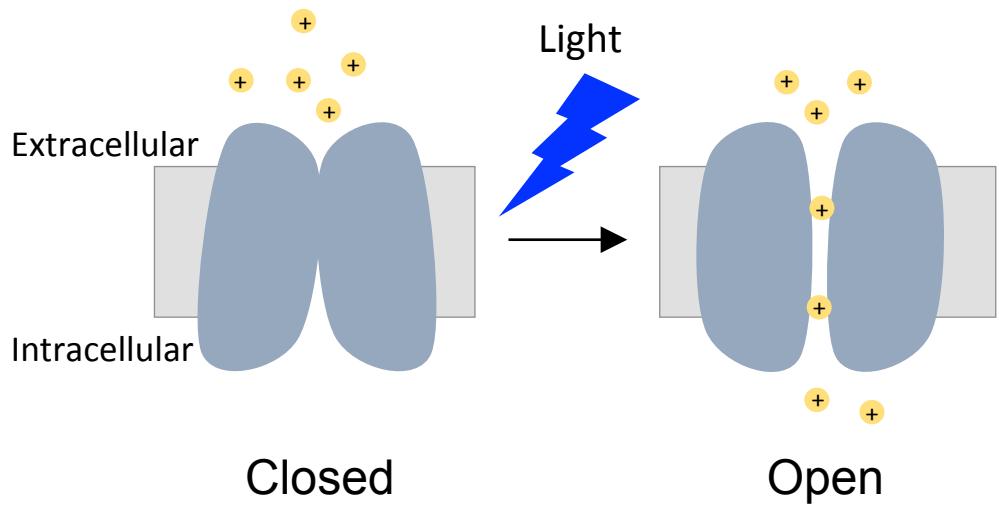
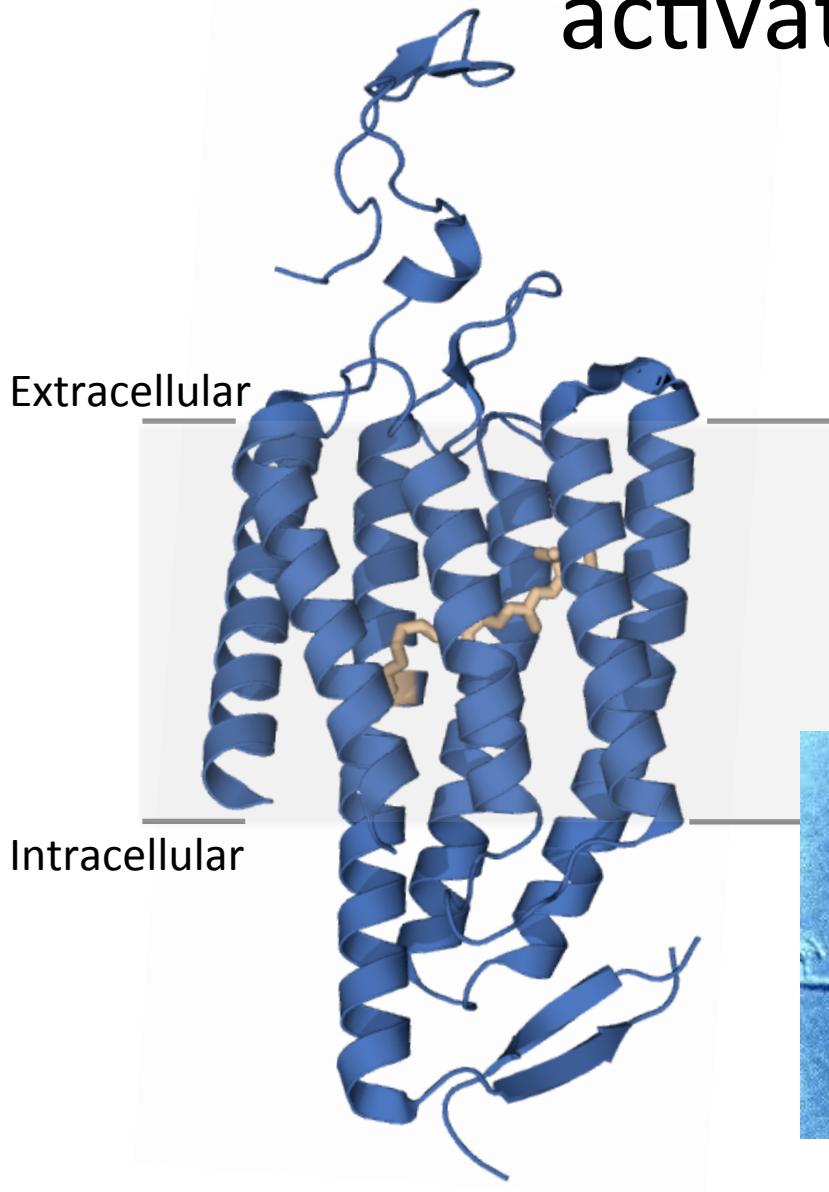
Methods for diversifying & screening membrane proteins



Channelrhodopsin (ChR): light activated channel

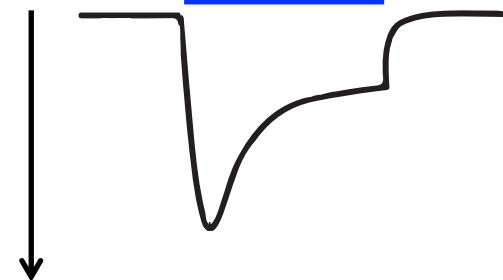


Channelrhodopsin (ChR): light activated channel

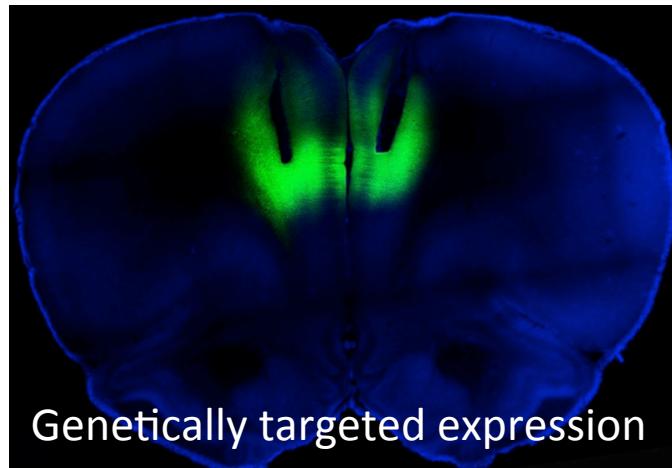
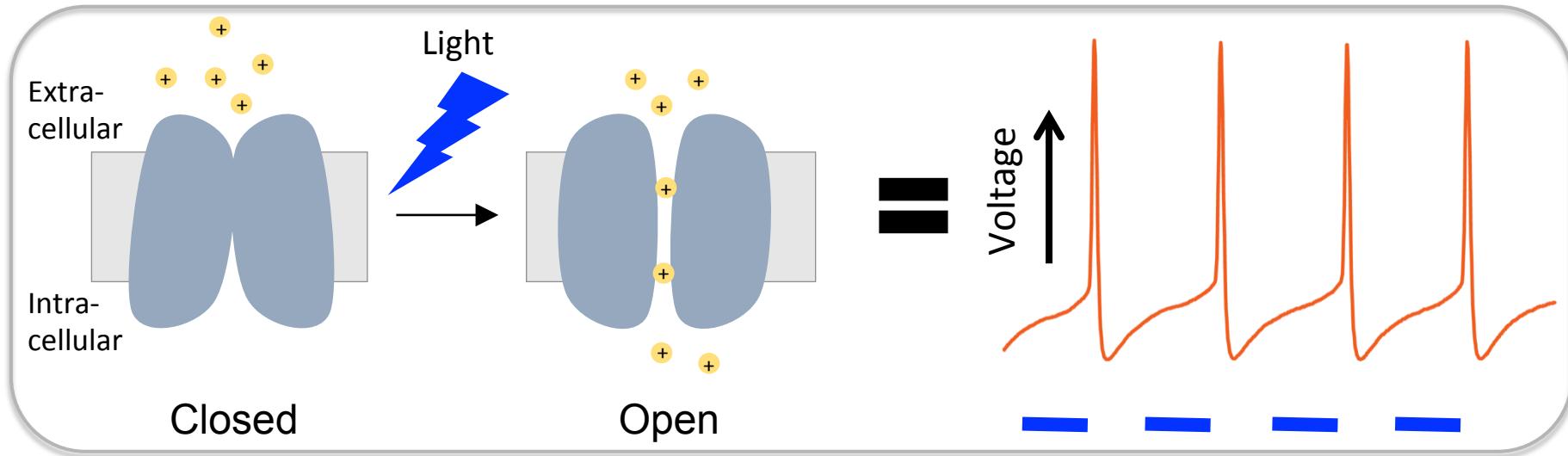


Current
[pA]

Positive ions into cell



Channelrhodopsins expressed in neurons induce action potentials with light stimulation



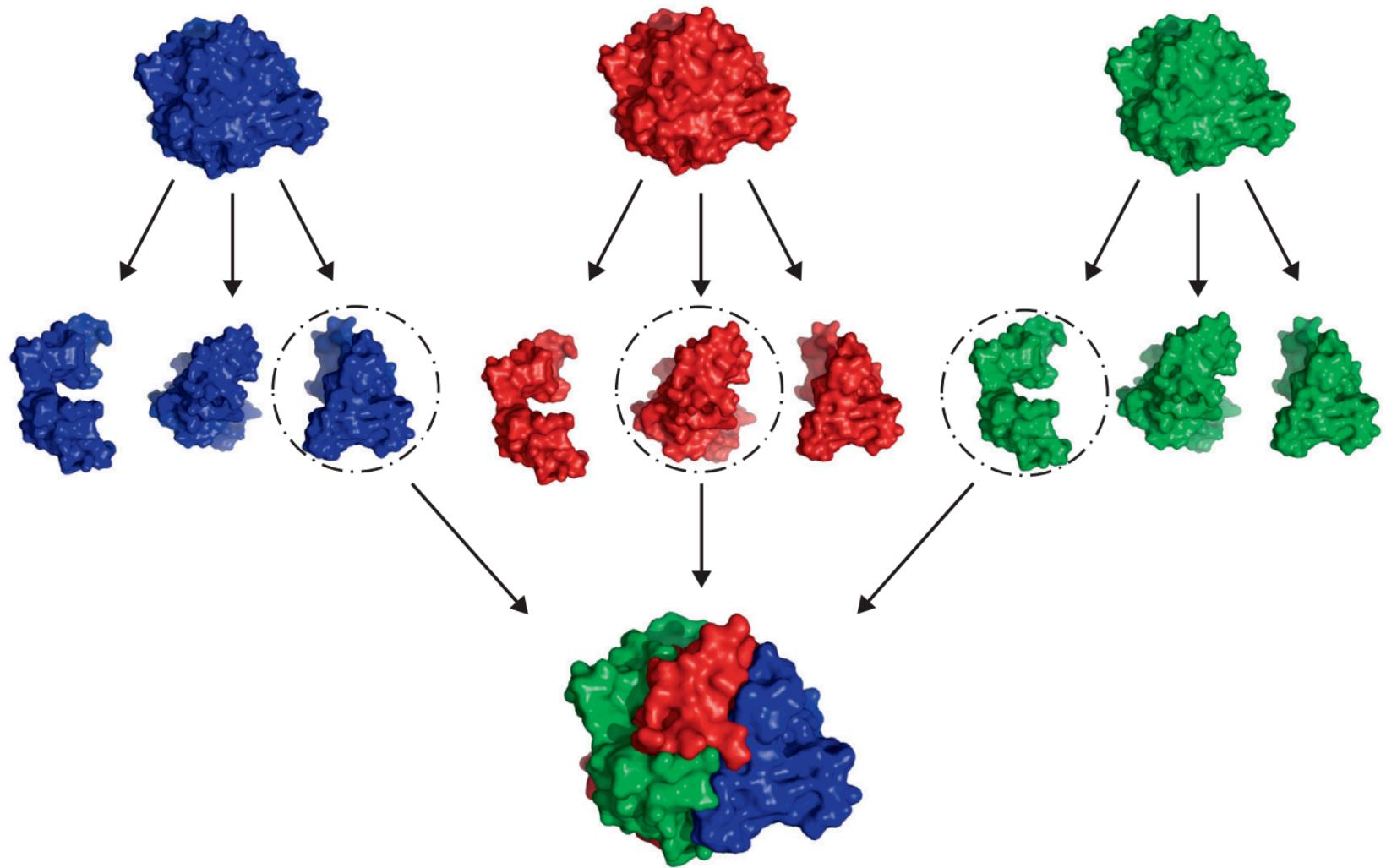
Methods for diversifying & screening membrane proteins

- Channelrhodopsin does not express in *E. coli*
 - Screening in mammalian cells
- Screening for channelrhodopsin function is done with electrophysiology NOT high-throughput
 - Cannot screen 100-1000's of variants for function

Need a method for **diversifying** and **screening functional** channelrhodopsins that does not require high-throughput methods

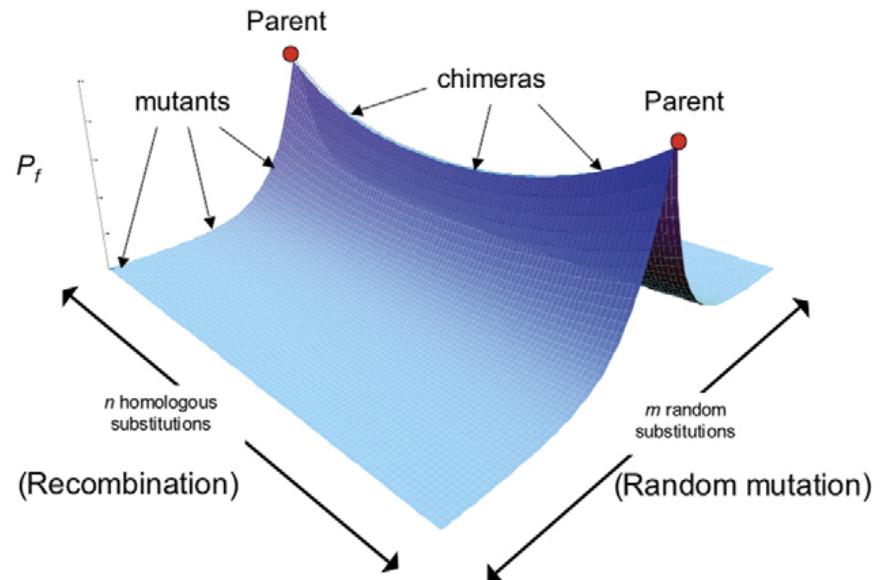
Structure Guided Protein Recombination

Fungal Cellobiohydrolase I's (Cel7a)

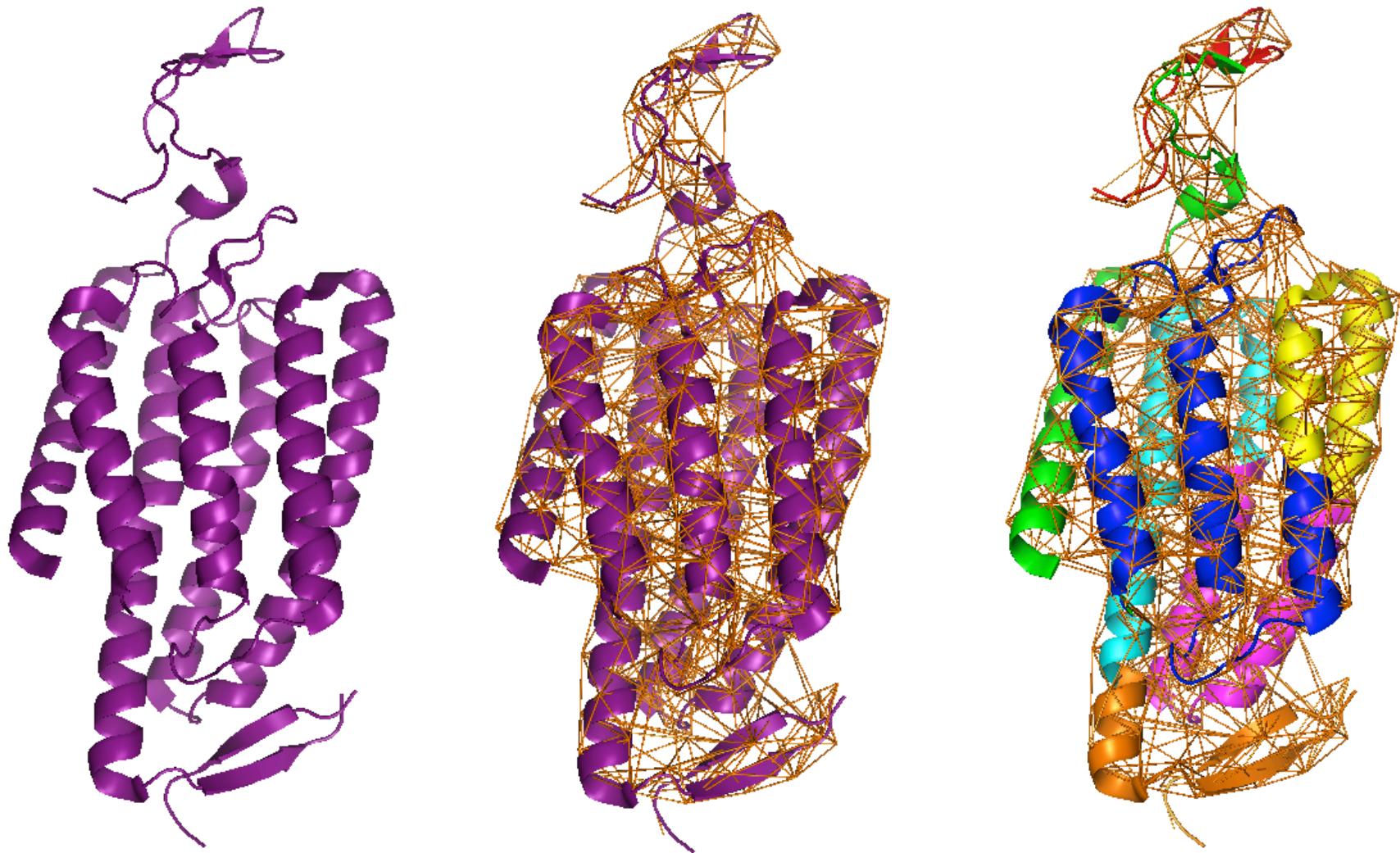


Benefits of using SCHEMA recombination for engineering ChRs

- Combines large amounts of diversity from parents with different properties
- Amenable for low throughput screening
- Predictive method that will facilitate machine learning

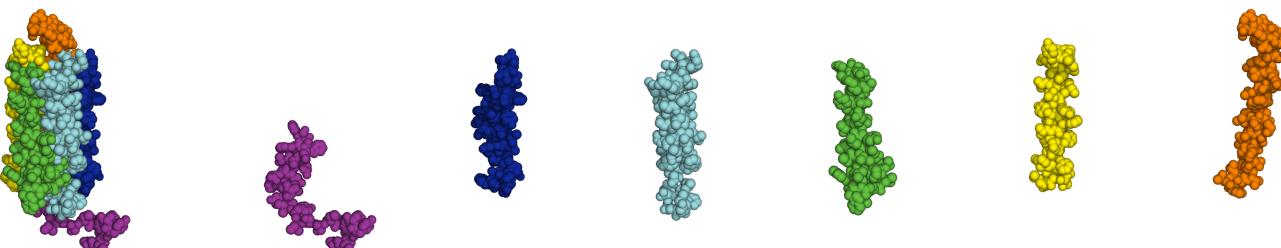


Applying SCHEMA to ChRs

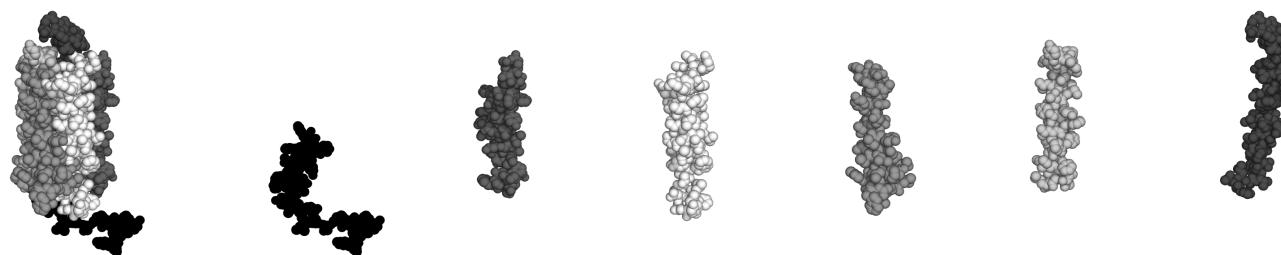


Block 1 Block 2 Block 3 Block 4 Block 5 Block 6

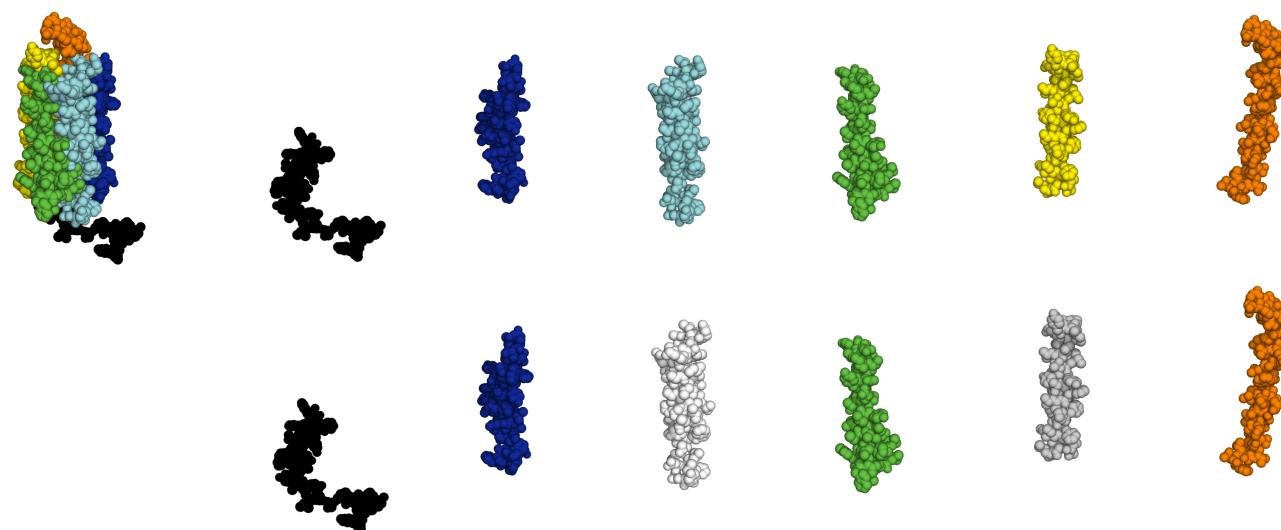
Parent1



Parent2



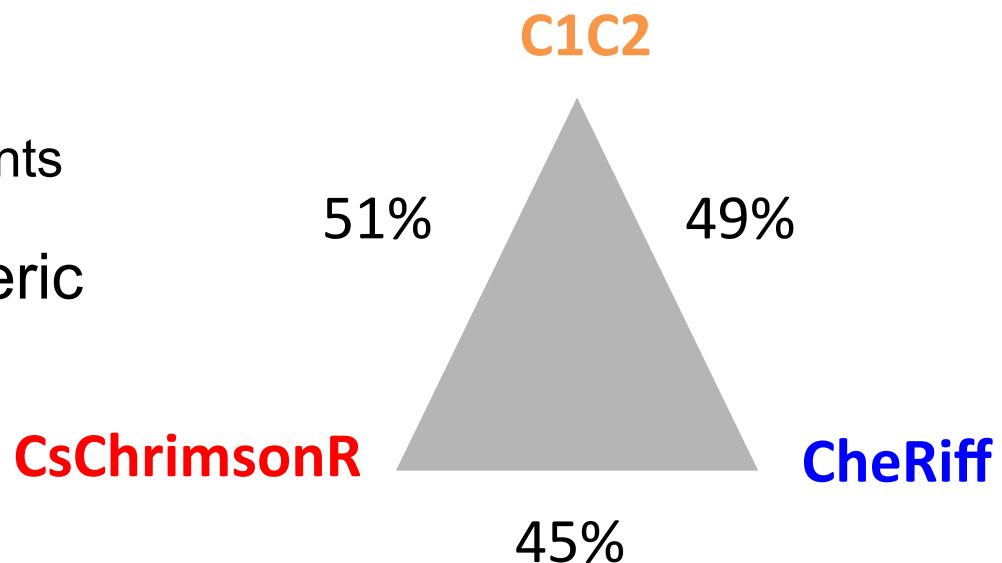
Chimera1



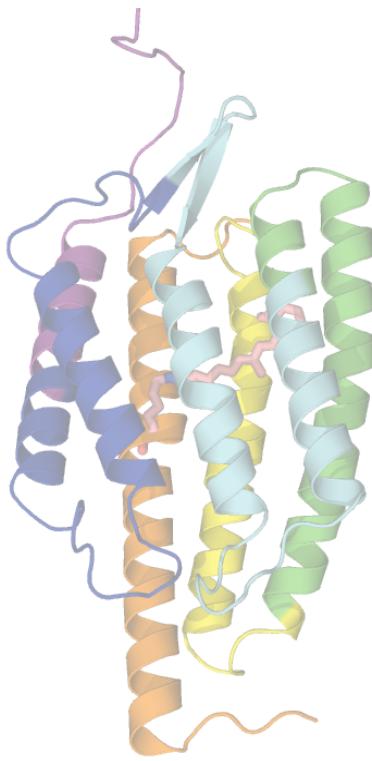
...

Parent ChRs for structure guided design

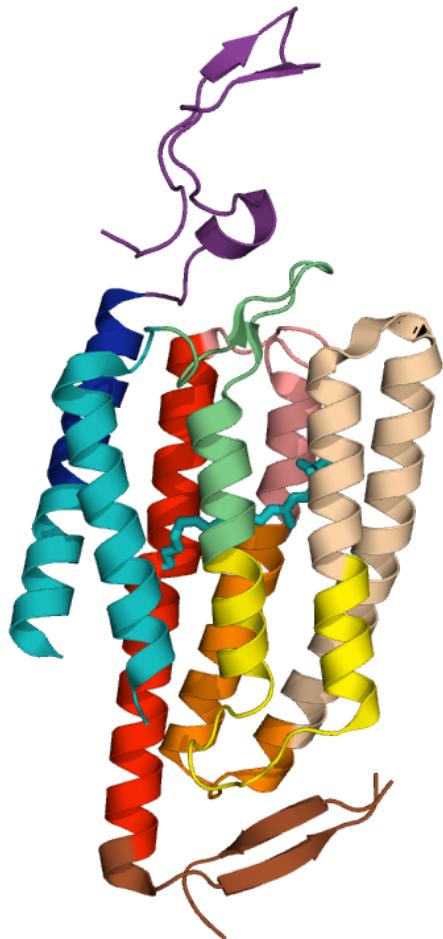
- Three parent ChRs
- 10 contiguous/
noncontiguous blocks
 - $3^{10} = 59,049$ possible variants
- Initial test set = 105 chimeric
variants per library
 - 60 single block swaps
 - 45 maximally informative
 - Ordered from Twist
Biosciences



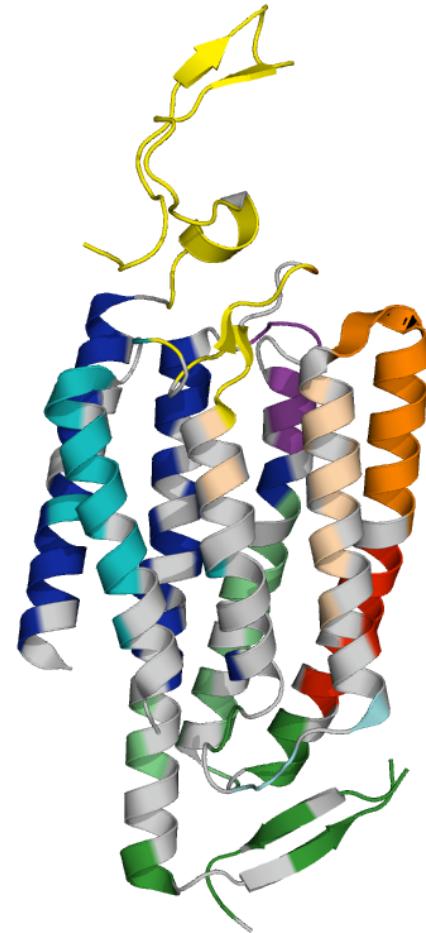
SCHEMA Block Designs



2nd Library
7-block contiguous
 $E = 23 / m = 47$

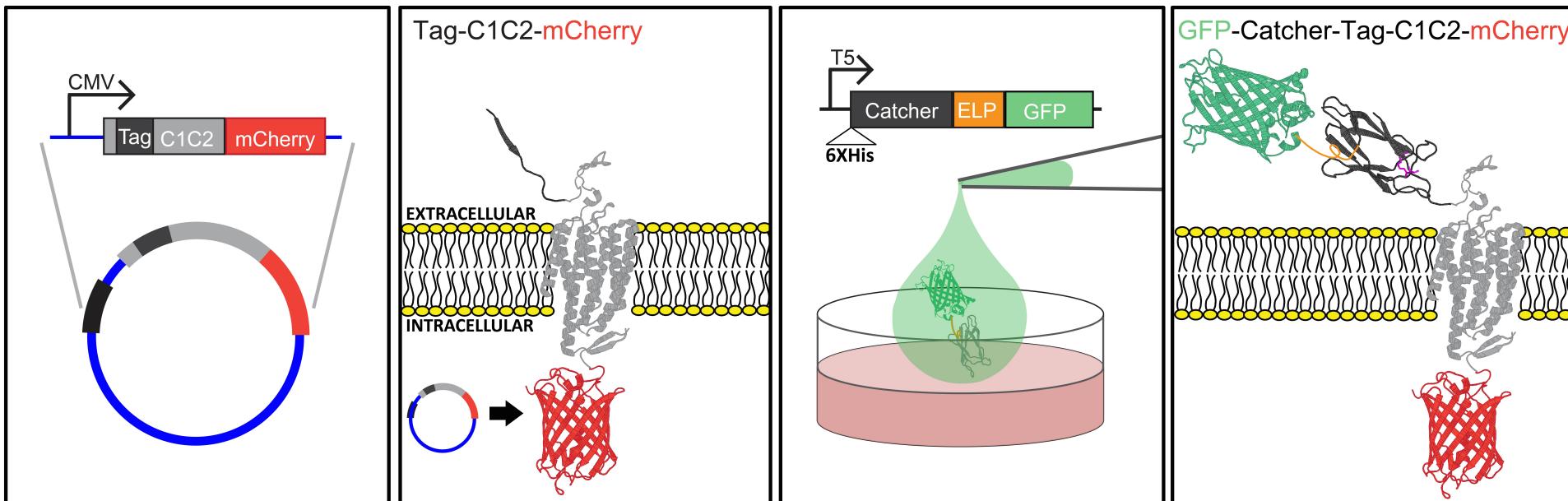


3rd Library
10-block contiguous
 $E = 25 / m = 73$

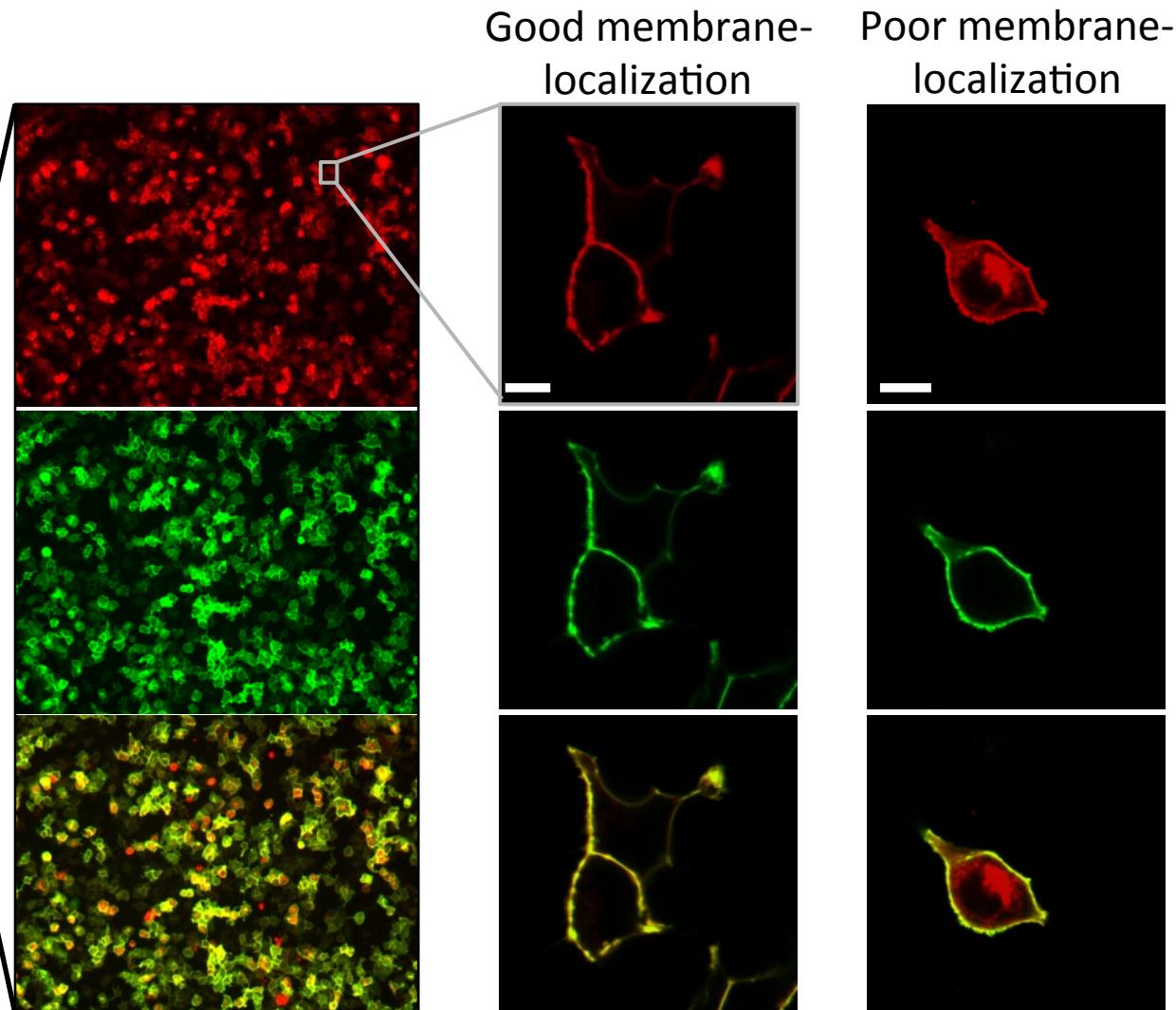
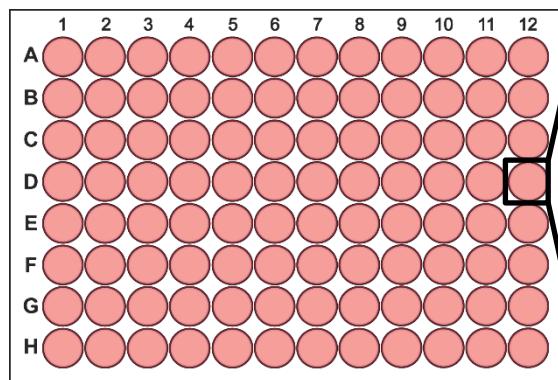


3rd Library Redux
10-block non-contiguous
 $E = 23 / m = 71$

How do we screen through hundreds of chimeras?

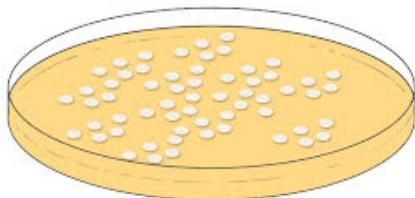


Prescreen for expression and membrane localization



Prepare DNA for each chimera

Streak out from
glycerol stocks



5 ml cultures



Miniprep

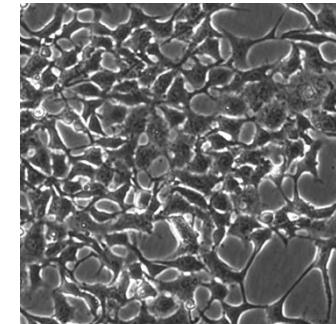


Prepare HEK cells in 96-well plates

Poly-D-lysine coat glass wells



Passage HEK cells at low
confluence



Transfect HEK cells with DNA for each chimera in duplicates



Wait 48 hours for expression

Add 20 uM SpyCatcher-GFP to the cells. Incubate for 1 hr.



Remove all media + SpyCatcher-GFP and wash 3x with PBS.
Then add fresh media



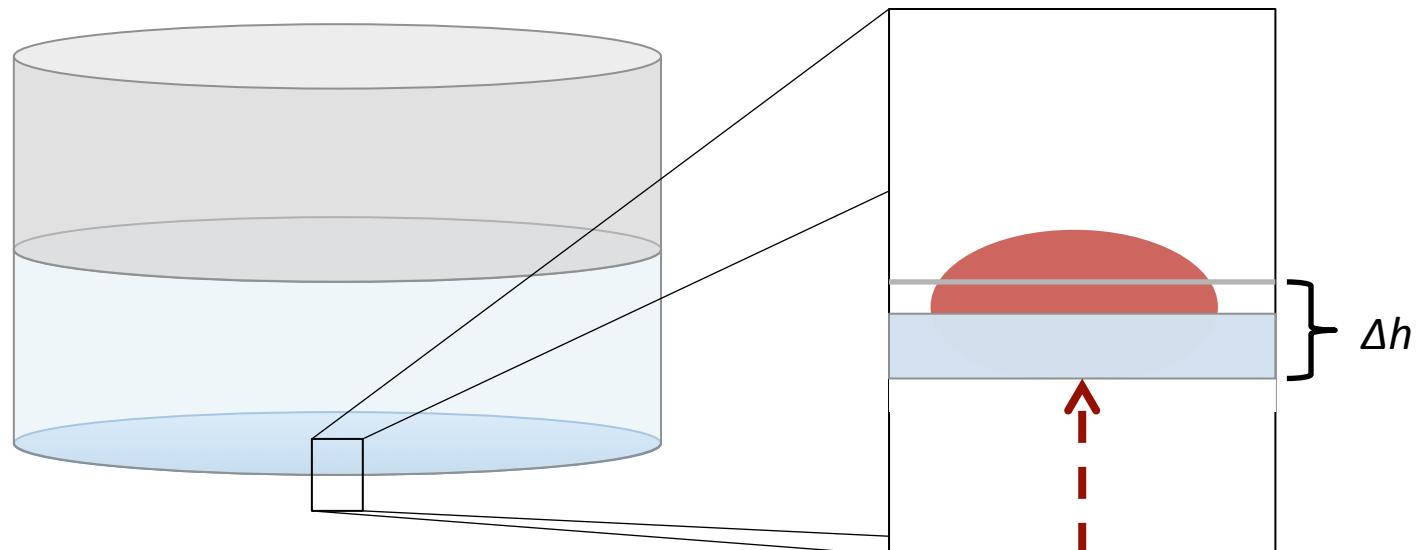
Incubated plates in fresh media for 1 hr



Replace media with extracellular buffer & image

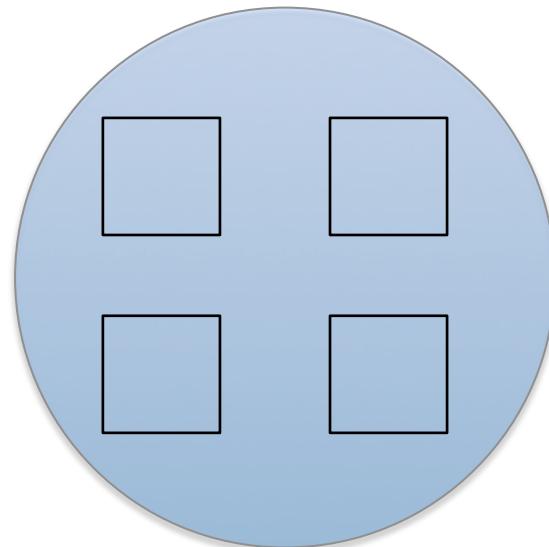
High throughput imaging requires autofocus!

- Imaging autofocus.
 - Infrared laser detects the glass bottom of well.
 - Autofocus goes to the same position relative to glass bottom (Δh).
- Impossible to compare expression level of different variants when they are not in focus



Imaging multiple positions per well

- Image four positions within each of the 96-wells
- Two duplicates per variant.
- Within each position should get 100's of cells



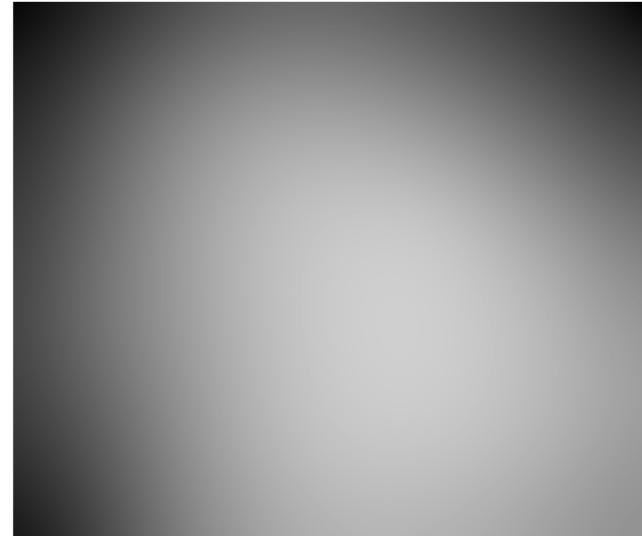
Parameters of interest from the membrane localization assay

- Overall expression of chimera
 - Mean intensity of mKate fluorescence
- Fraction of expressed protein that is membrane localized
 - Ratio of total pixel intensity of GFP over total pixel intensity of mKate

SpyTag/SpyCatcher membrane localization assay quantification

1. Background subtraction
2. Threshold the image and create a mask of pixels above that threshold
3. Calculate the mean intensity of mKate pixels within the mask
4. Sum the pixel intensity of all GFP pixels and all mKate pixels within mask and take ratio

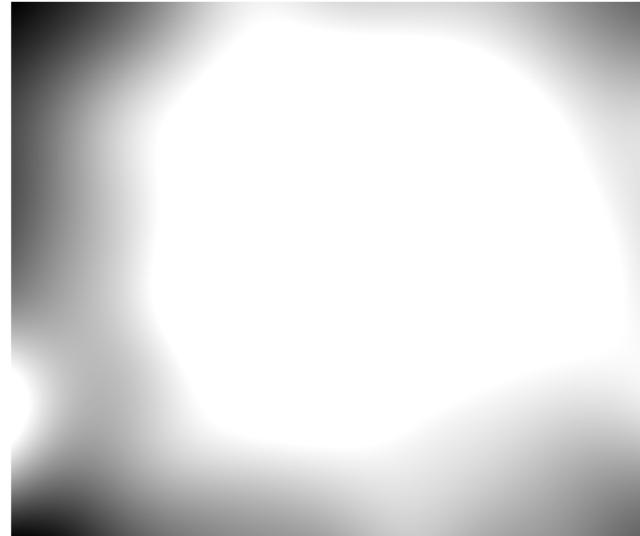
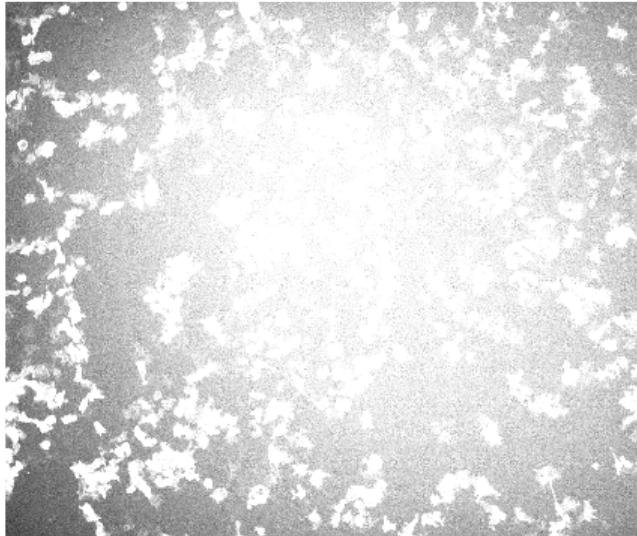
Gaussian filter



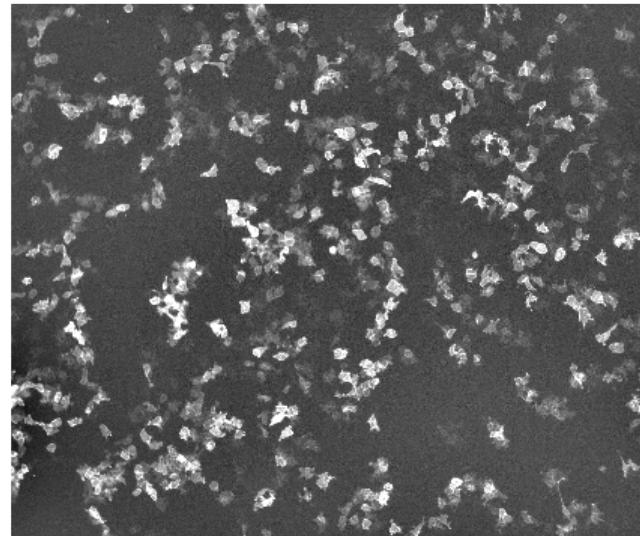
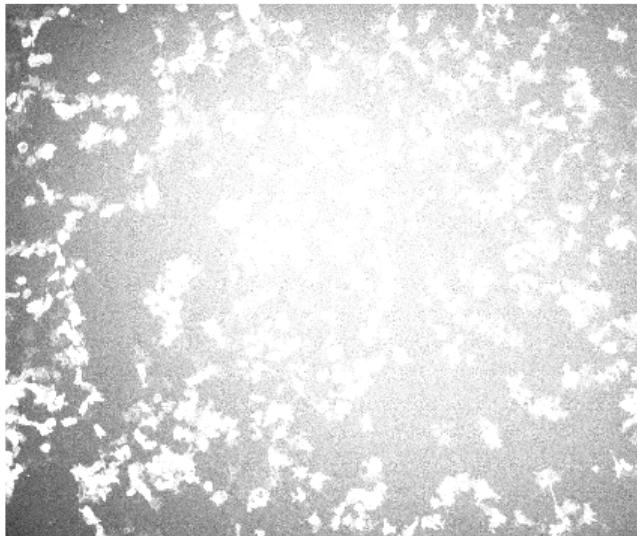
Background subtraction of
Gaussian filtered version of the image



Gaussian filter



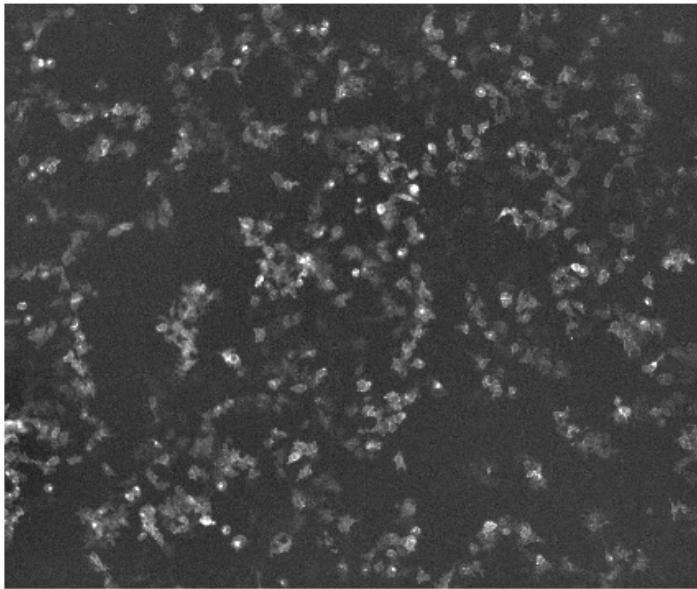
Background subtraction of
Gaussian filtered version of the image



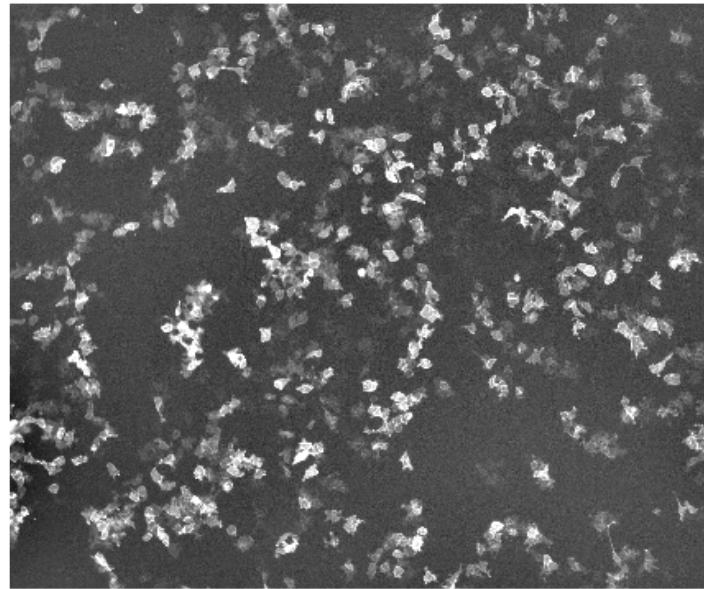
SpyTag/SpyCatcher membrane localization assay quantification

1. Background subtraction
2. Threshold the image and create a mask of pixels above that threshold
3. Calculate the mean intensity of mKate pixels within the mask
4. Sum the pixel intensity of all GFP pixels and all mKate pixels within mask and take ratio

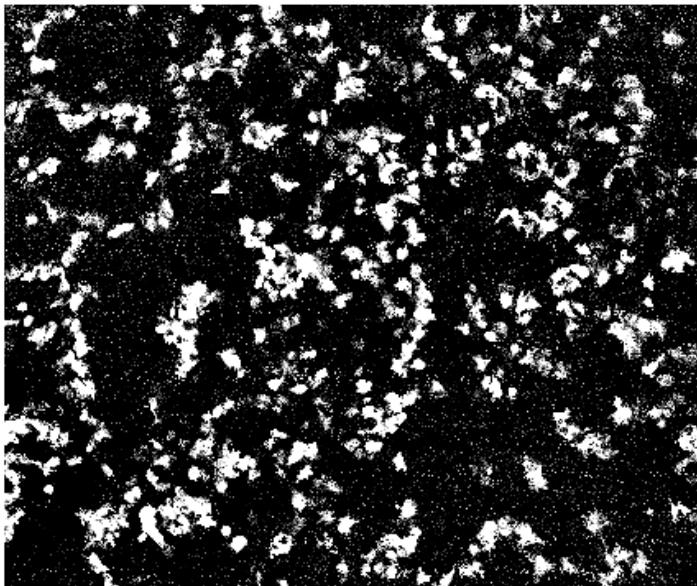
mKate background subtracted



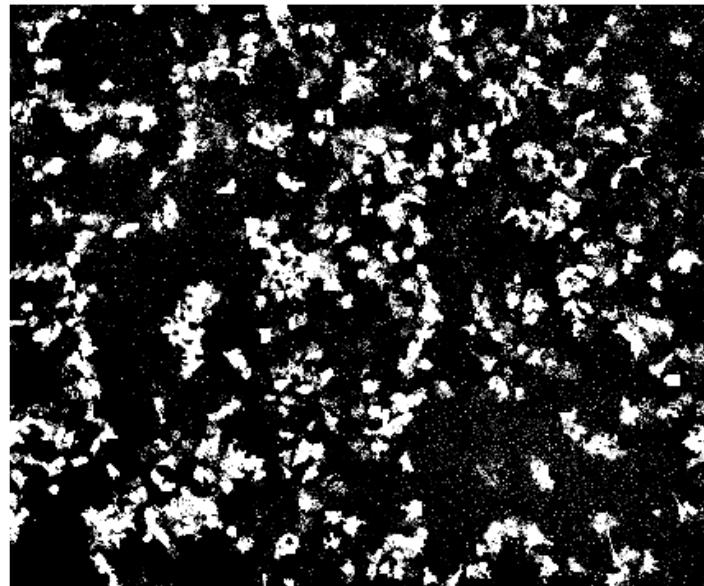
GFP background subtracted



mKate mask



GFP mask



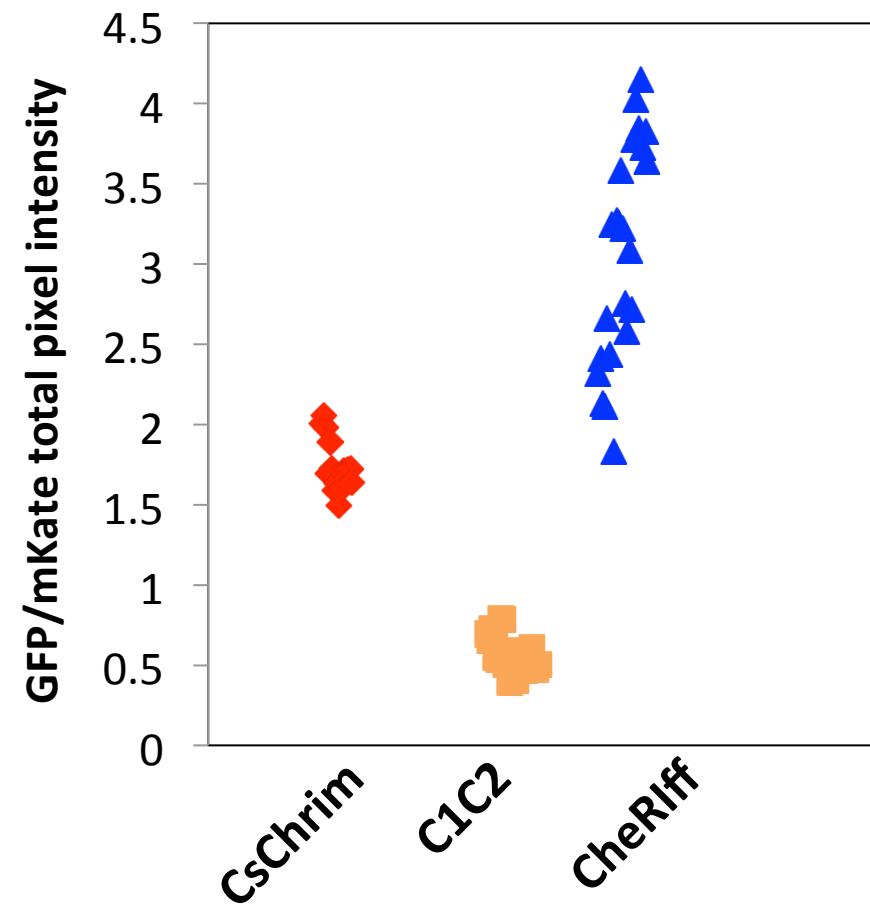
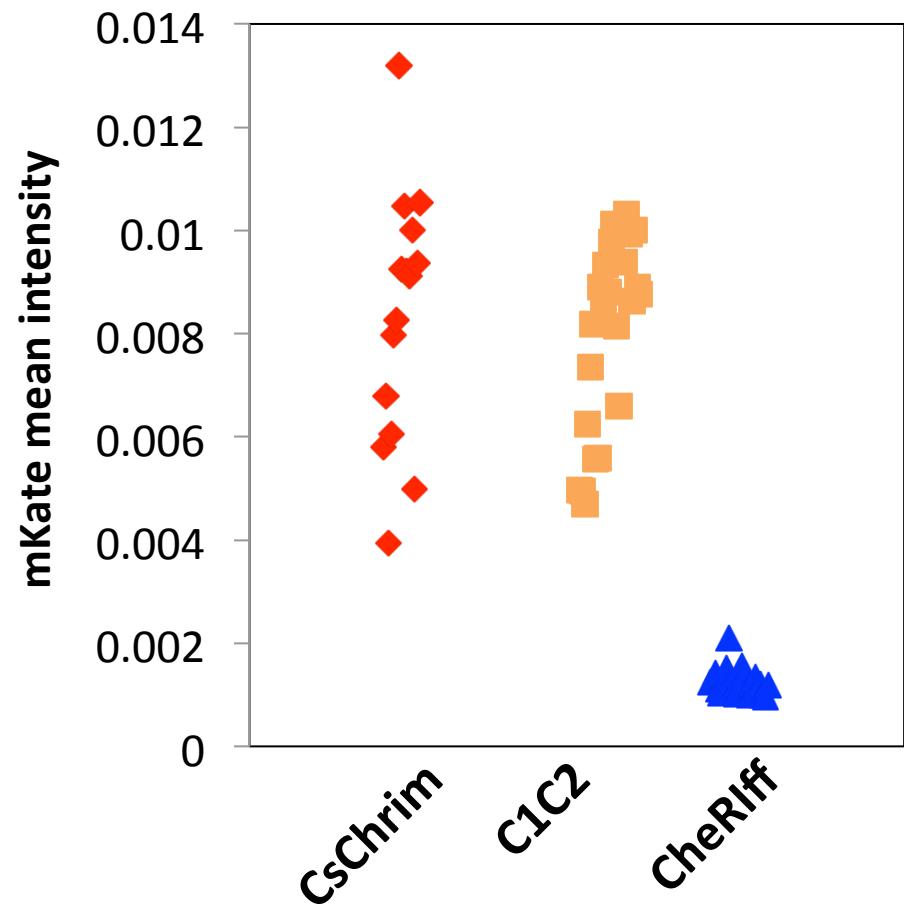
SpyTag/SpyCatcher membrane localization assay quantification

1. Background subtraction
2. Threshold the image and create a mask of pixels above that threshold
3. Calculate the mean intensity of mKate pixels within the mask
4. Sum the pixel intensity of all GFP pixels and all mKate pixels within mask and take ratio

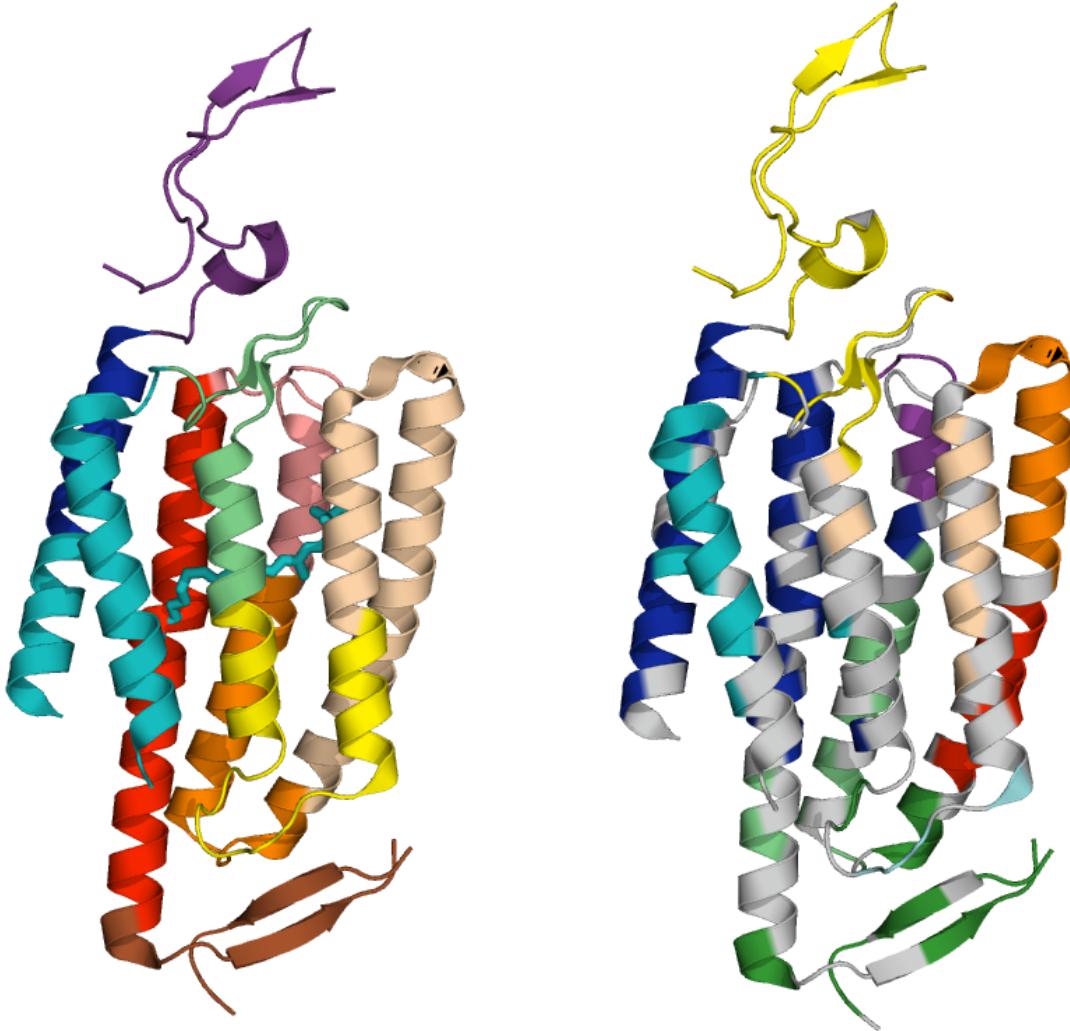
SpyTag/SpyCatcher membrane localization assay quantification

1. Background subtraction
2. Threshold the image and create a mask of pixels above that threshold
3. Calculate the mean intensity of mKate pixels within the mask
4. Sum the pixel intensity of all GFP pixels and all mKate pixels within mask and take ratio

SpyTag/SpyCatcher Membrane Localization Assay Quantification



Do we see expression in the contiguous or noncontiguous library?



#TheDirtyDozen

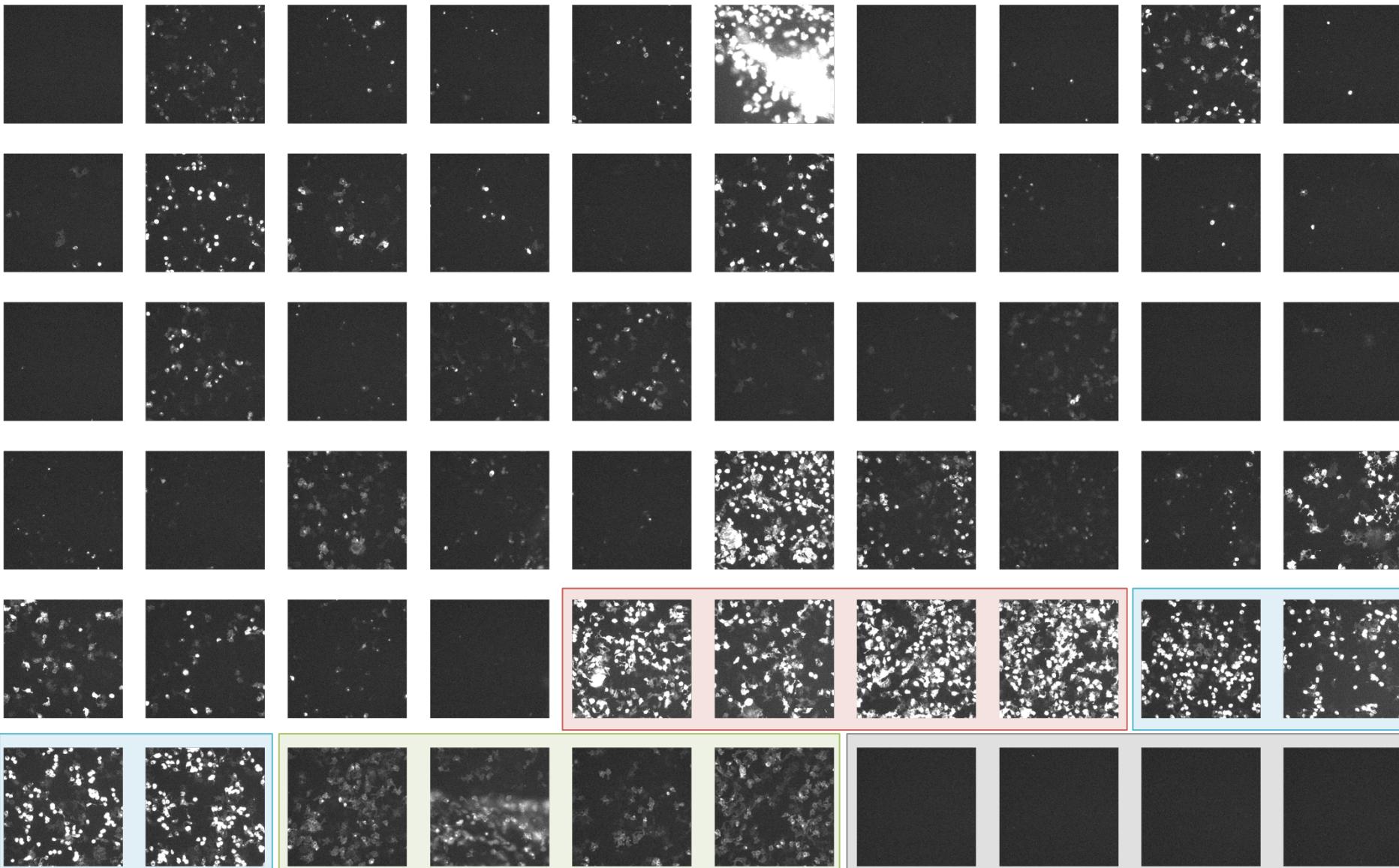
Construct ID	E	m	Expression?
ChR n21	29	57	0
ChR n75	43	103	0
ChR n76	20	79	0
ChR c79	39	101	0
ChR n80	17	93	0
ChR c80	40	89	0
ChR c83	32	95	0
ChR n84	30	81	1
ChR c84	42	95	1
ChR n88	29	86	0
ChR c88	47	82	0
ChR c92	36	95	1
Average	34	88	

No opsin-
just mKate

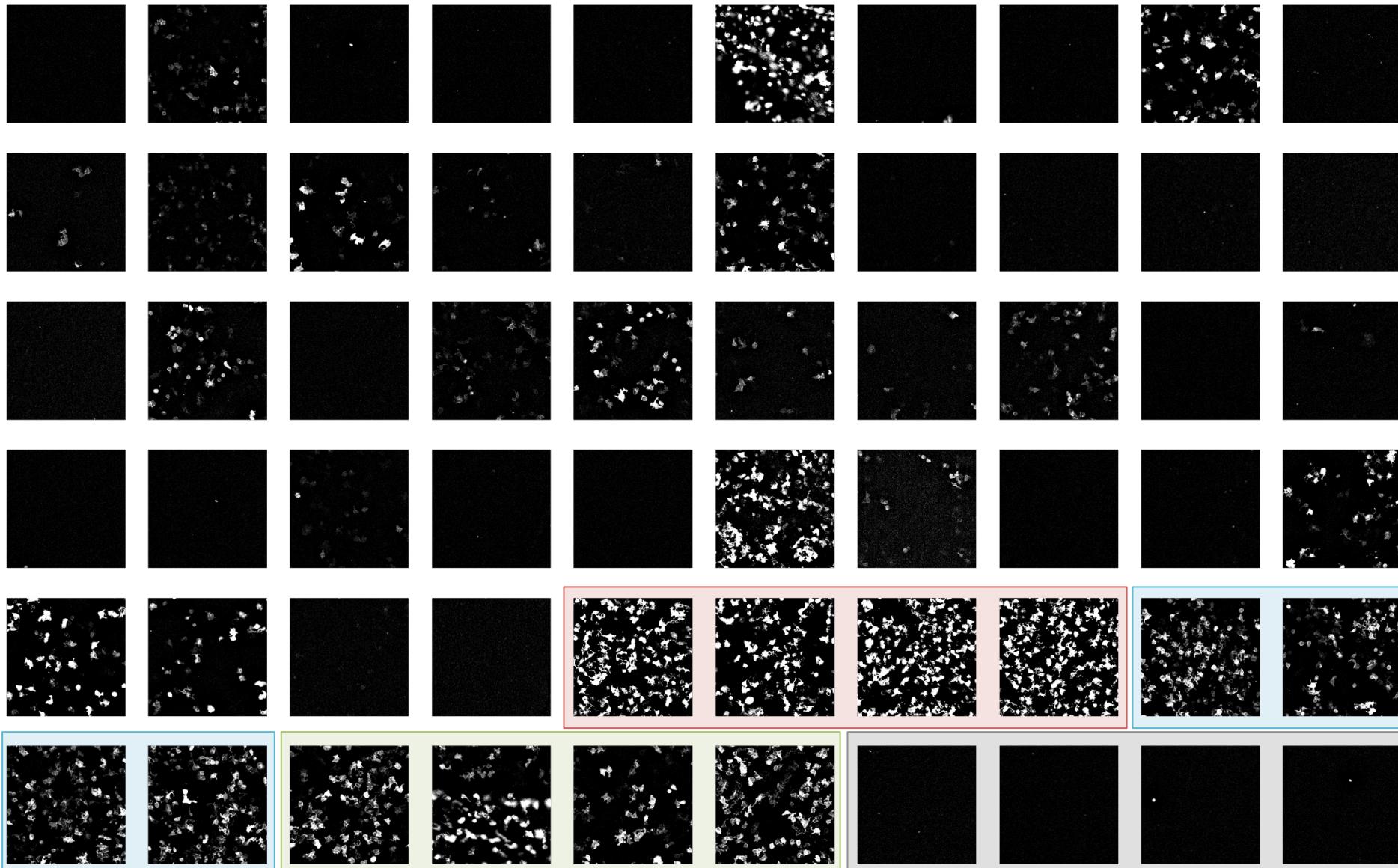
#TheDirtyDozen

Construct ID	E	m	Expression?
ChR n21	29	57	1
ChR n75	43	103	0
ChR n76	20	79	1
ChR c79	39	101	-
ChR n80	17	93	1
ChR c80	40	89	1
ChR c83	32	95	1
ChR n84	30	81	1
ChR c84	42	95	1
ChR n88	29	86	1
ChR c88	47	82	1
ChR c92	36	95	1
Average	34	88	

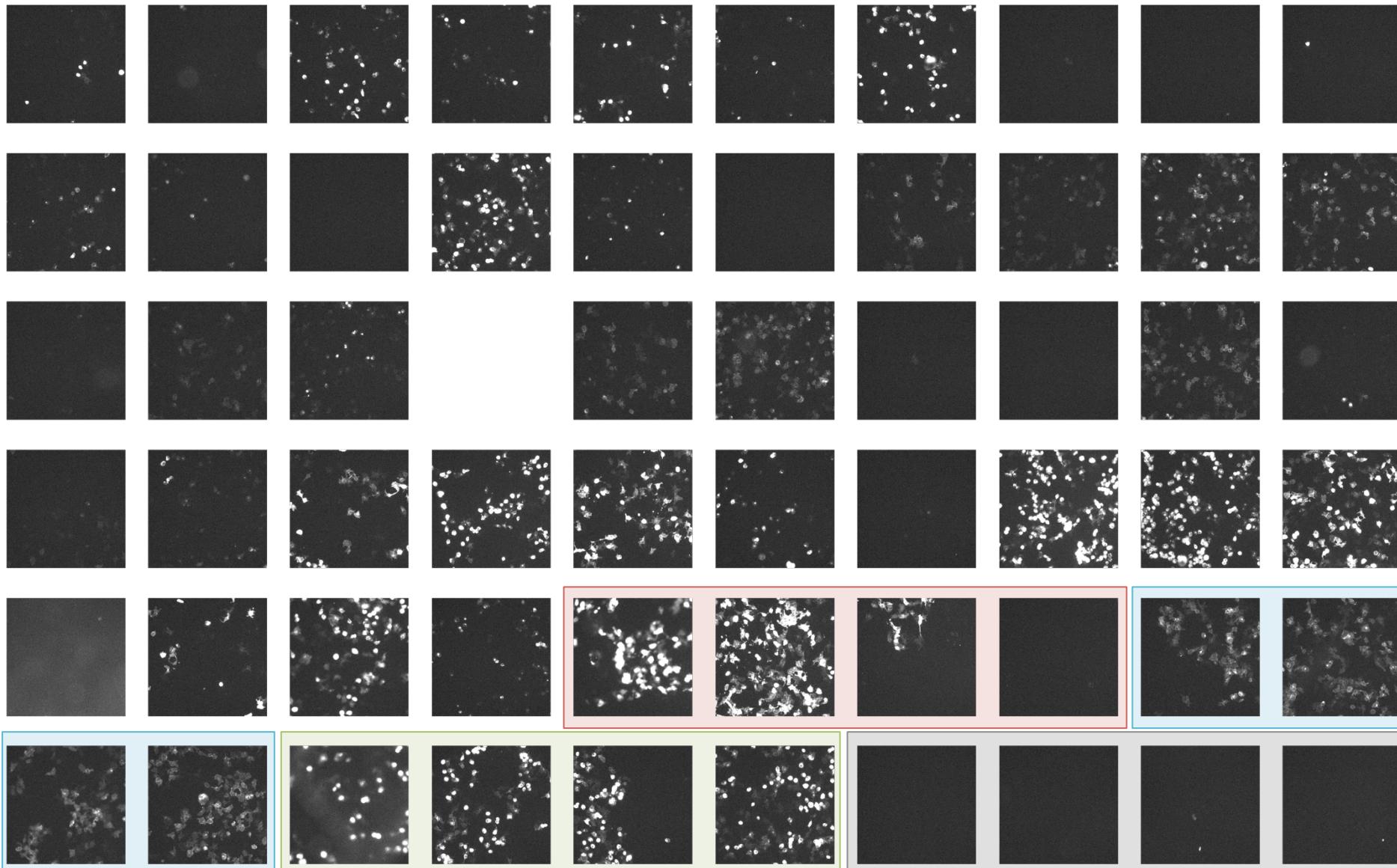
mKate expression



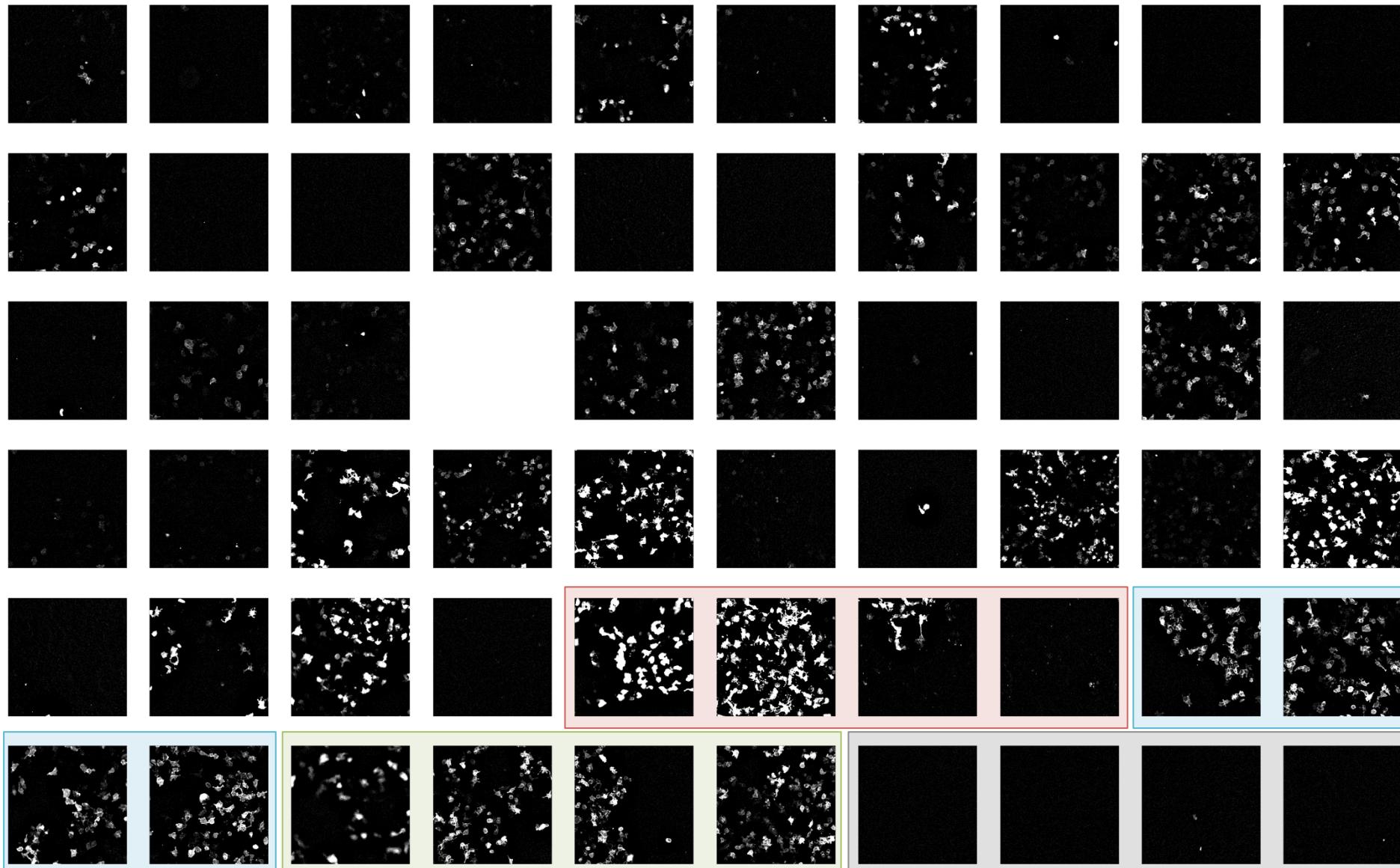
SpyCatcher-GFP labeling



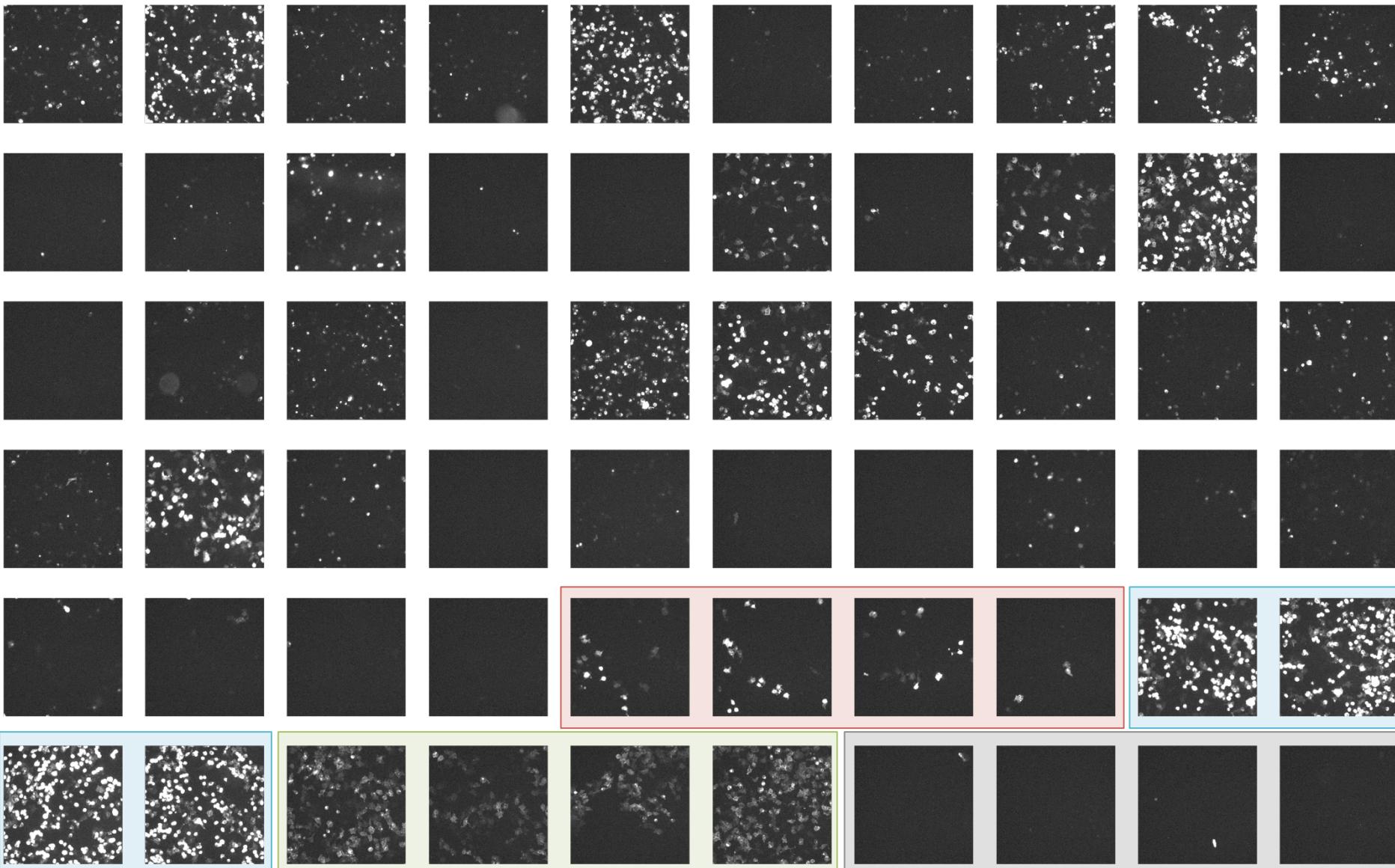
mKate expression



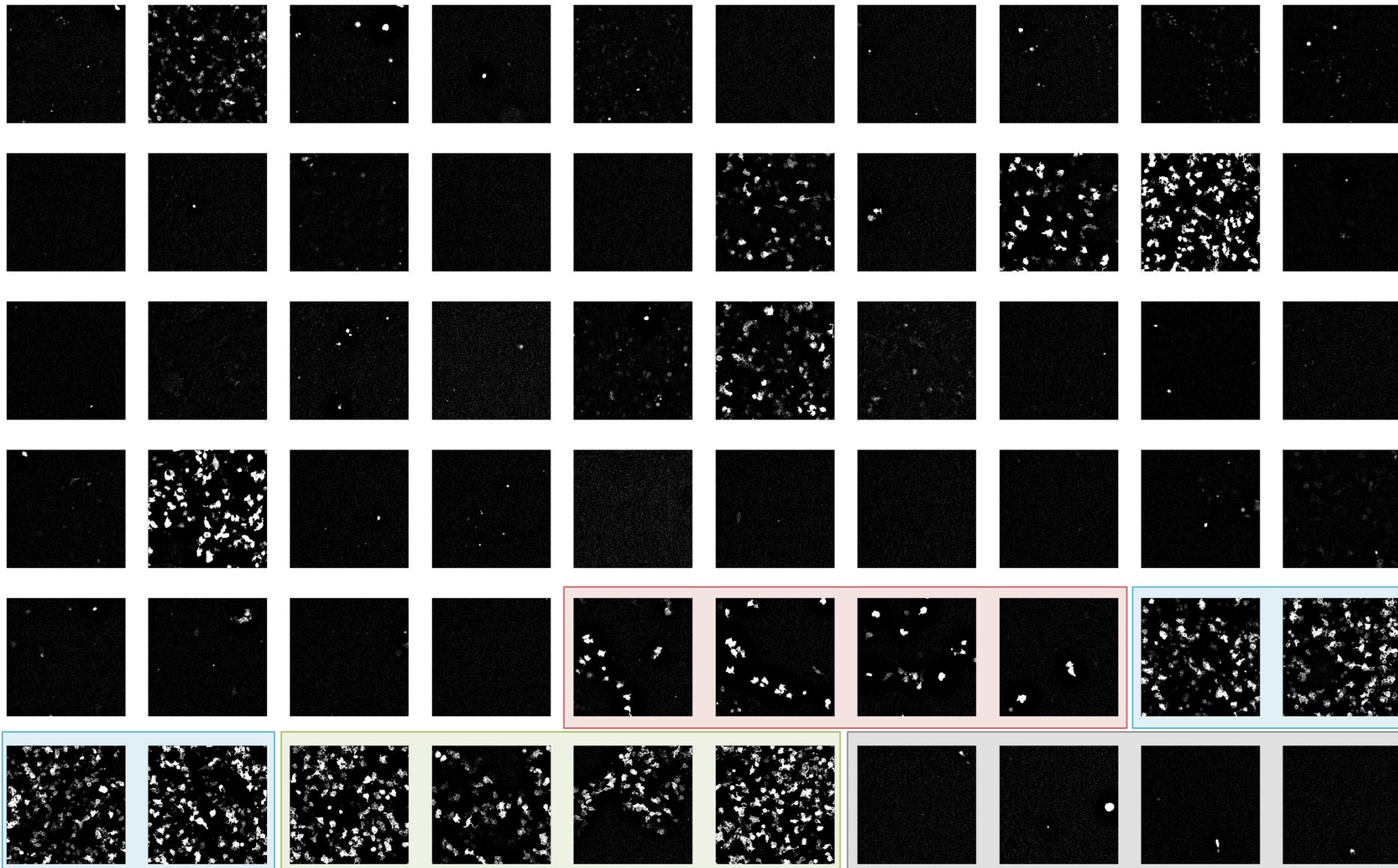
SpyCatcher-GFP labeling



mKate expression



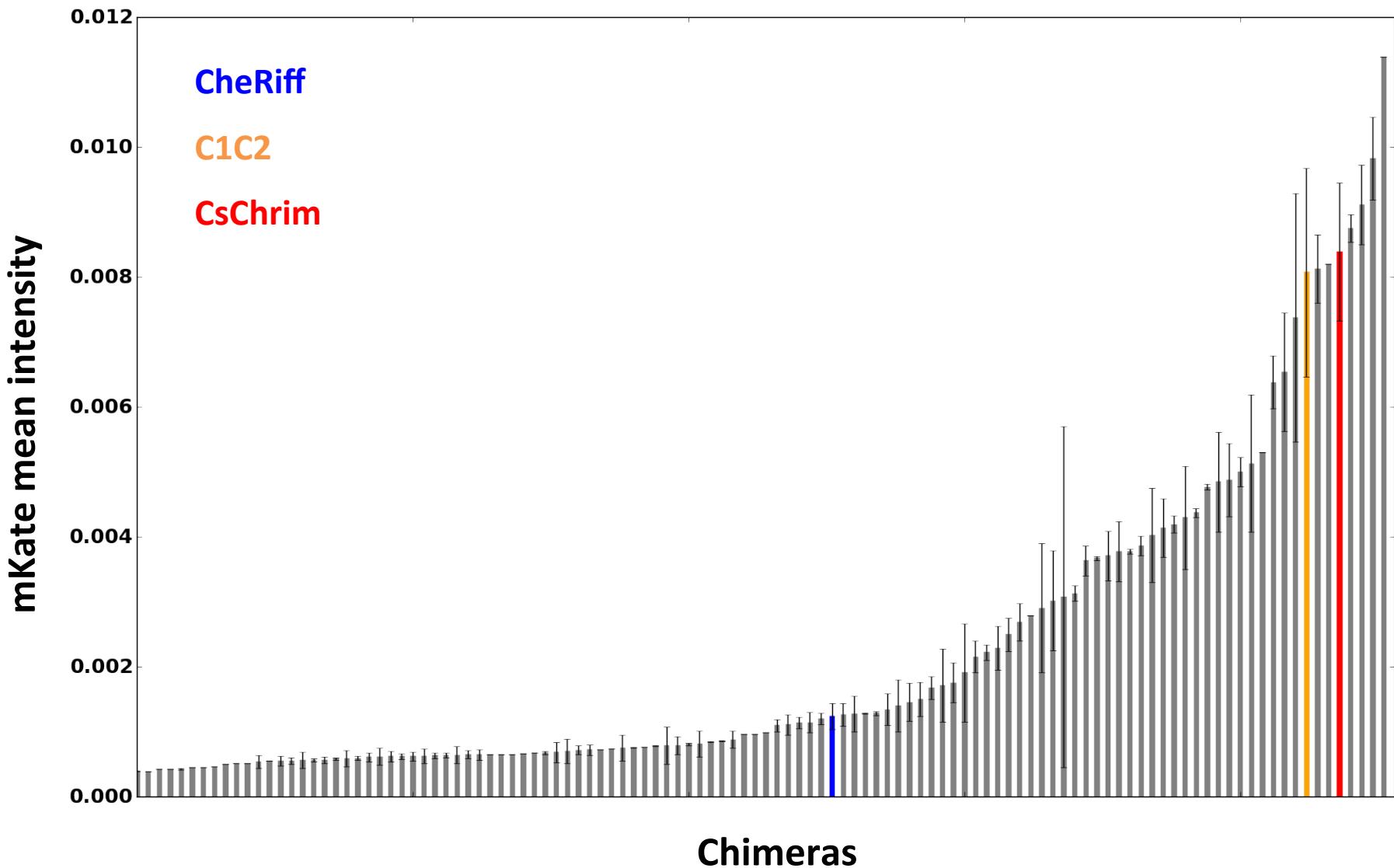
SpyCatcher-GFP labeling



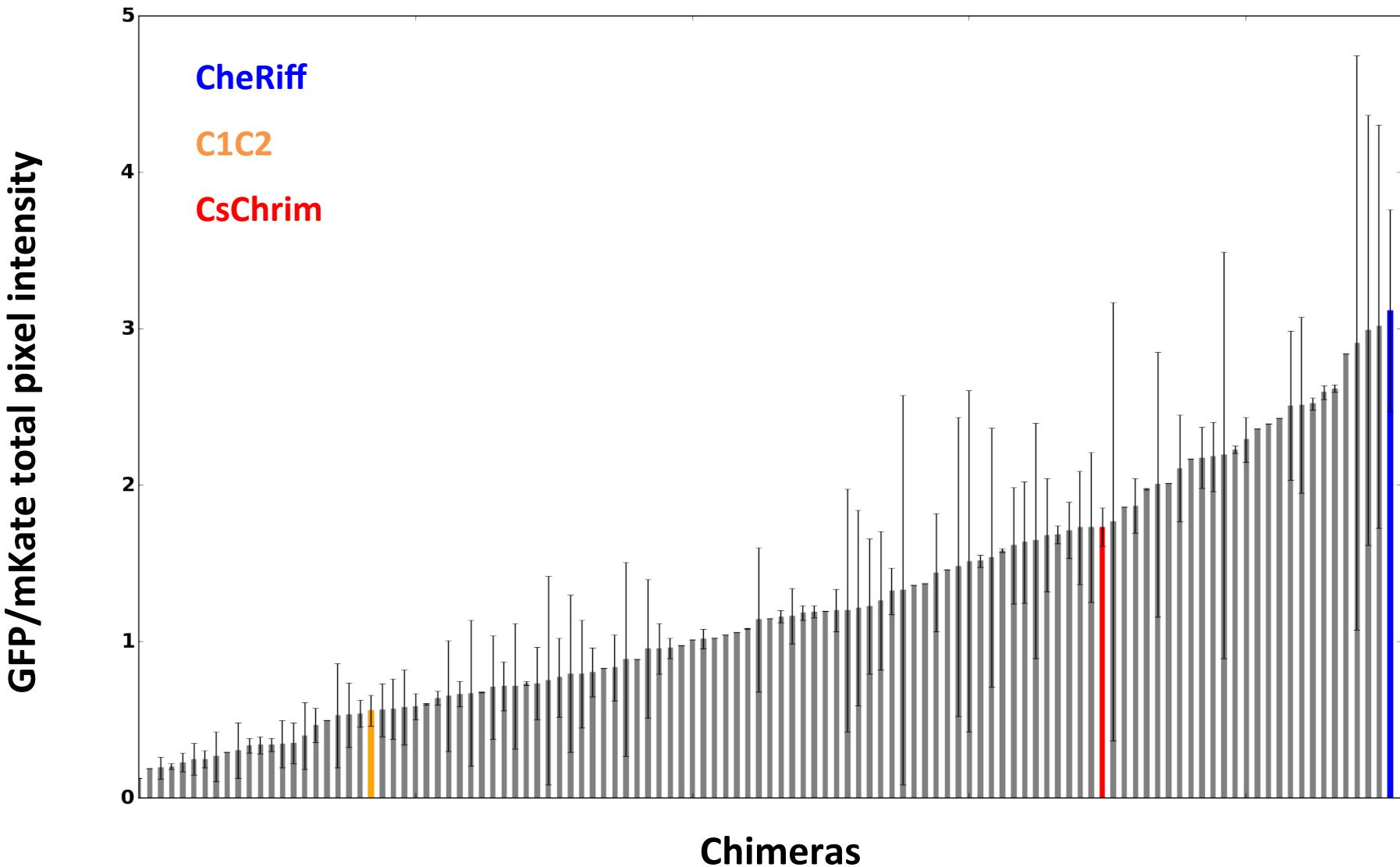
Overall results from the 131 chimeras tested

	Contiguous	Noncontiguous
Chimeras tested	68	63
Single-block-swaps (sbs)	39	37
Maximally informative (mi)	29	26
% of library that expresses	89.7%	90.5%
Chimeras not expressing	7 (3 mi, 4 sbs)	6 (5 mi, 1 sbs)

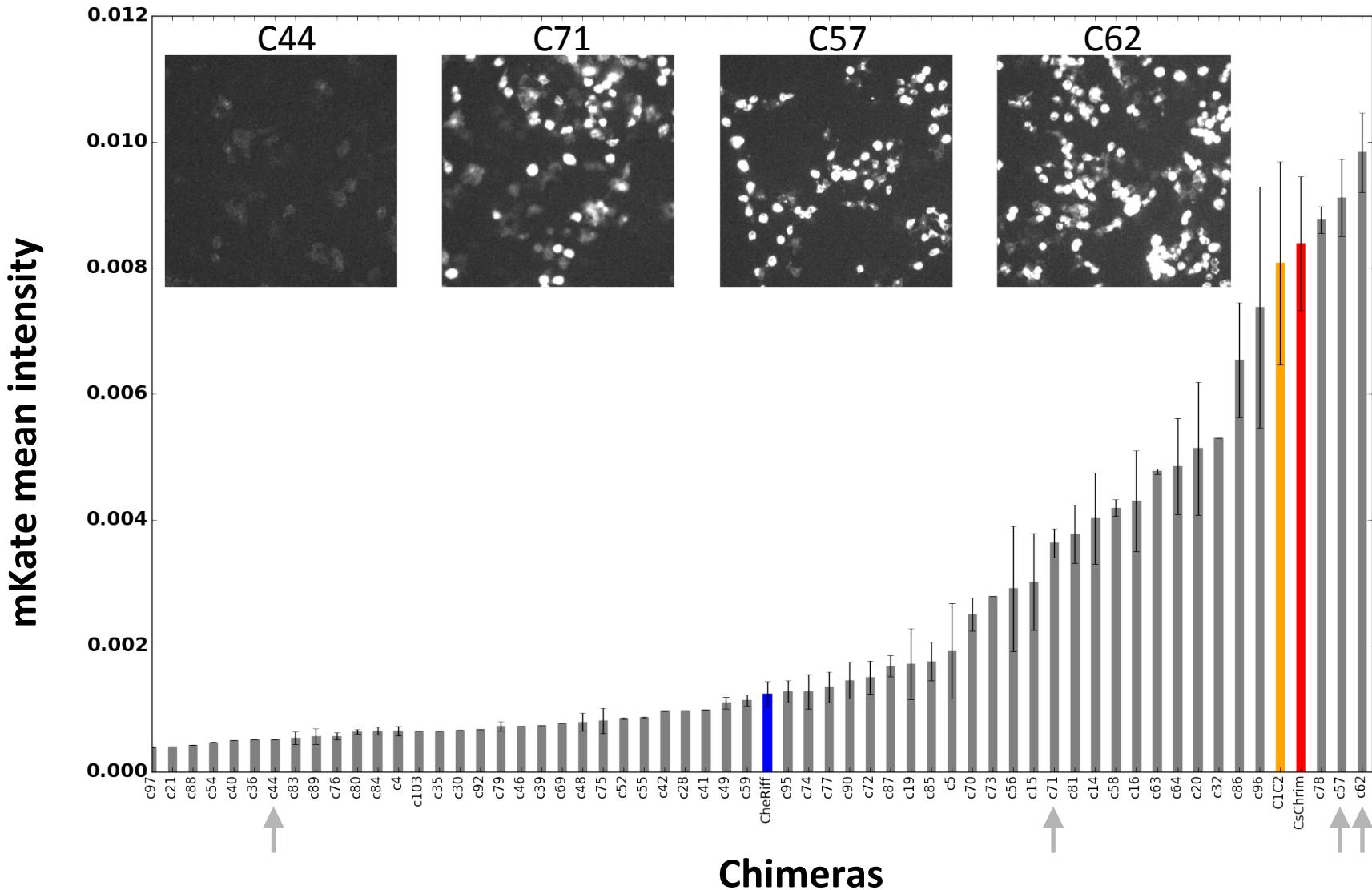
Distribution of mKate mean intensity for both libraries



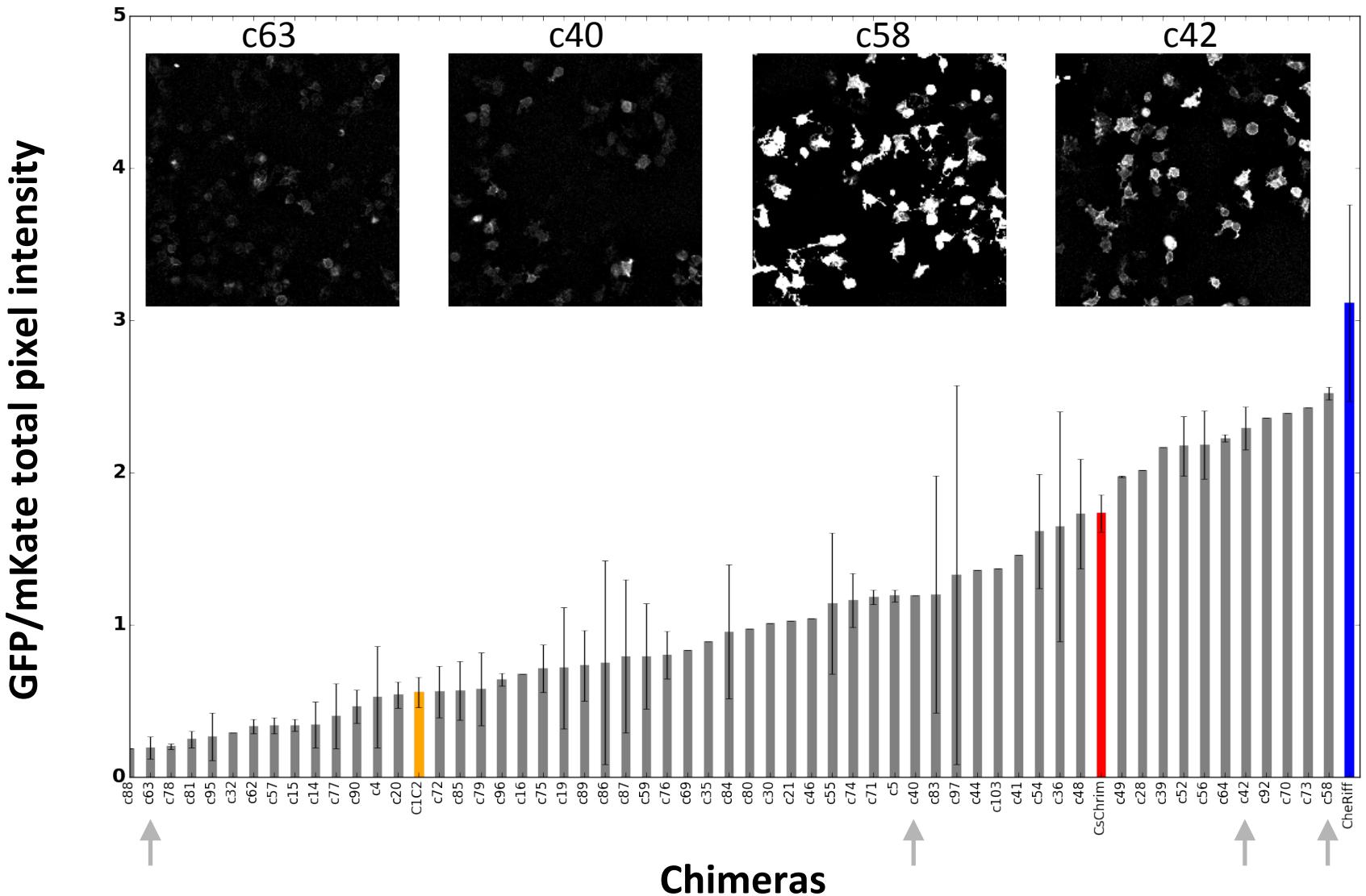
Distribution of GFP/mKate for both libraries



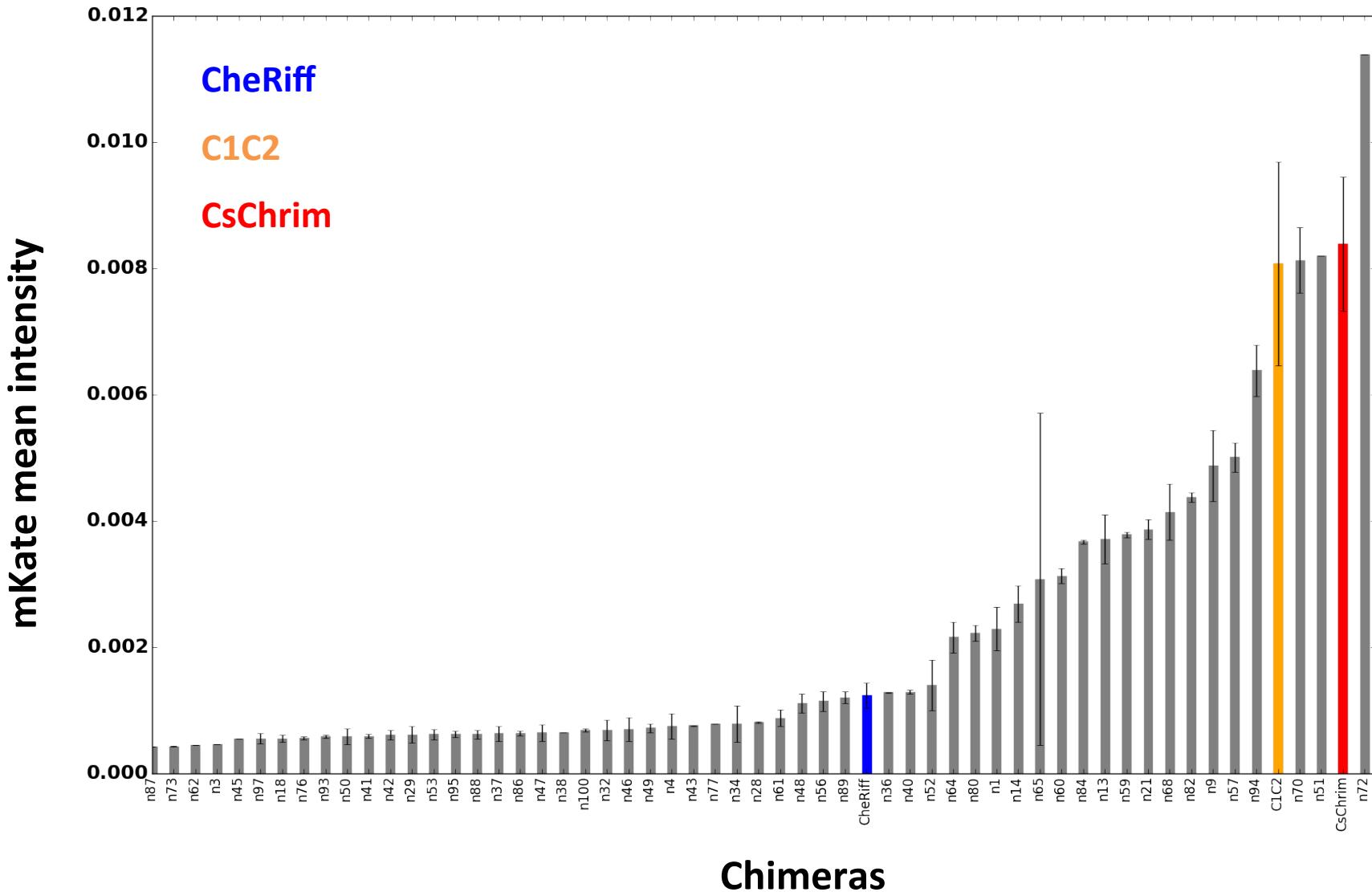
Distribution of mKate mean intensity for contiguous libraries



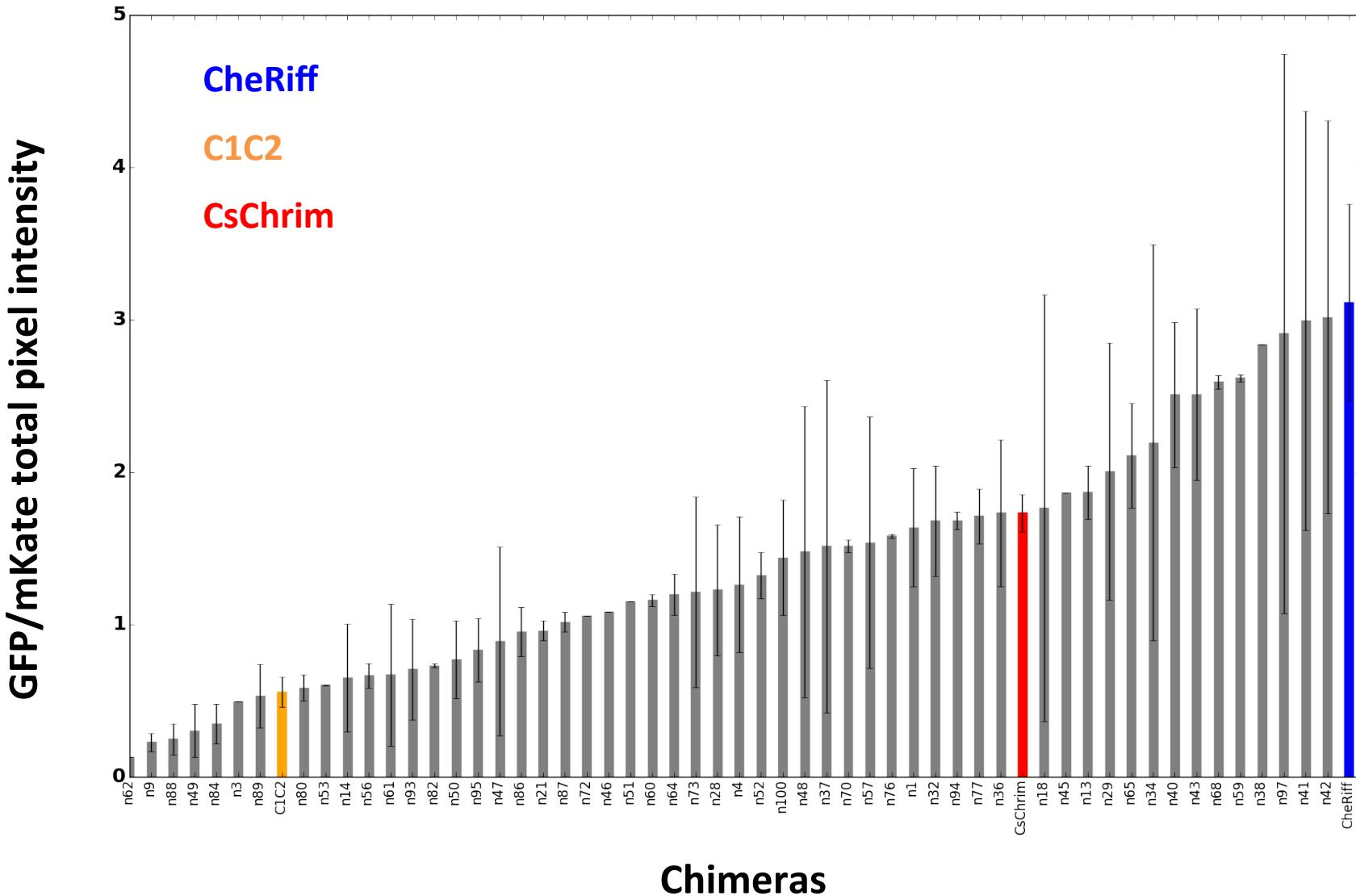
Distribution of GFP/mKate for contiguous libraries



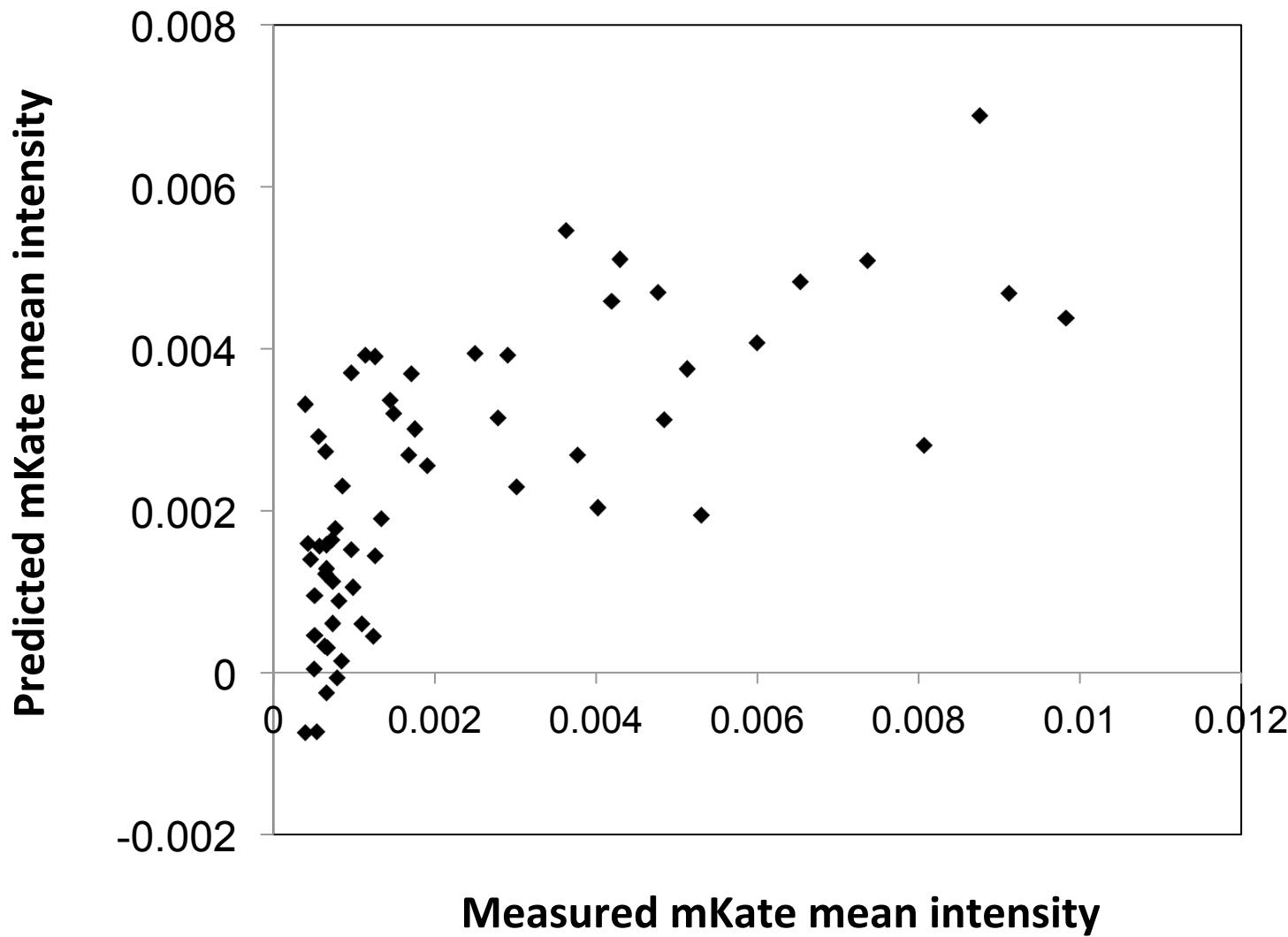
Distribution of mKate mean intensity for noncontiguous libraries



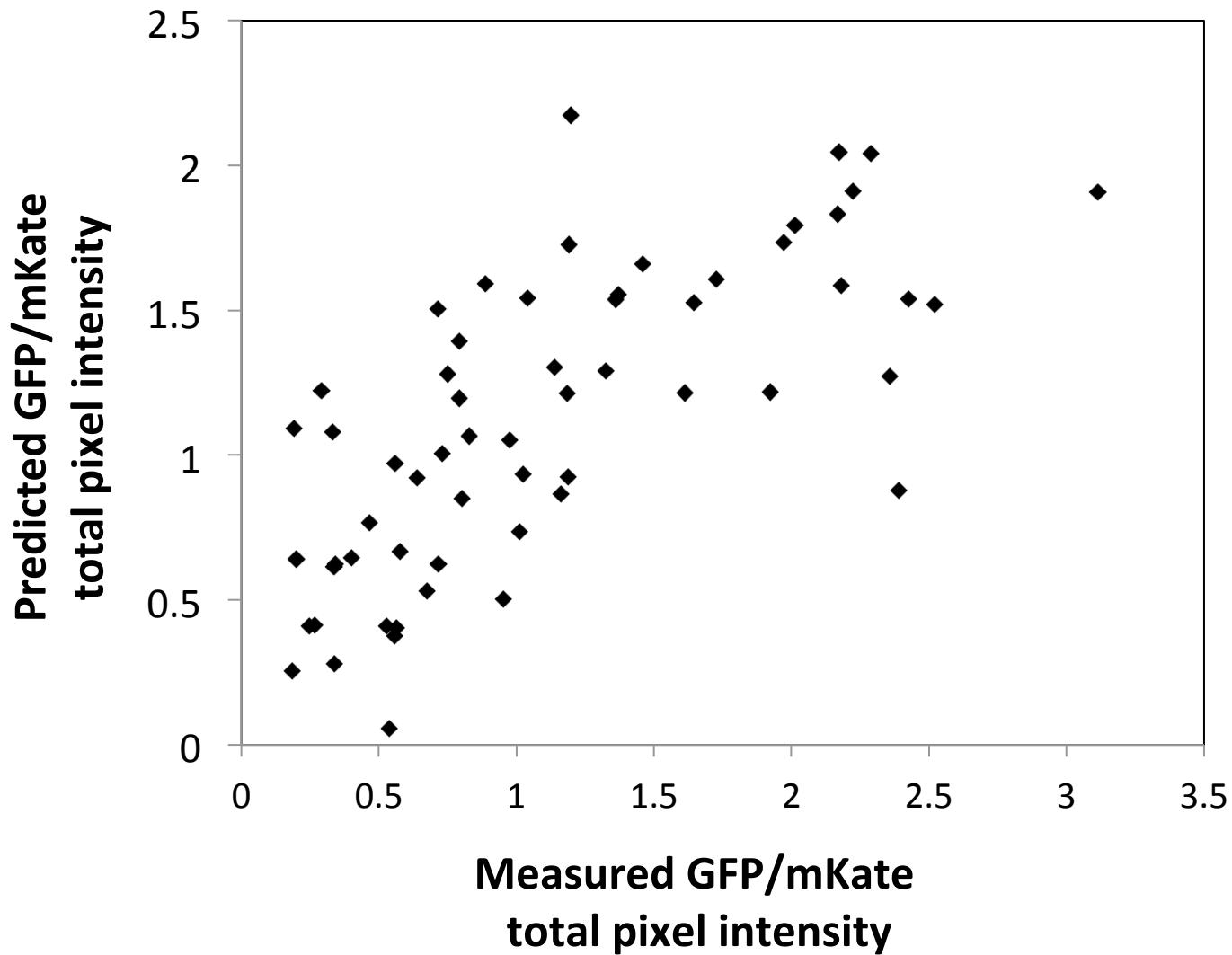
Distribution of GFP/mKate for noncontiguous libraries

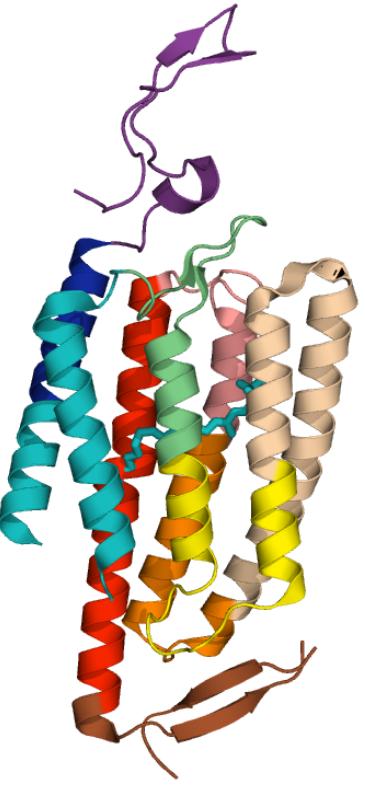


Is expression level a linearly-additive property?

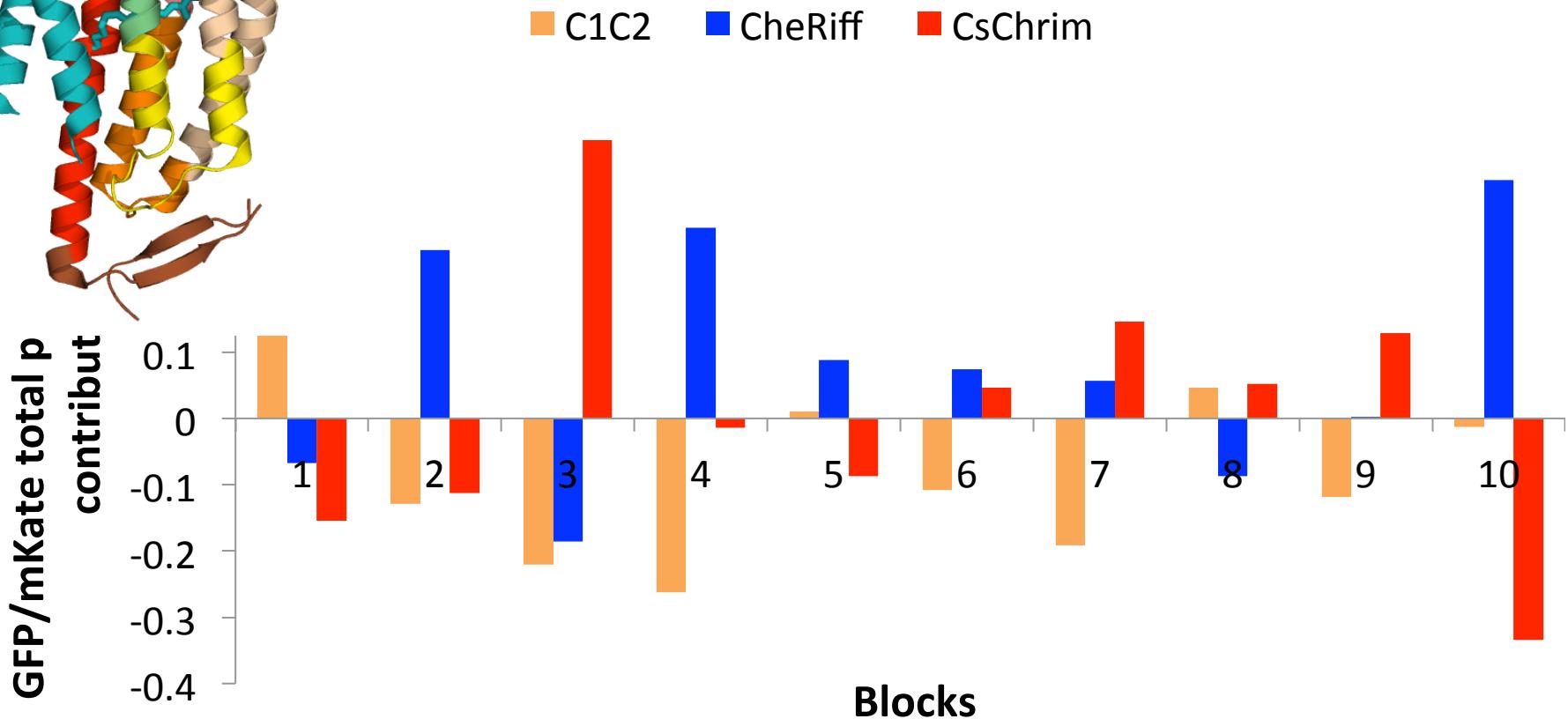


Is membrane localization a linearly-additive property?



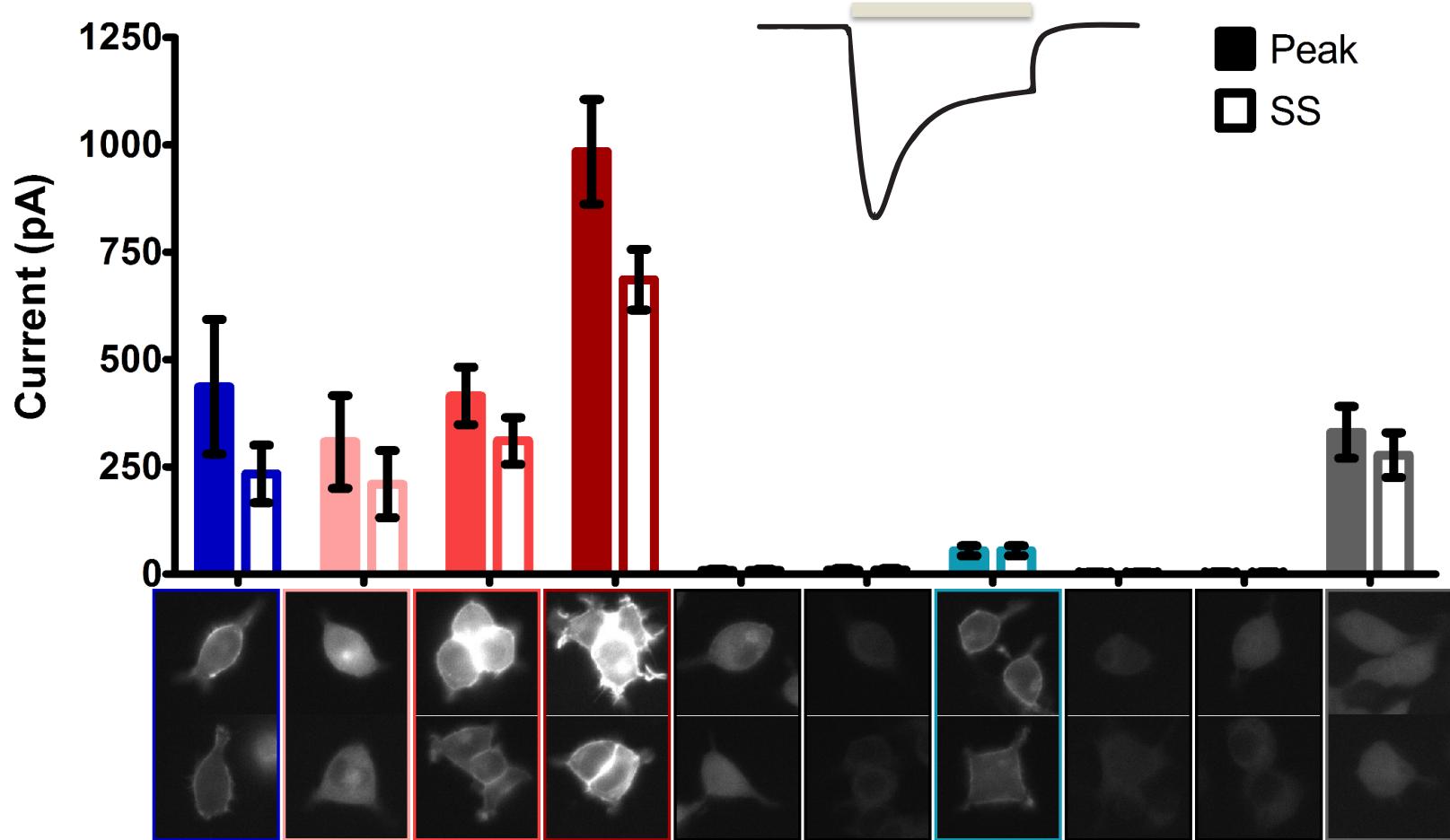


Membrane localization a linearly-additive property?

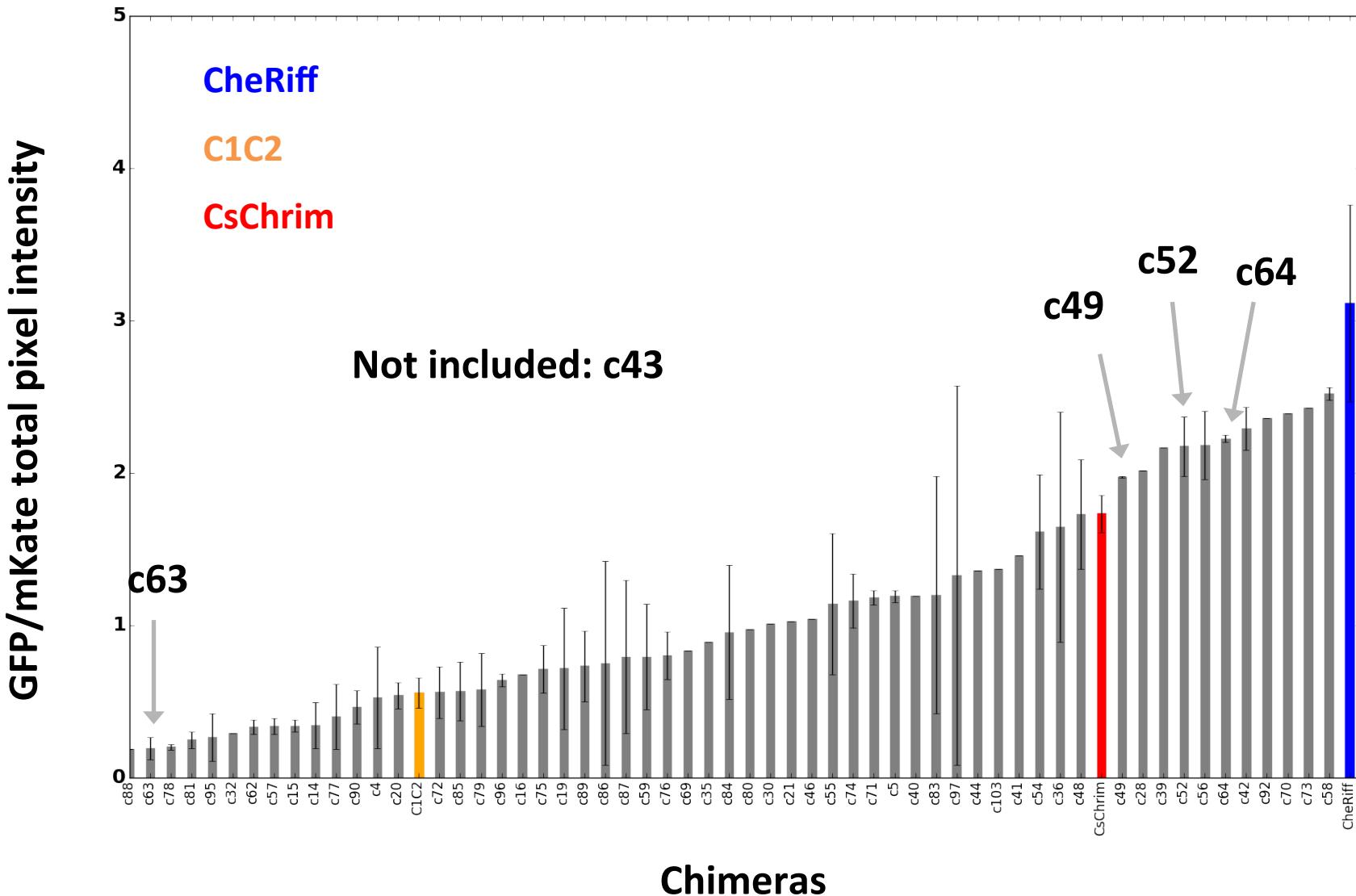


If chimeras have good membrane localization are they functional?

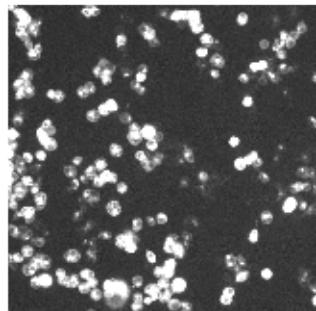
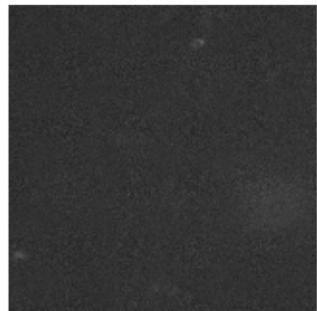
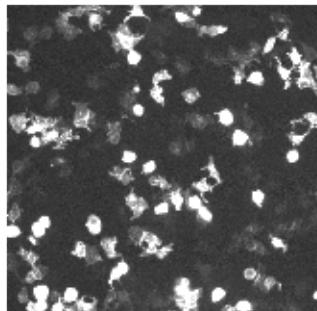
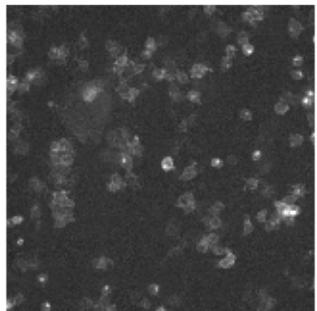
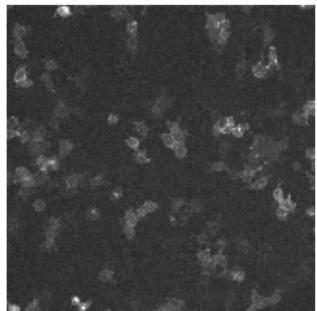
Chimeras vs parents' peak and steady state currents upon light induction in HEK cells



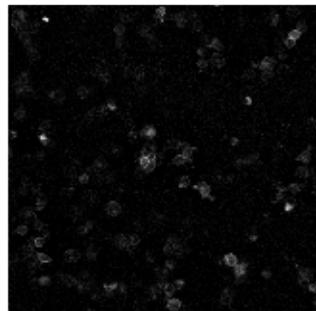
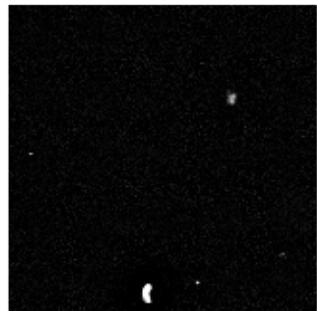
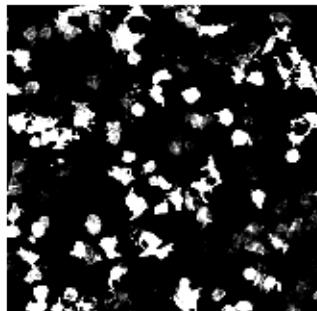
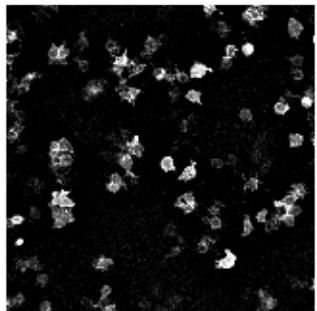
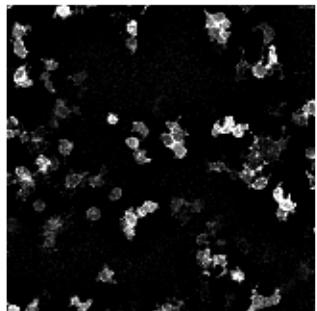
Distribution of GFP/mKate for contiguous libraries



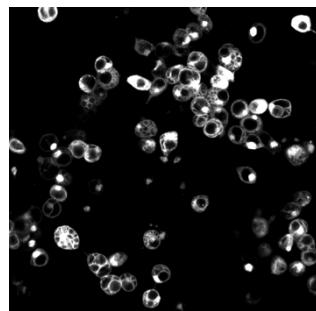
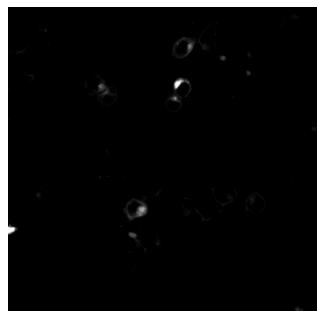
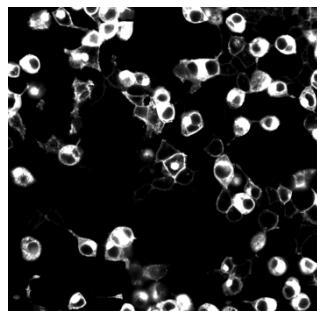
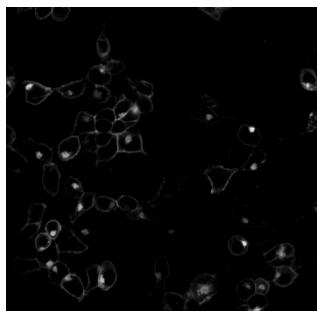
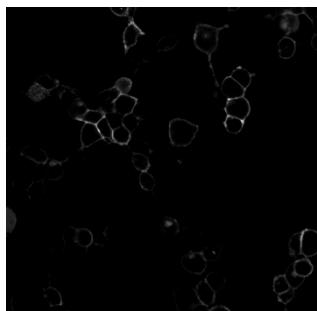
mKate



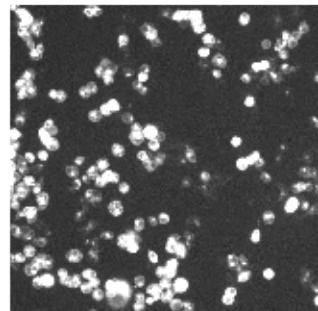
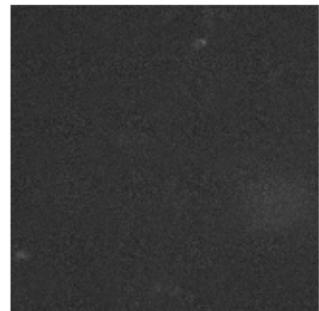
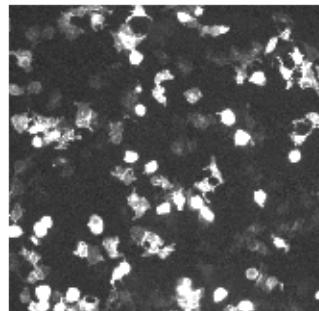
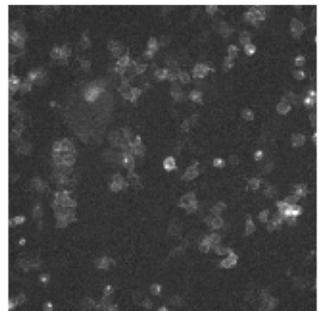
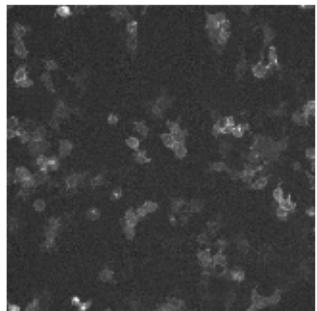
SpyCatcher-GFP



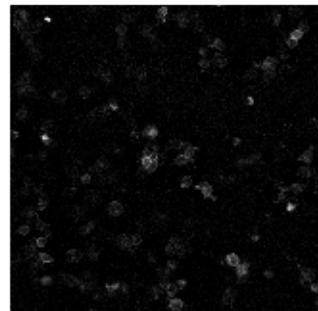
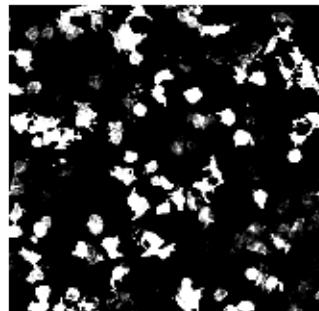
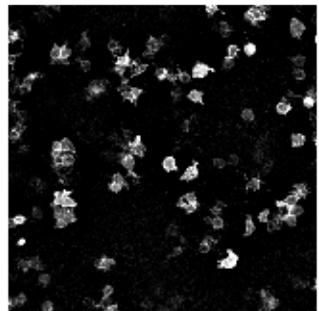
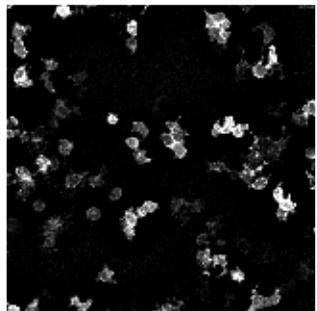
Confocal Images



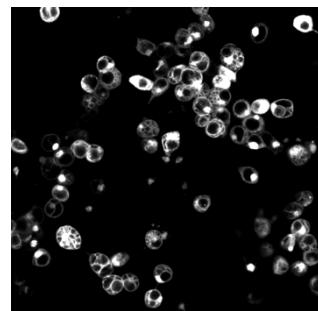
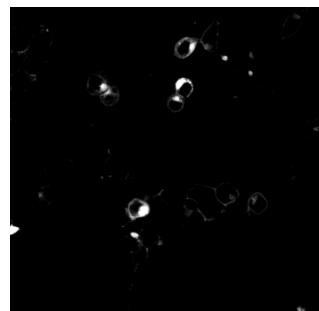
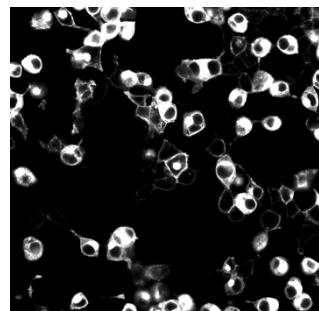
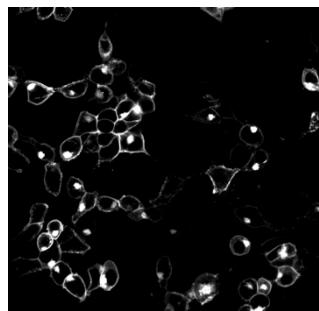
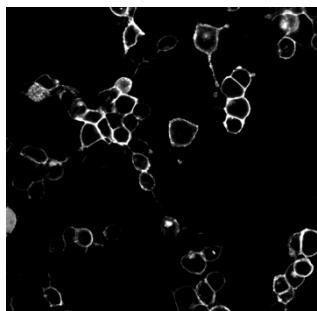
mKate



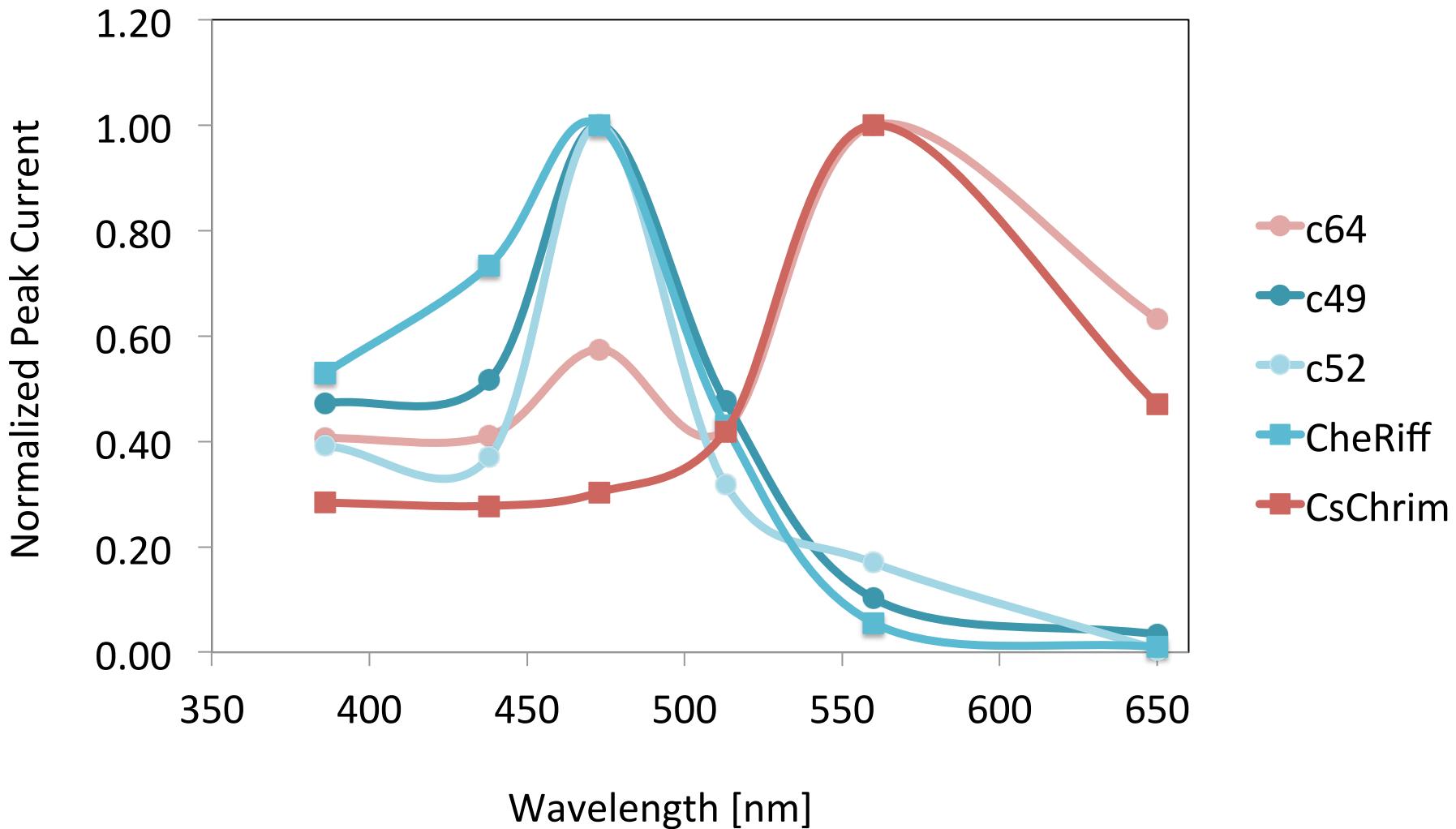
SpyCatcher-GFP



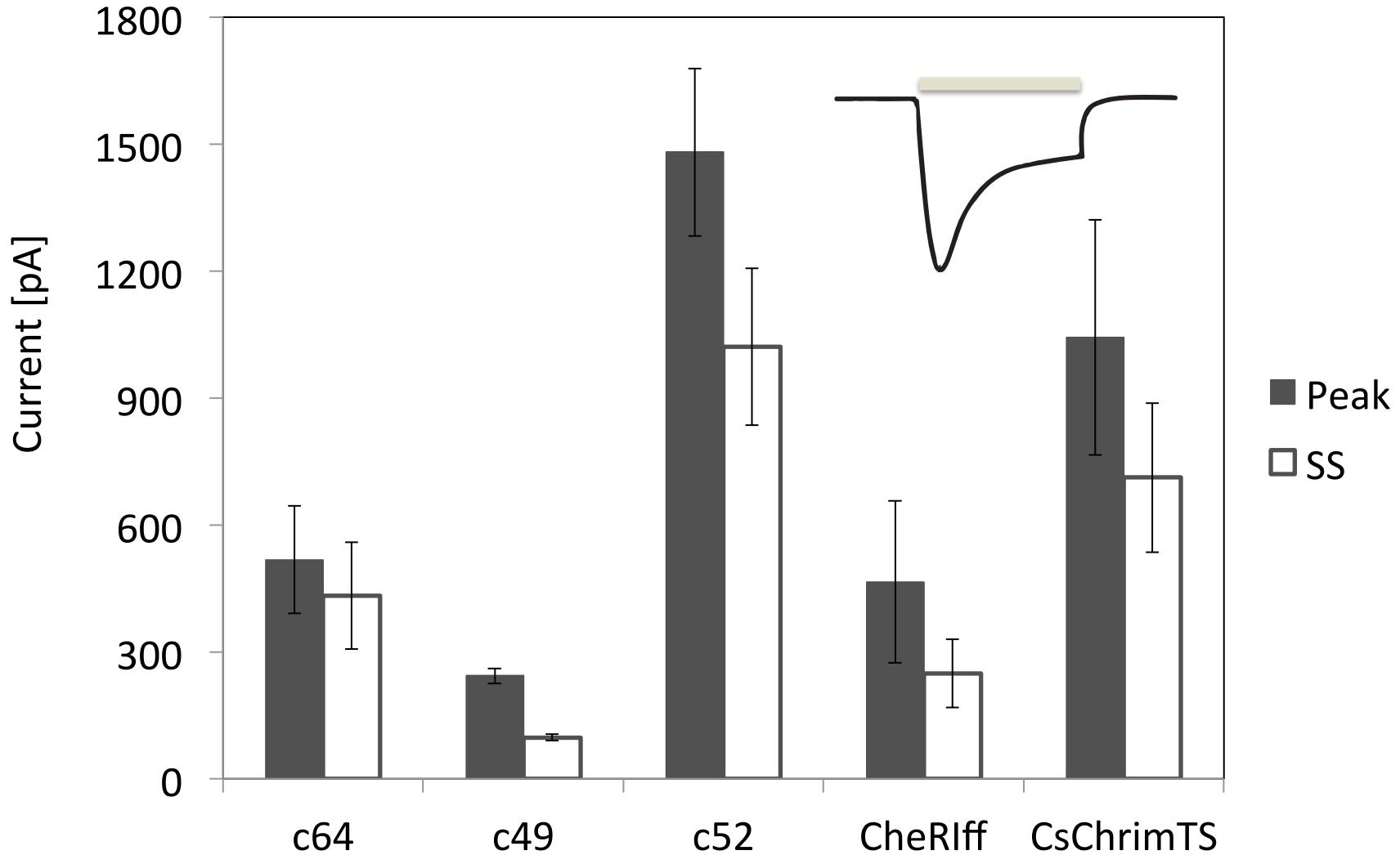
Confocal Images



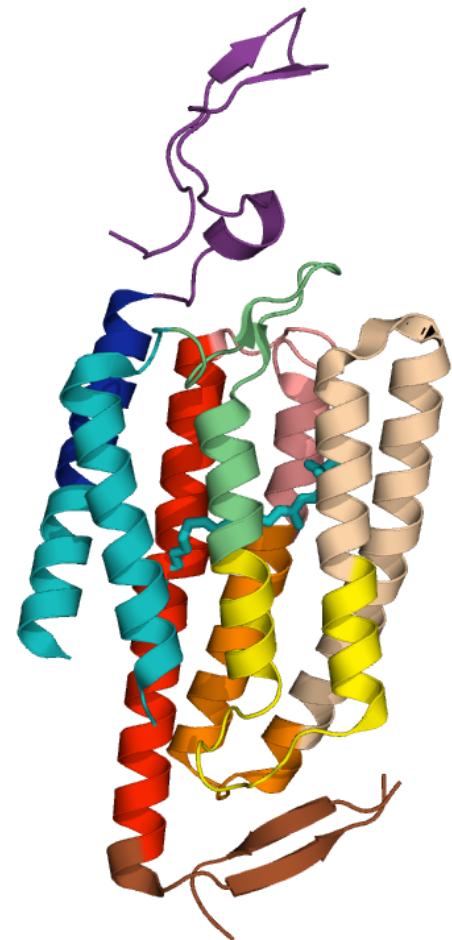
Spectral characterization



Peak and steady state currents



chimera	blocks	mutations	E
CheRiff	1111111111	-	-
CsChrim	3333333333	-	-
c52	1111111311	10	7
c49	1111311111	10	8
c64	3333333331	17	7
c45	3111111111	25	10
c63	3333333313	20	16



Summary of results

- Membrane localization assay that works in medium-throughput; but could be further optimized for low expressers
- Structure guided recombination can predict cut sites to generate a library with a high fraction of chimeras that expressed (& fold) for a transmembrane protein
- Both contiguous & noncontiguous libraries produce a high fraction of folded proteins
- Chimera properties are predominantly distributed between parents
- It does not appear that expression level or membrane localization is strongly linearly additive

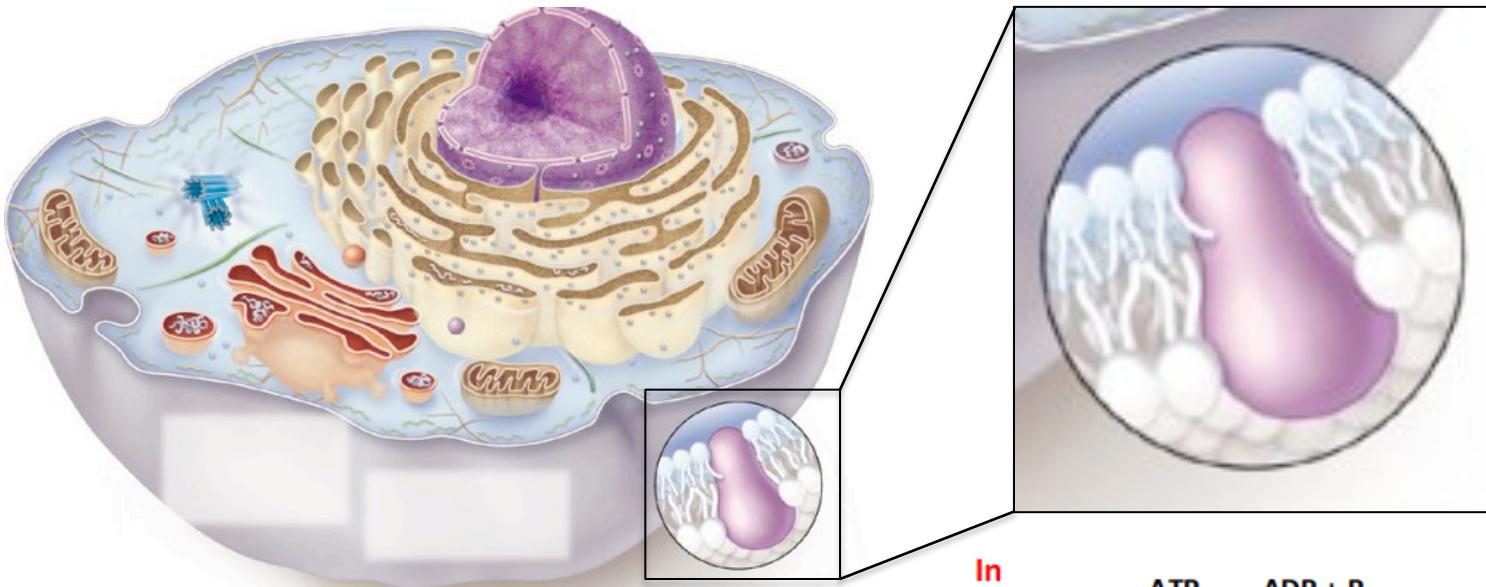
Summary of results

- Re-screening of a couple of chimeras is consistent with screening results
- Hypothesis that good membrane localization = functional seems to be holding up!

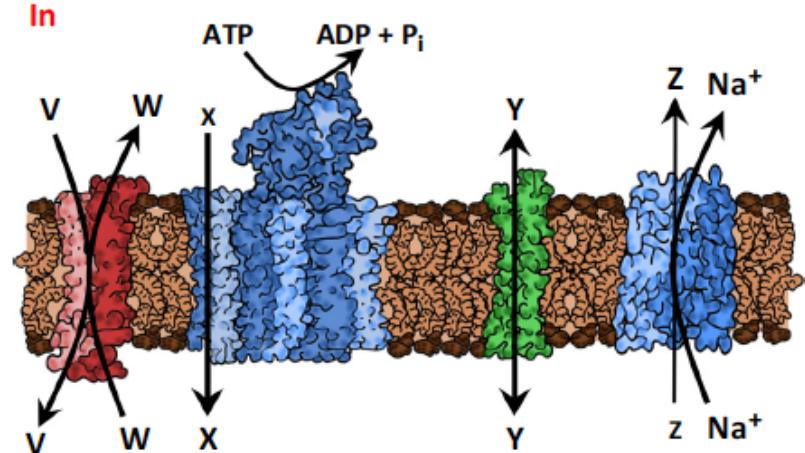
Next steps...

- More repeats of each construct: quadruples instead of duplicates
- Improve quantification method (colocalization)
- Test the remaining Twist constructs
- Use Gaussian Process model to predict new, improved chimeras
- Further activity characterization of top variants

Engineering of membrane proteins



Methods for diversifying & screening membrane proteins



Acknowledgments

- Frances
- Viviana
- Recombination project: Austin & Kevin
- G-lab & Arnold lab

