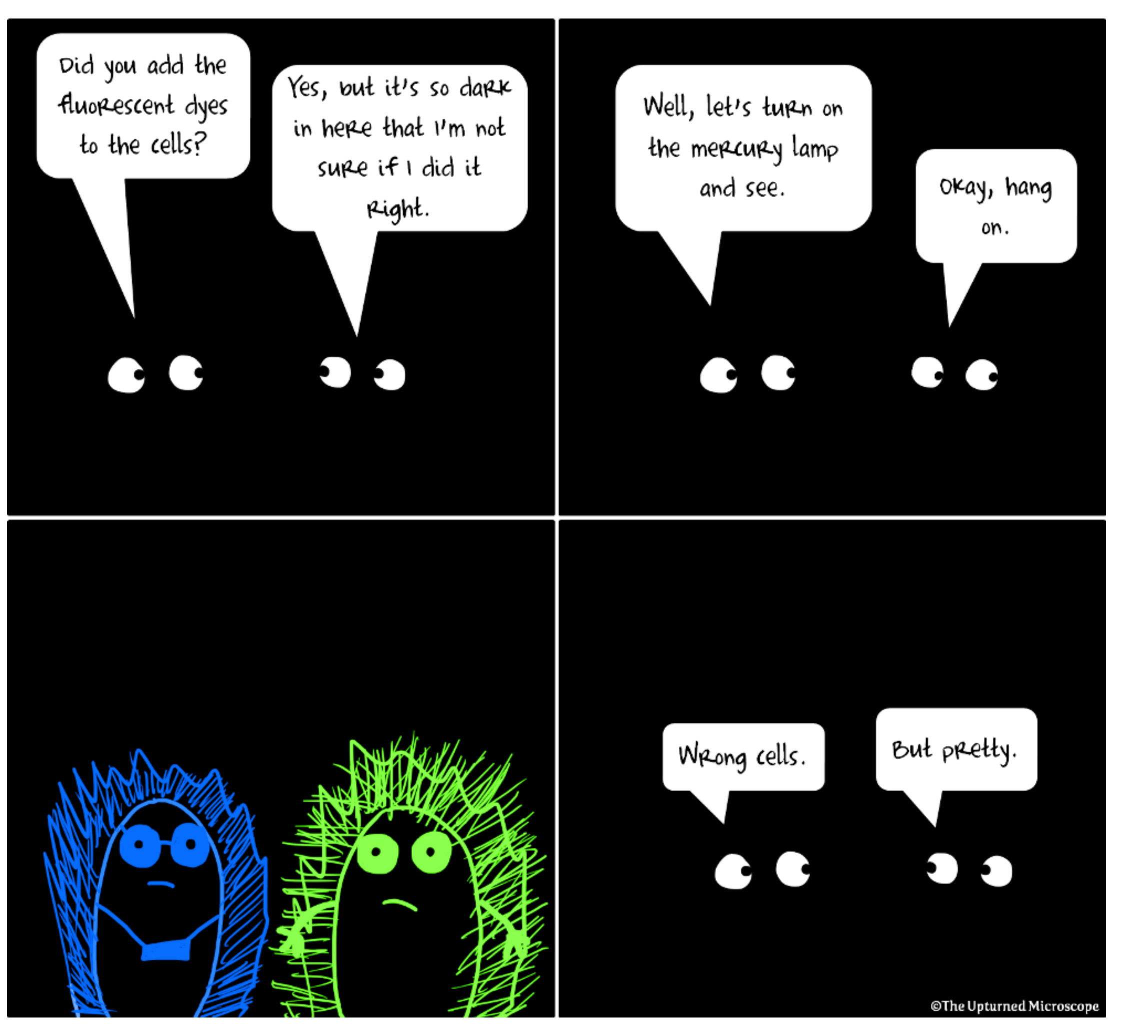
M2D5: Immunofluorescence staining

1. Prelab
2. Fix yeast
3. Antibody staining of Fet4_mutant



Overview of Mod 2 experiments

Last lab:

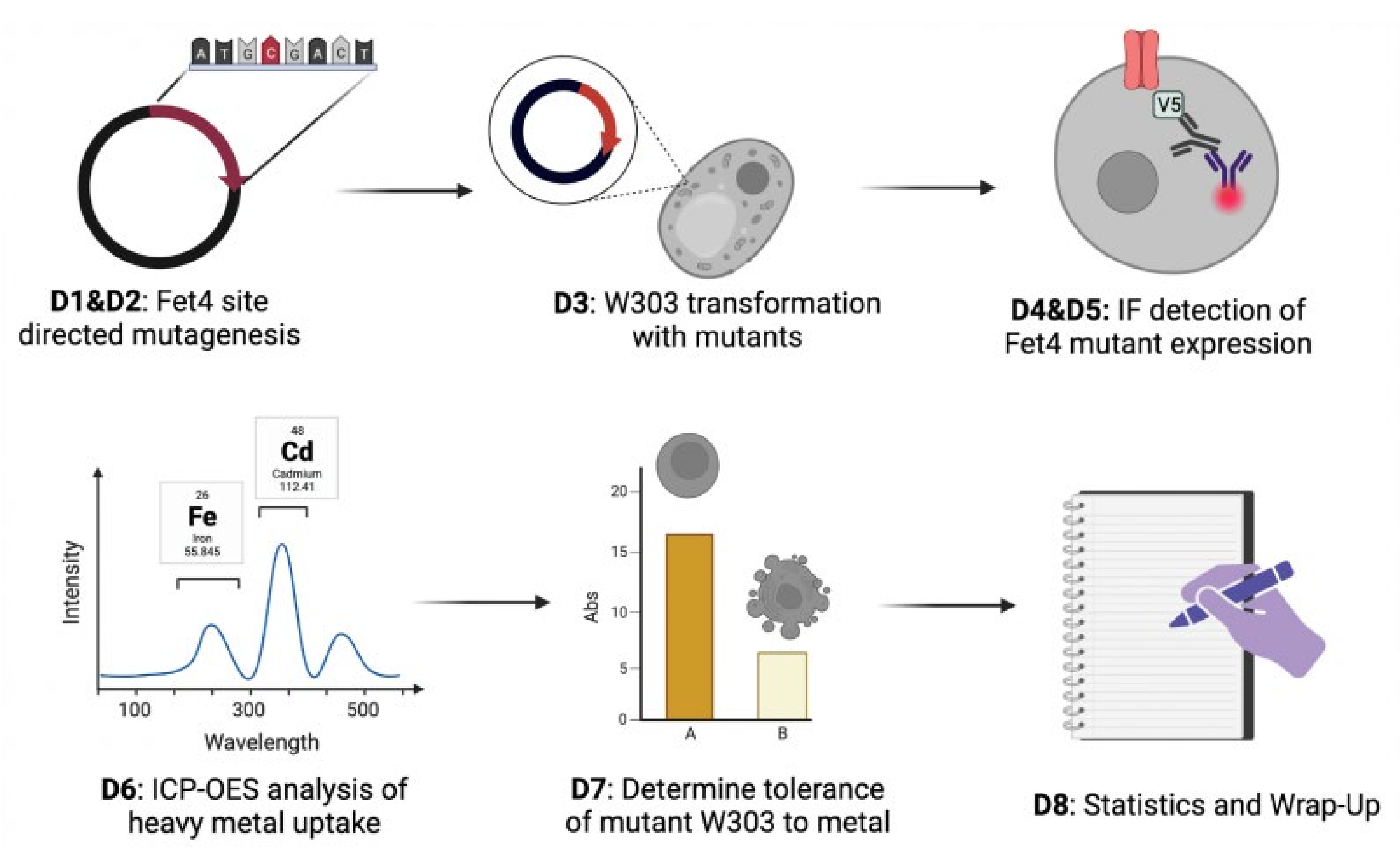
Transformed W303 yeast with mutated FET4 plasmids

This lab:

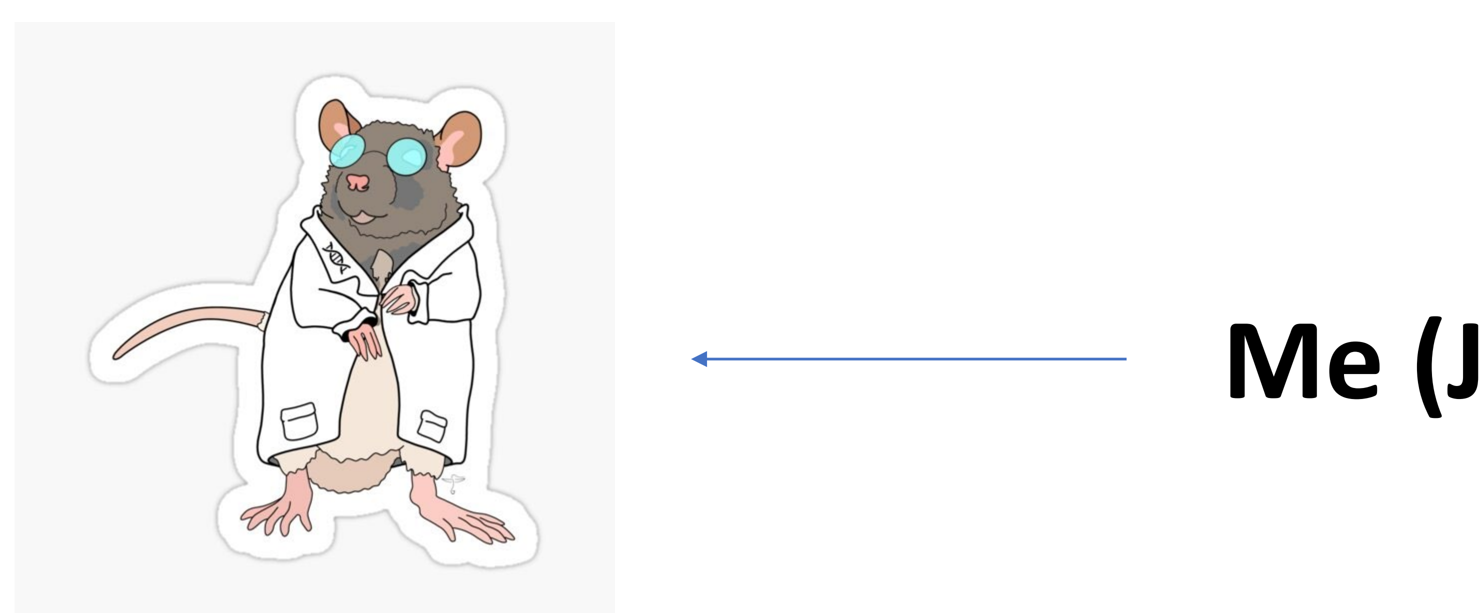
Perform IF on FET4 expressing yeast

Next lab:

Prepare Metal Uptake, analyze IF data



A rational basis for amino acid mutation in Fet4



Me (Jamie)

Goals for a rational FET4 Mutation

Ultimate goal: To perform a point mutation that shifts Iron transport preference to Cadmium Transport

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1) Selected amino acid should bind iron

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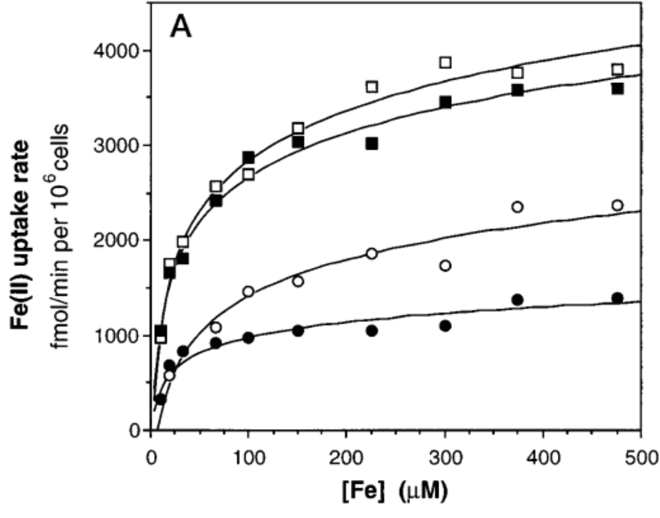
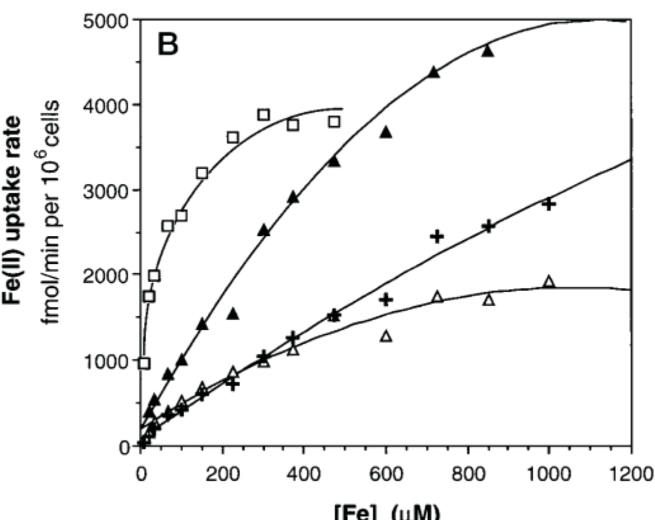
How do we narrow down 500+ amino acid candidates down to just 1-2?

Residues that are important for iron uptake are known by alanine screen in Dix paper

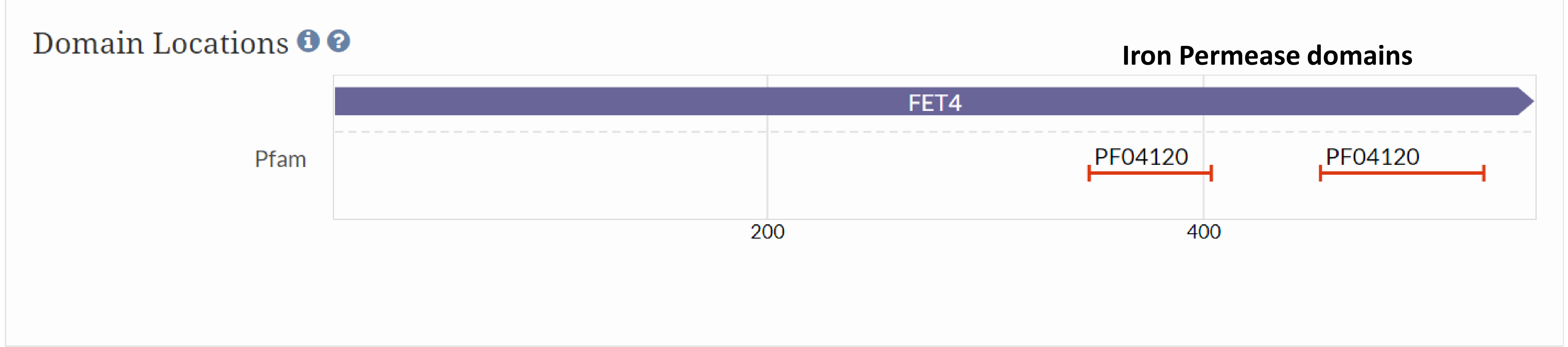




Order from Highest Fe Intake to Lowest

1) WT
2) Y222A (Dark Squares)
3) Y276A (Dark Triangles)
4) D271A (**+**)
5) Y392A (Empty Circles)
6) Y352A (Empty Triangles)
7) Y408A (Dark Circles)

Where are the important domains located?



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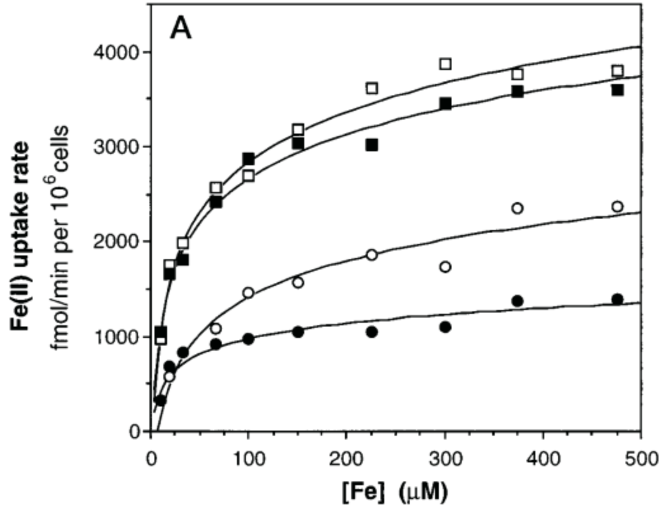

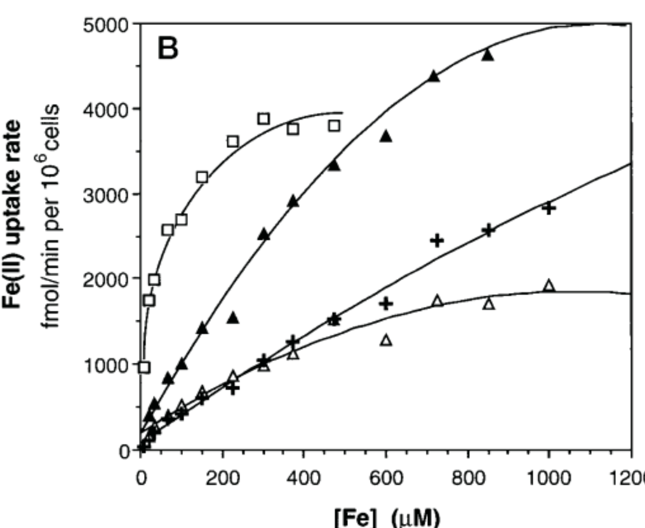

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Permease Domains are: 348- 404 / 454 – 529

Bolded mutations are within the permease domains

The least impactful mutations being outside these permease domains is internally consistent.

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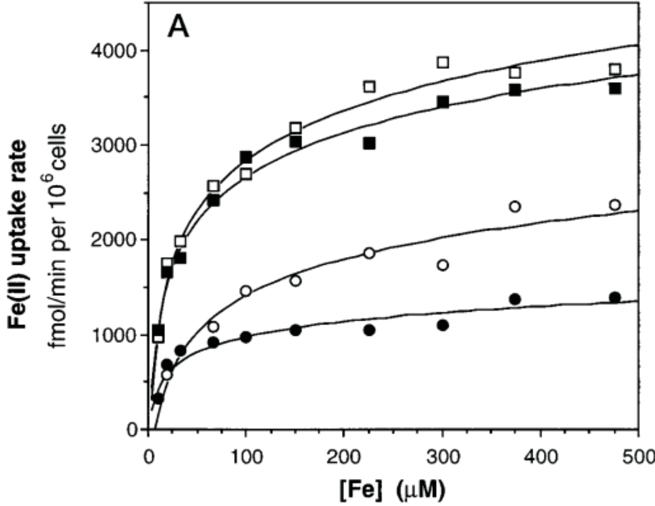

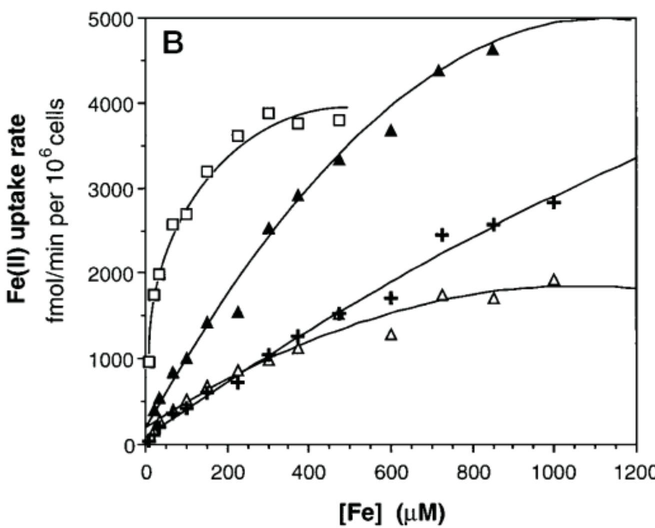

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What characteristics are typical of good mutation candidates?

Sun et. Al performed an alanine screen in SFM1 to design hyperaccumulators

ARTICLE

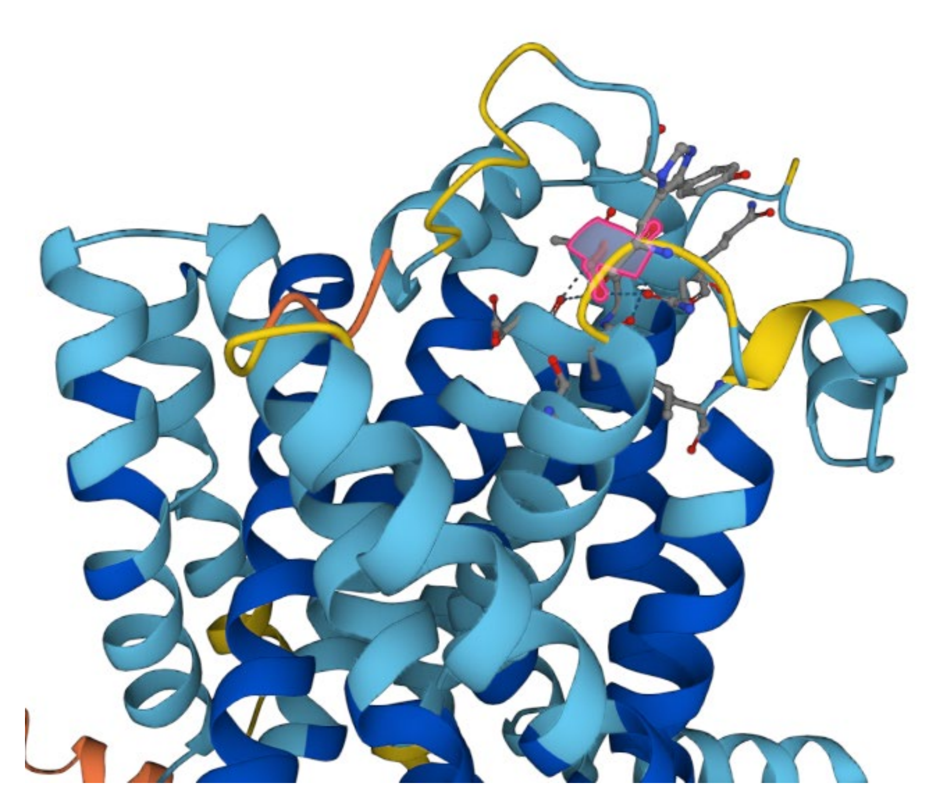
https://doi.org/10.1038/s41467-019-13093-6

OPEN

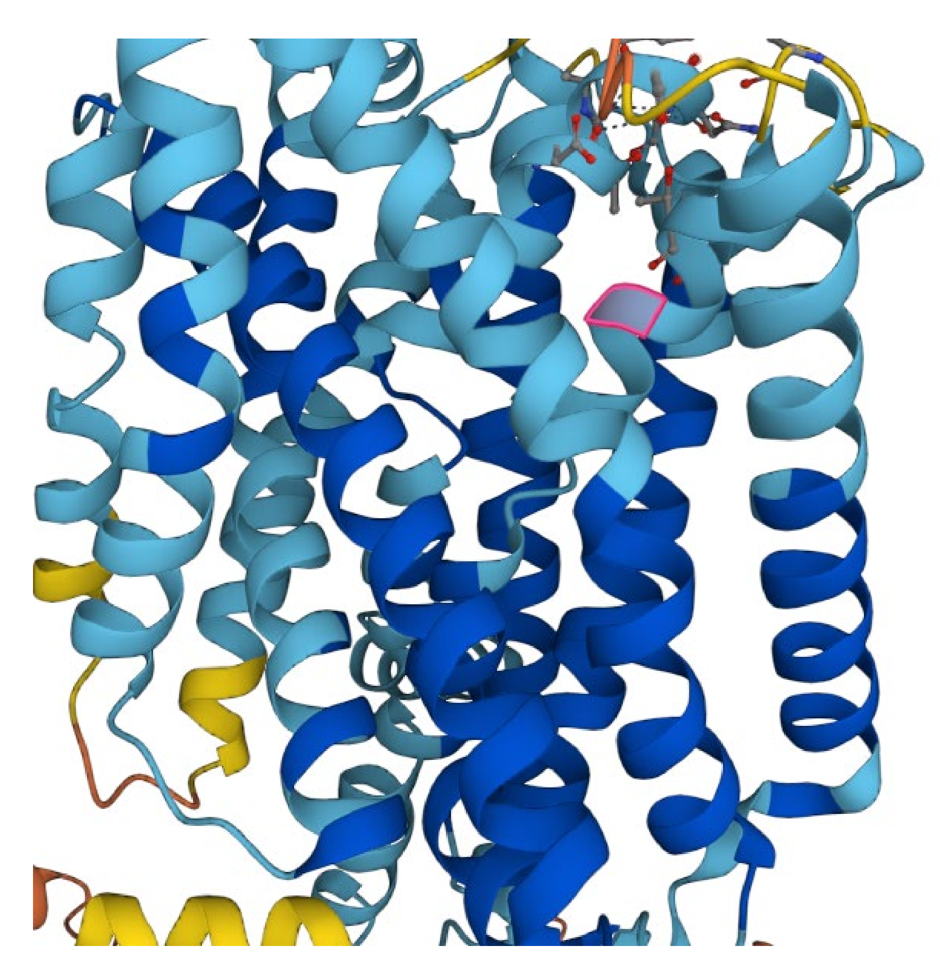
Designing yeast as plant-like hyperaccumulators for heavy metals

George L. Sun[1,2], Erin.E. Reynolds[3] & Angela M. Belcher[1,2,4*]

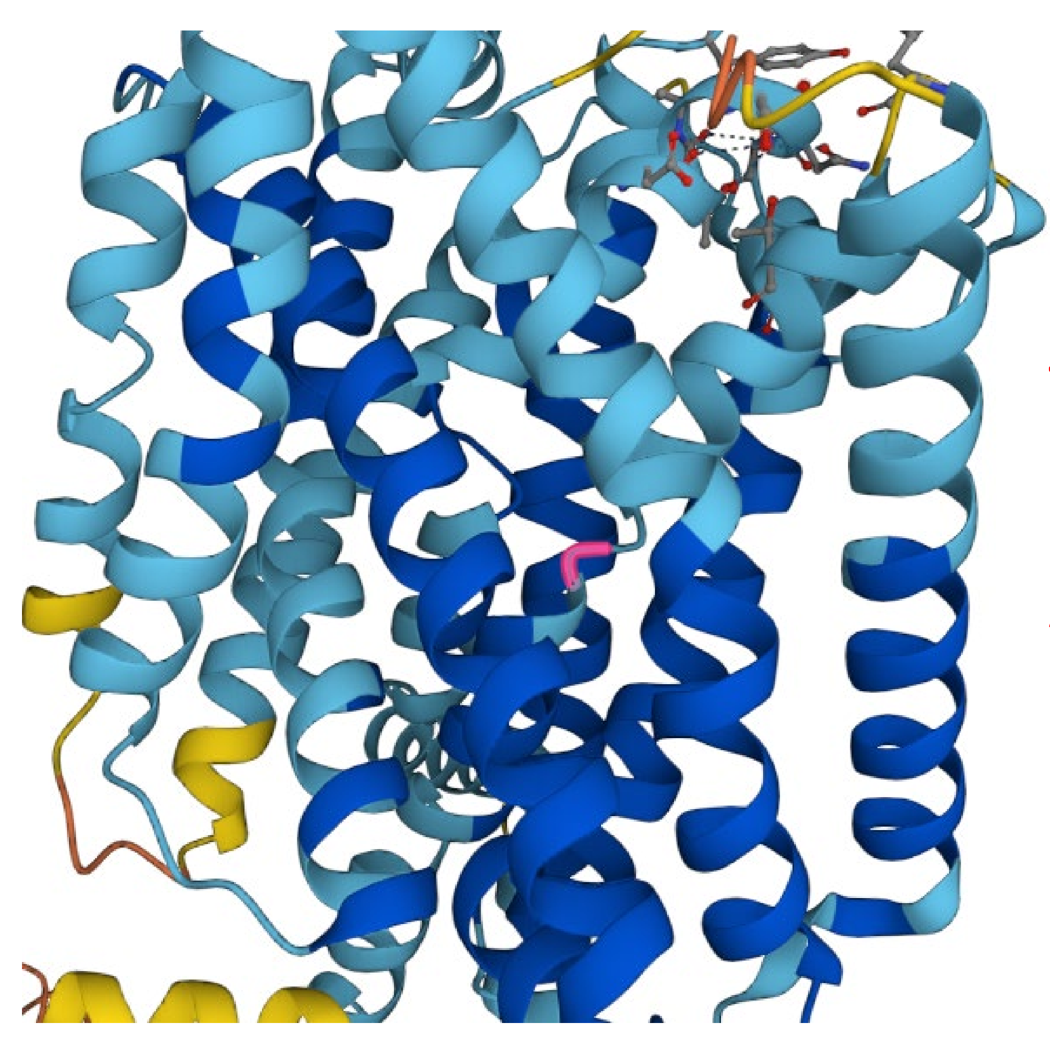
In SMF1 S105 is points into the pore



In SMF1 S269 is in the pore



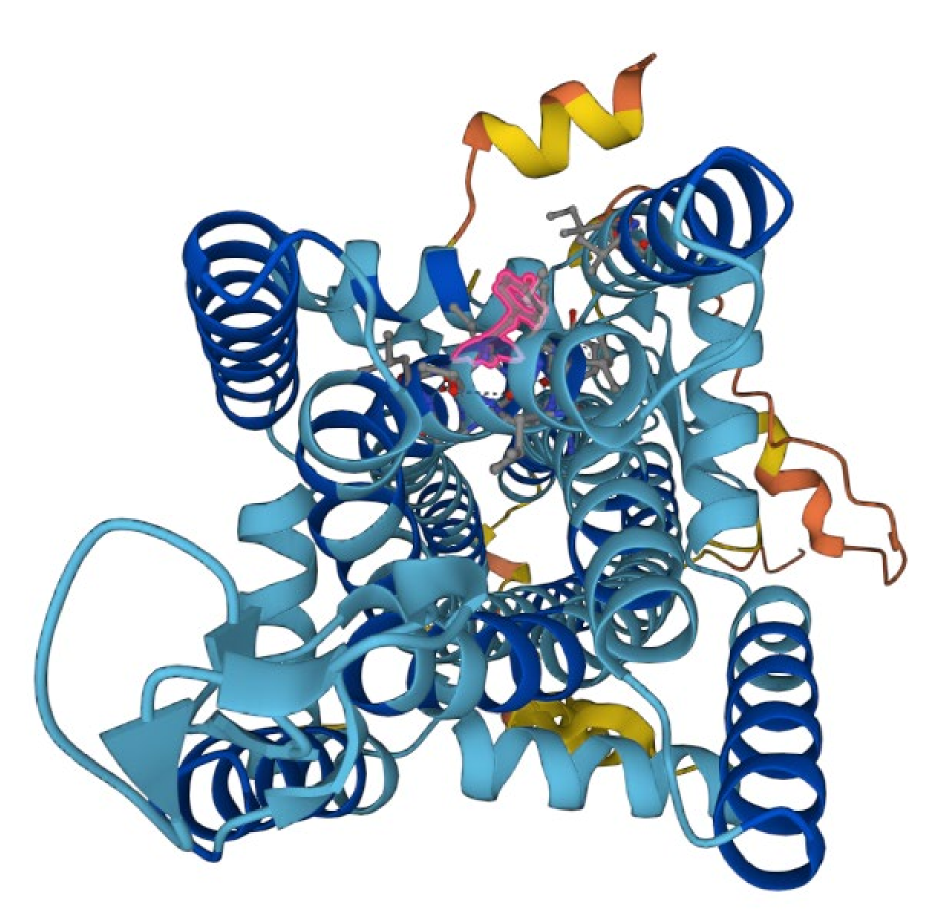
M276 is straight up in the pore (rationally chosen due to conservation) in SMF1



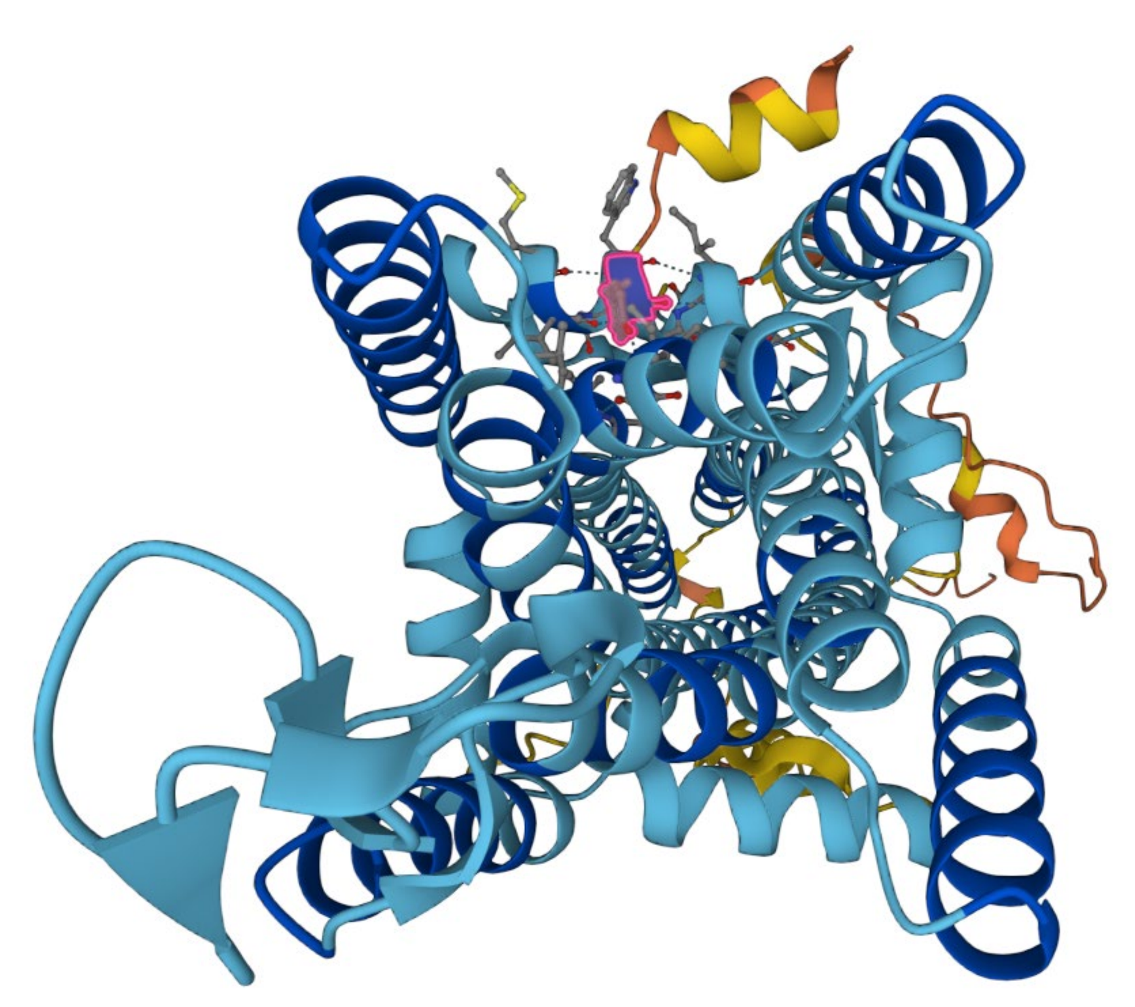
The best residues to mutate in SMF1 were all in the pore

Are there Iron-binding residues in the pore of the FET4?

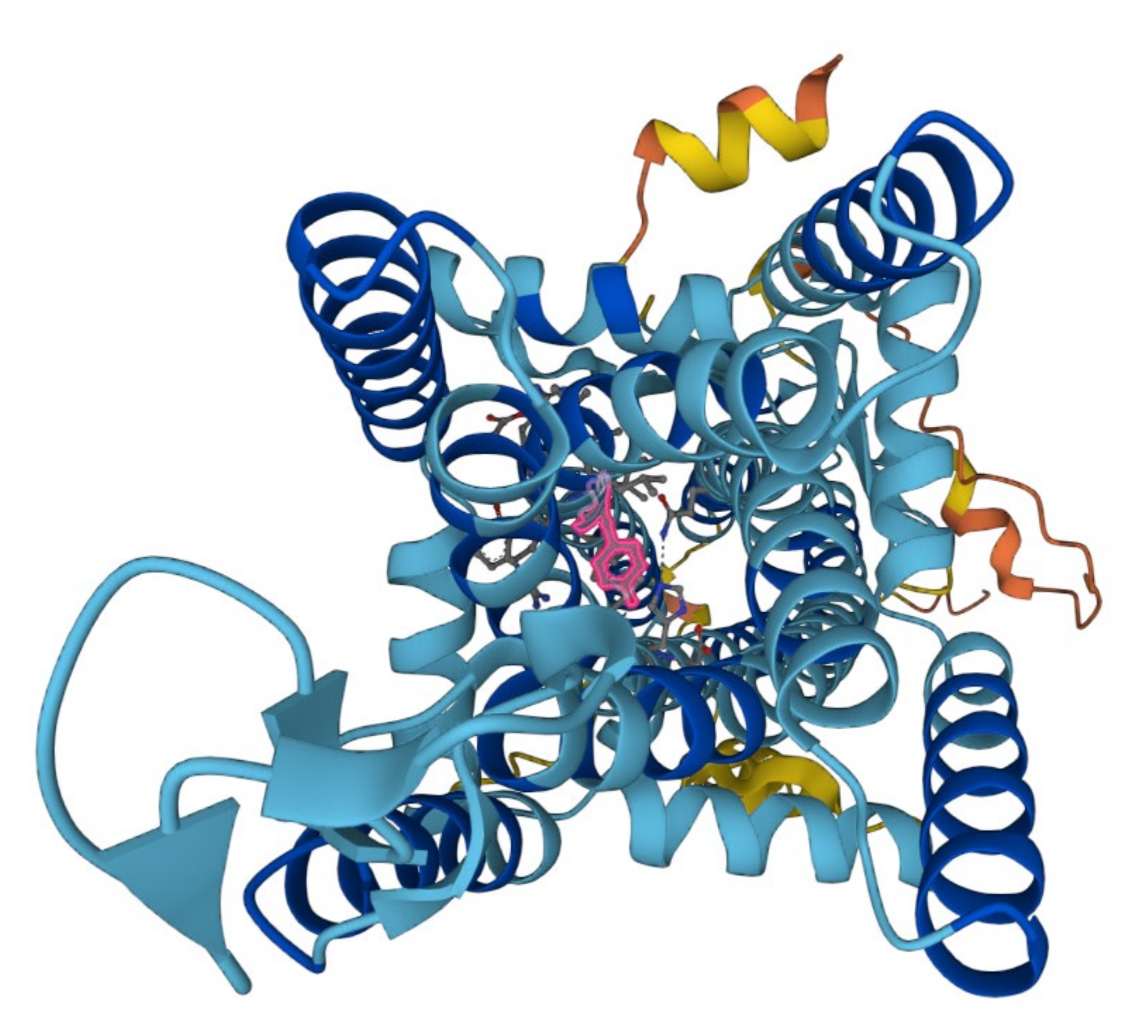
Y392 is a tyrosine that points to the outer edge of the protein



Y352 is a tyrosine pointing inward, potentially interacting with the helix that makes up the pore(?)



Y408 has a tyrosine jutting out into the pore



What to mutate the amino acid to?

Sun et. Al screened mutations in a different protein for HM binders

Cadmium

105 S-> C (Polar, Uncharged -> Terminal Sulfur)

276 M-> C (Hydrophobic -> Terminal Sulfur, also,

This one was rationally designed)

269 S->T (Polar -> Polar)

Strontium

G->R (Kink -> Positive)

T->S (Polar -> Polar)

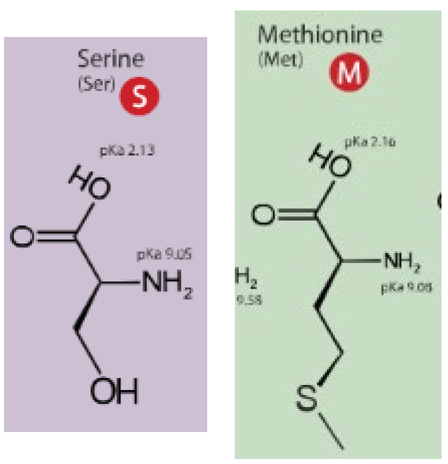
M->C (Hydrophobic -> Terminal Sulfur)

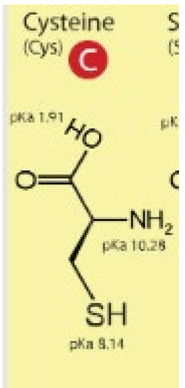
G->Q (Kink -> Polar Uncharged)

Importantly, no amino acid conferring affinity for cadmium or strontium are hydrophobic residues.

None are negatively charged

More than half of them are -> cysteine mutations if you don't count the glycine kink mutations





Cysteine has a very high stability constant w/ cadmium

Amino acids with a sulfur-containing side chain		
S-Methyl-cysteine	3.79	7.04
Methionine	3.65	6.76
Cysteine[a,b]	12.82	21.71

Strategy #1: Mutate the Tyrosine

Take Y408 and turn it into C

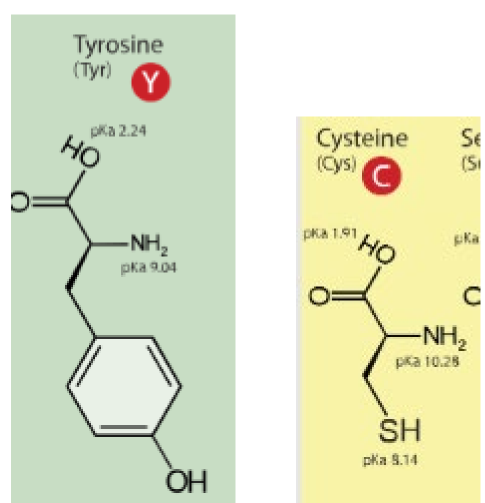
1) Removes the most important Fe complexing tyrosine (with regards to uptake rate)
2) Changes it into a good binder

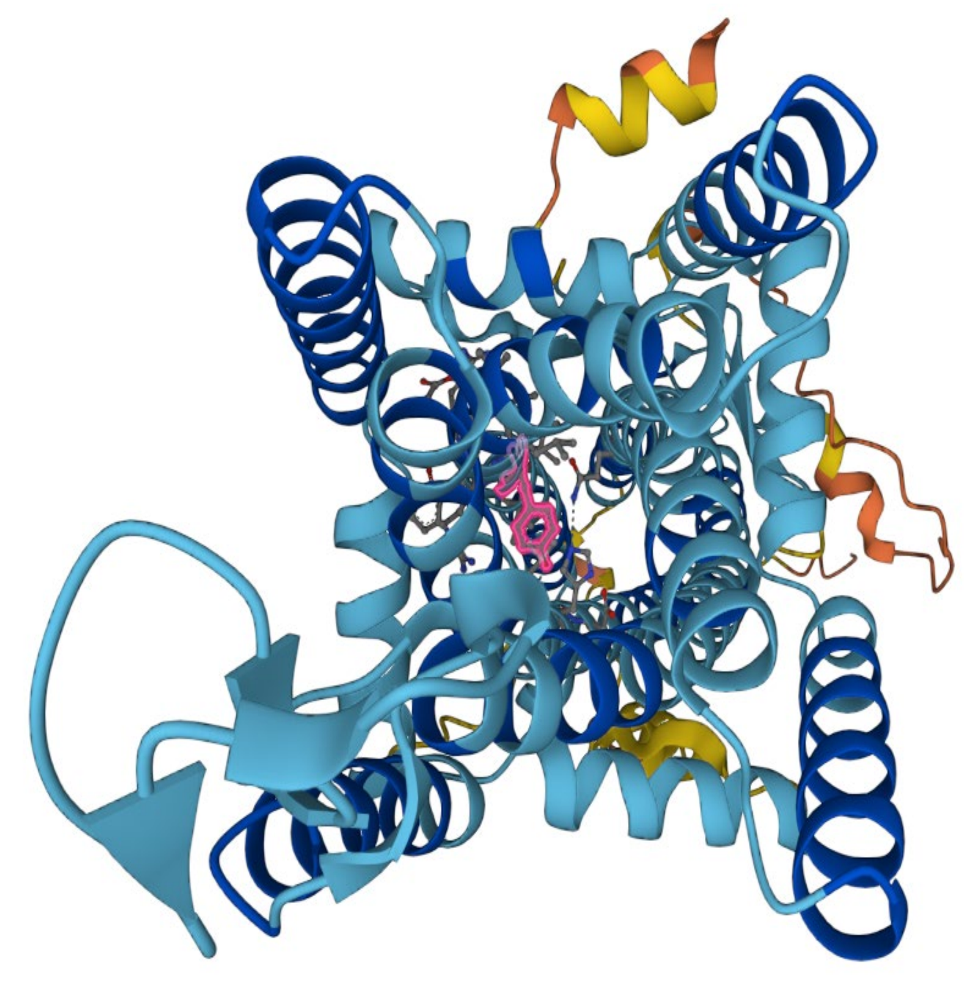
For cadmium

3) -> C mutations consistent with SFM1 work

4) Potential

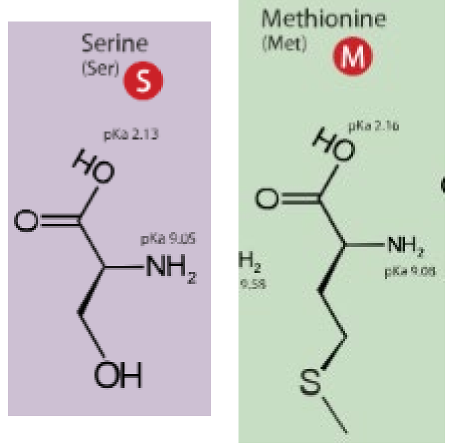
Structure issues

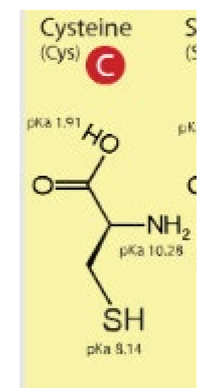




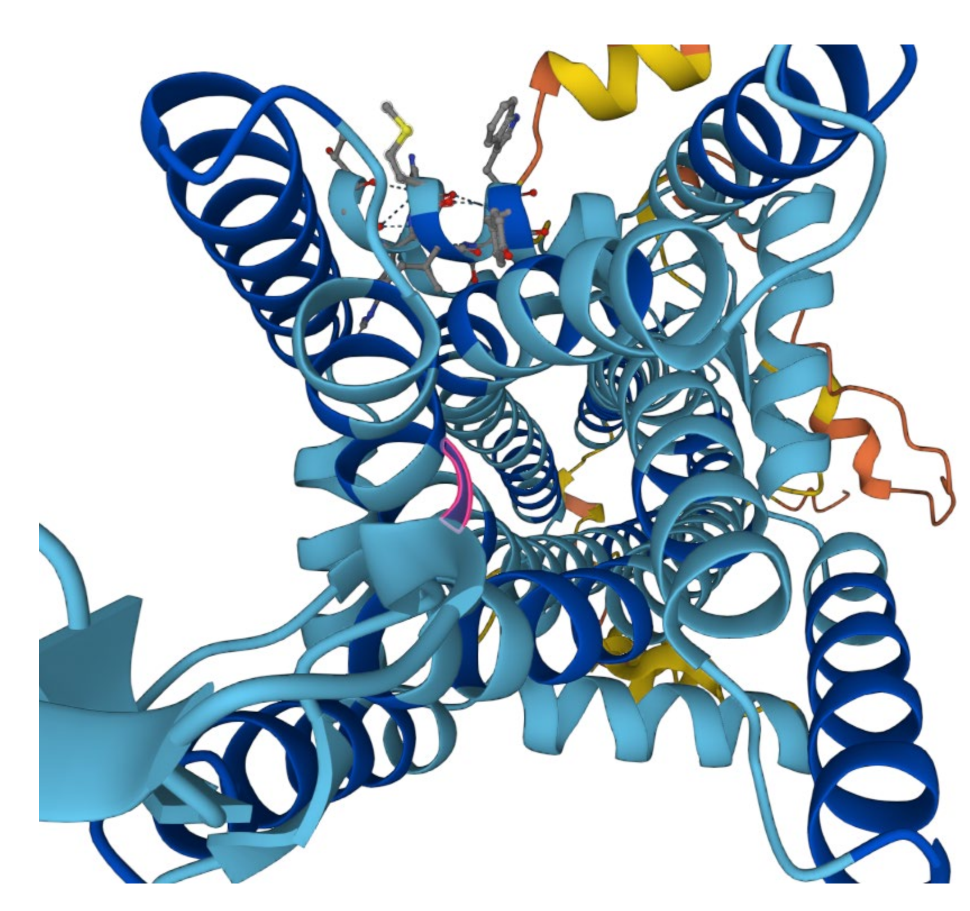
Strategy #2: Turn a nearby S or M into a C

Pro: Looks like the SMF1 work
Con: Iron affinity might still be too strong





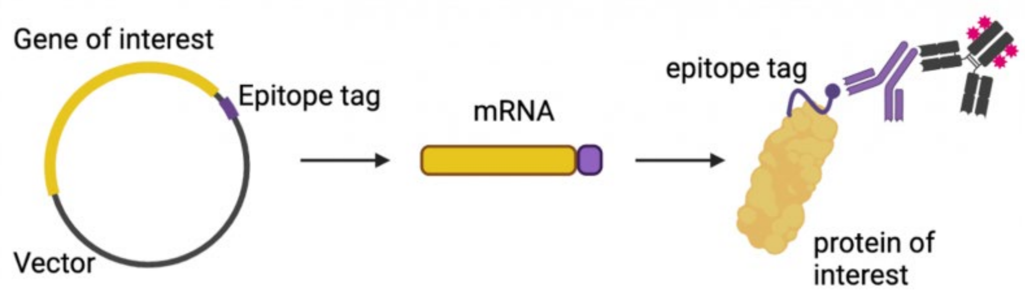
M501 is within the permease domain and sticks out into the pore.



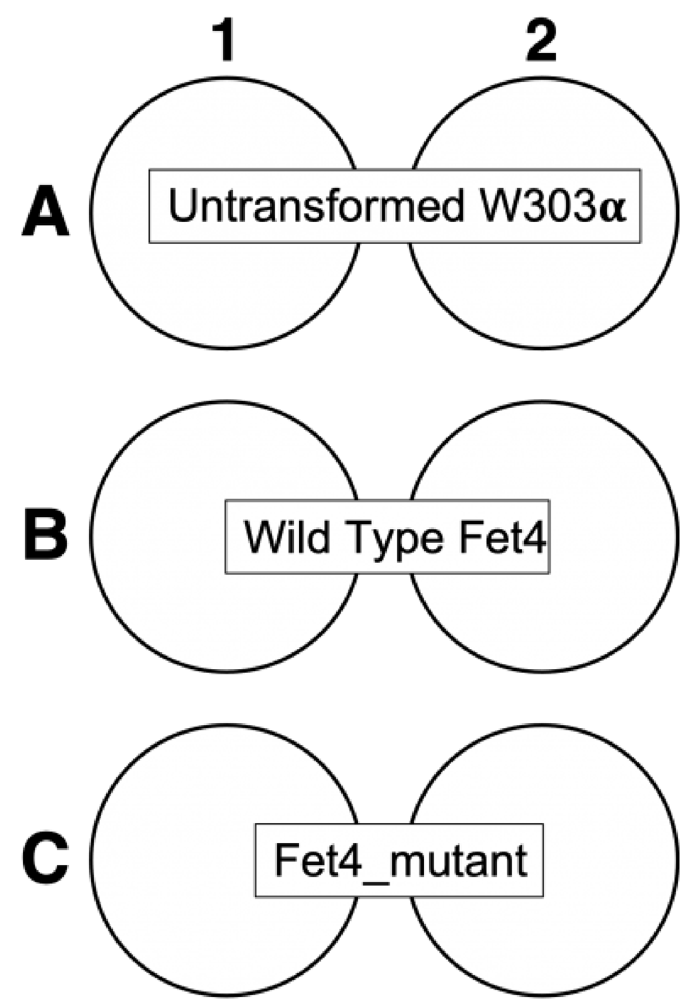
Area containing that methionine *seems* pretty well conserved?

Using immunofluorescence: Expression of Fet4_mutant in yeast

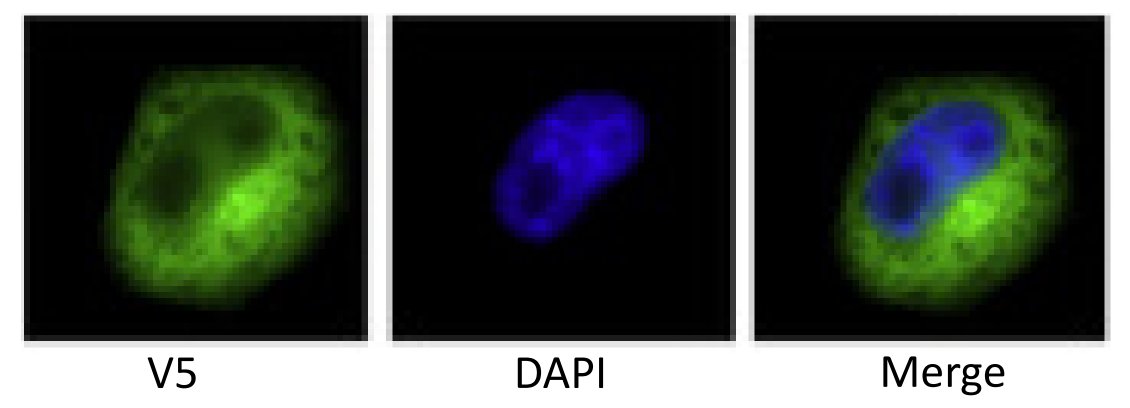
- Yeast cells transformed to express Fet4_mutant protein

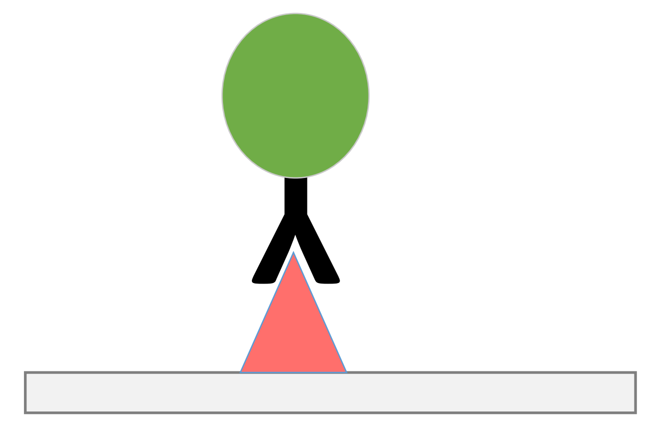


- Why do immuno at all?
- Why untransformed cells?
- Why wild-type Fet4?



Using immunofluorescence: Identification of Fet4_mutant expression





protein of interest	V5
primary antibody	mouse anti-V5
secondary antibody	none
Fluorophore (conjugated to secondary antibody) exc./ em. wavelengths	488/525 nm

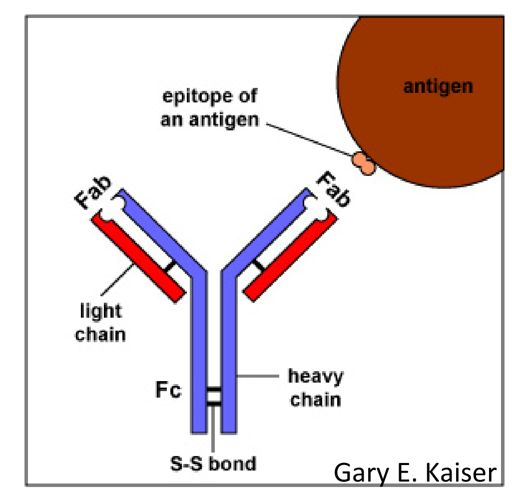
Considerations for using antibodies in the lab

Epitopes

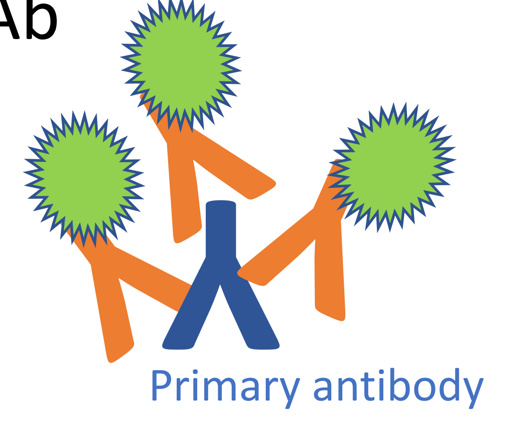
Antigen

- Antibodies bind to specific epitopes on antigens
  - Antigens may have multiple epitopes

- Primary antibody recognizes the antigen
  - Specific protein sequence
  - Specific conformation of protein
  - Specific state of protein (i.e. phosphorylation)



- Secondary Ab recognizes the species of the primary Ab
  - Often conjugated to tag for visualization
    - Enzyme or fluorophore
  - Amplifies signal through multiple bindings
  - Consider sample species when choosing antibodies!

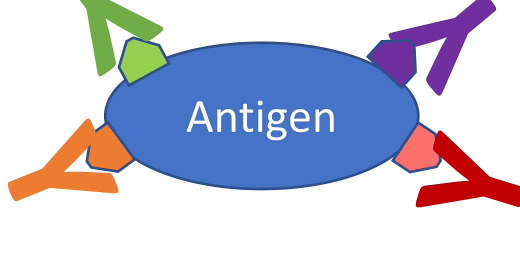


Secondary antibody conjugated to a fluorophore

Polyclonal vs. monoclonal antibodies

Polyclonal

- How it's made: animal (often rabbit) immunized with antigen of interest then antibodies collected from blood sera and affinity purified
- Advantages:
  - Less expensive and faster to produce than monoclonal
  - Multiple antibodies in one polyclonal mixture can increase antigen recognition by binding multiple epitopes
    - Especially useful for proteins with low expression
- Disadvantages:
  - Variability from lot to lot

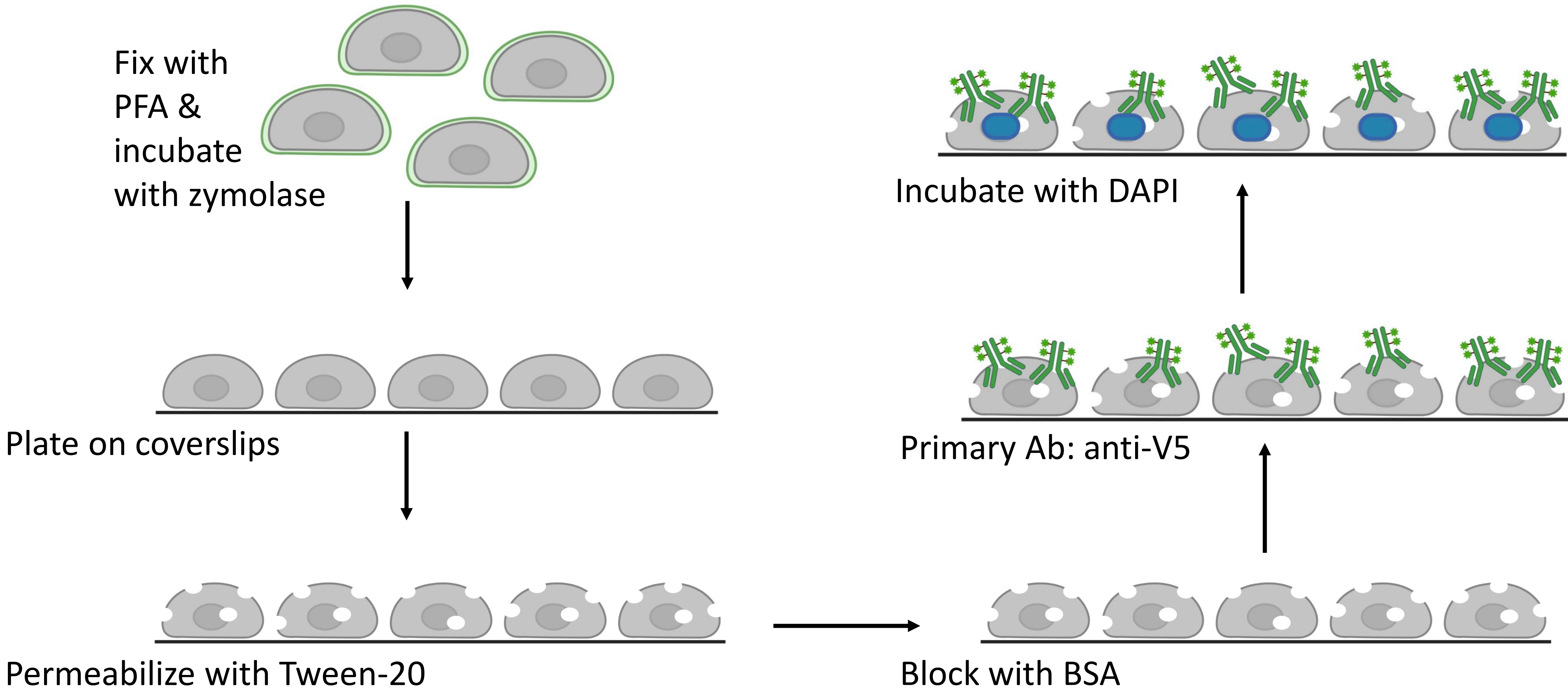


Monoclonal

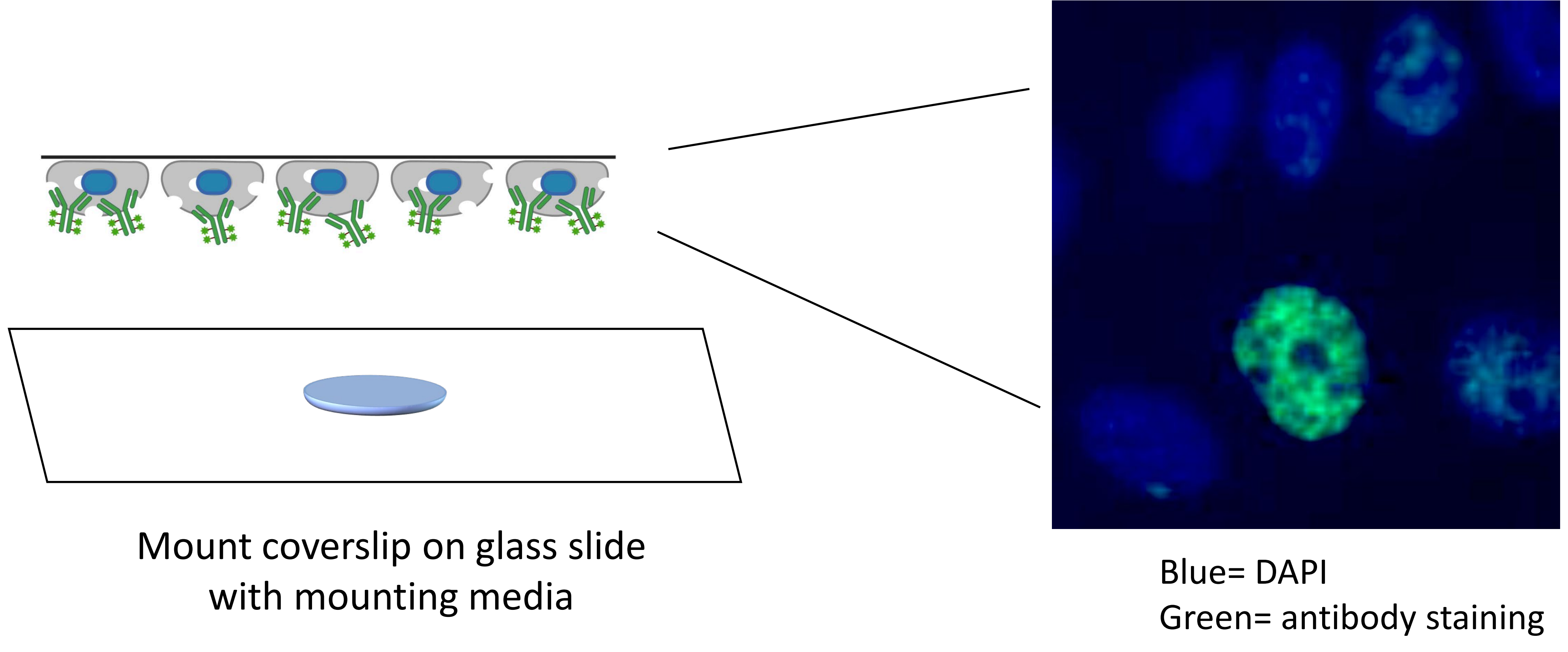
- How it's made: animal (usually mouse) immunized with antigen of interest then B cells from spleen are harvested and fused with myeloma cells to create hybridoma cell line that will continually produce single antibody clone
- Advantages:
  - Very consistent
  - Binds single epitope (can also be disadvantage)
- Disadvantages:
  - More expensive and requires animal sacrifice

Antigen

Using immunofluorescence (IF) in yeast: steps in protocol



Finish IF by mounting coverslips on slides



For today:

1. Fix yeast samples
2. Perform IF
    1. Downtime: Look at new alignments on Dropbox that show the mutations
3. Mount coverslips on slides for imaging

For M2D6

1. Write methods for M2D2-M2D5

*** Individual Assignment ***