

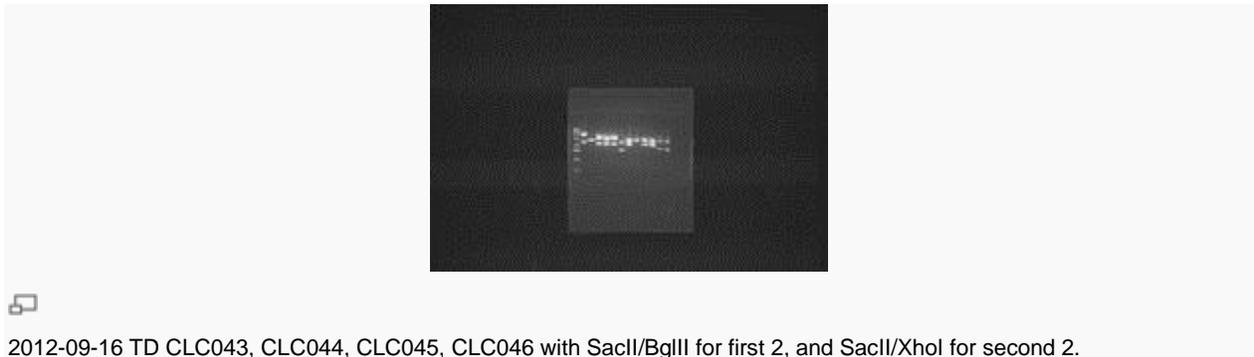
Thomaschow 20 September 2012 (PDT)

Meeting:

1. Change CP images to illustrate the step (color).
2. A lot of the techniques we want to apply requires a large data set regarding the properties of organelles. If we wanted to do this during the summer, we would not have time to build anything. We wanted to get started and develop relatively simple tools that could perform image recognition of our MiCodes. Of course, in order to optimize this procedure, we would definitely have to take the next step and gather this data for machine learning.

[edit] Thomaschow 16 September 2012 (PDT)

1. Colonies grew, about 40-50% colonies were red. Picked 3 colonies for each plasmid.
2. Miniprep, run test digest:
pCLC043: SacII and BglIII. Expect ~3900, 1700. NEB 2
pCLC044: SacII and BglIII. Expect ~3900, 1700. NEB 2
pCLC045: SacII and XhoI. Expect ~2800, 1700. NEB 4
pCLC046: SacII and XhoI. Expect ~2800, 1700. NEB 4
3. Test digests confirmed; pCLC043 #3, pCLC044 #2, #3, pCLC045 #1, #3, and pCLC046 #1 worked.



[edit] Thomaschow 15 September 2012 (PDT)

1. Ran BsaI for pCLC043, pCLC044, pCLC045, pCLC046. Ran out of pWCD0520, waiting for Robert to grow more for pCLC042.
2. Transform into TG1, on Lb-Amp. Grow ON.

[edit]Thomaschow 14 September 2012 (PDT)

Meeting on 9/20 at 5:30pm with Terry.

Meeting on 9/27 at 5:30 with Terry.

[edit]Thomaschow 12 September 2012 (PDT)

Ideas of engineering phenomena that can only be screened under microscopy.

Subcellular localization:

Morphology:

-differentiation/groups organized cells. Cancer/developmental biology -ras ratio-> whole exp differentiation

-stem cells/ scaffolding

-fruiting body

-cancer cells.

Motility:

Quality control (industrial):

1. Assign MiCodes to strains of yeast to ensure no mistake in experimentation. Check to see if colonies are always correct.

[edit]Thomaschow 7 September 2012 (PDT)

Presentation:

Slide 2: No yeast phenotype images. Get yeast phenotype. Replace microscope cartoon with real one.

Slide 4:

1.FACS, Output = "whole cell fluorescence".

2.Add animations, to show logic of explanation.

3.Yeast 2 hybrid, output = "whole cell color."

4. Pull-down, output = "binding/not binding to substrate."

5. FACS, information loss = have brightest cells pass through cell.

6. Yeast Two-Hybrid, information loss = get picture of plate off internet, depicts colorimetric data.

7. FACS, information loss = "gated threshold".

Threshold gating, another is based on effort. Info loss depends on experiment. Depends on plate number, but if 1 million in library, cannot cover enough square footage. Very low throughput, if you don't want info loss.

"Other screening assays out there. All suffer from these problems, either not high throughput, or lose info, or both."

Don't say everything, leave audience questions.

COME UP WITH NONWHITE BACKGROUND SLIDE. NEED TO MAKE SURE IMAGES GO WELL WITH BACKGROUND.

Slide 4: delete title.

Slide 2: Delete microscope, make images all same height or width, put at same x-y axis.

"Microscopy can give you morphology, fluorescence intensity, fluorescence location. Describe things that you get out of microscopy. Lots of interesting reasons to apply microscop. to other problems. We wondering in lab if we could apply microscop. to screening tech. <- punchline. Segue to next slide: what to apply microscopy to screening- but who are our competitors?"

Slide 3: Transition question to Slide 4: "So why do we need a new technique? How does microscopy give something new that current things don't?"

Slide 4: "Because there are issues with current tech. that we think can/need to be dealt with."

- Emphasize spatial information on organelle level, distinguish between whole cell fluor. and organelle fluor.

- Instead of saying we want to achieve Syn Bio trends, we say "Think of what this can do in syn bio."

- Be consistent with "Data Retention" and "Spatial info" terms.

- Spatial information -> intracellular processes, organelle size (Will's).

You may be interested in assay that reflects subcellular output.

Nothing is thresholded, this is high throughput. But, there's one issue. This is high throughput only if we have a link between genotype and phenotype. The goal here is to make this a useful screening tech, so we can look at a cell's phenotype and tell its genotype. 2 ways: Locate interesting cell, isolate it,

sequence. Not high throughput. We lose this advantage of microsc. if we do this. We need to make sure there's a visual phenotype associated with its genotype.

Slide 7: kill title.

"The other option is if we barcode the cell. (Show the arrow with question mark)."

We've chosen a variety of yeast cells. In this example, there's varying levels of fluorescence. We can identify a particular interesting cell that has a particular level of fluorescence. We chose an intermediate green.

Slide 8: Title:How do we barcode these cells?

We want a way to barcode these cells. We call this Micoding, which are microscopic barcodes.

Slide 9: Title:MiCodes can accommodate large libraries.

We micode cells by taking various fluorescent proteins and target them to organelles.

[[edit](#)]**Thomaschow** 6 September 2012 (PDT)

To identify Nuclei:

1. Threshold of 2.8 for typical PNG images, with Nuclei expressing RPL18b. Use RobustBackgroundAdaptive.
2. Attempting to process Harneet's isolated cell images.

Do not show math of increasing library size,

Part of our workflow is designed to identify the nucleus.

Have a miCode box, have it update the miCode box as CP finds the organelles.

Can build large libraries. 10 to the 6th. "We can make miCodes for that many colors. It's not high throughput if it's not identifiable all at once. We've been working on software that can help us perform this automation.

"Goal slide."

- Create a 1,600 member miCode library.
- Verify MiCode identification by user
- Verify MiCode identification by computer
- Screen a 2-member library of leucine zippers with MiCodes
- Screen a 1600-member micoded library of leucine zippers.

iGEM meeting:

1. Leucine zipper proof of concept: Test to see if the zippers work, and that the miCodes are accurate in describing the interactions.

- use 2 sets of zippers, different Kd's, and run binary miCode.

- If there's in vivo assay measuring transcriptional output of GFP for the zipper, why do miCodes?

- advance of project is to use miCodes as a microscopy technique that connects to interaction of zippers. In this way, we have a modular assay that can be used for a number of simultaneous tests or others.

- how to connect miCodes to in vivo assay? Measure pixel intensity of cytosolic GFP.

- improve dynamic range of evaluation.

-

2. Proof of concept of micodes: mix random number of miCodes with random genotypes.

-how many micodes?

-can people identify these correctly?

-

3. Small number of miCodes

4. Everything together.

Presentation:

1. Everyone has screened a library...

2. asked ourselves what if we could screen cells on a microscope, by seeing the cells visually, and determining the genotype.

Microscopy is versatile, can search for a variety of phenotypes. Show the power of microscopy (images of various phenotypes). What's missing from microscopy is that you can't tell the genotype from the phenotype.

There are other screening techniques available, but they have cons.

Biologists are interested in trends, so determining intermediates are necessary as well.

Throughput

Information loss

Thomaschow 31 August 2012 (PDT)

Presentation practice with Terry Johnson:

Choose consistent font. Titles = 1 font, other text = another font.

Keep font at minimum at size 18.

Clothing analogy:

Library does not isolate global optimum, it thresholds.

We need to talk about our problem specifically ->

1. Don't know why losers were losers, after screen. We lose information about some members in library.
2. Don't know if we truly explored every possibility.

Need to explain that we deal with genomic libraries, going from outfit libraries.

Genotype linked to observable phenotype. Show image of
DNA double helices <-----> for each strand, have a specific phenotype.

[[edit](#)] Thomaschow 29 August 2012 (PDT)

miCode library design:

40 plasmids:

LZa-mKate

3 * 5 * 3 = 45 possible half miCodes

Nucleus - CFP, YFP, PAmCherry

on/off, on/off, on/off

Actin -

40 plasmids:

LZb-linker-PTS1

VM CP

[\[edit\]](#) Thomaschow 21 August 2012 (PDT)

Ran nucleus test on total of 9 images consisting of ~115 cells. Out of these, cellprofiler incorrectly identified 19 times, giving approximately 85% accuracy. This procedure took approximately 13 minutes.

Reran nucleus test with adjustments to robust background adaptive threshold. Changed max threshold to 0.7. CP incorrectly id's 9 cells, giving ~92% accuracy.

Reran nucleus test with robust background adaptive threshold adjustment factor changed to 3. Gives ~95 % accuracy. The majority of wrong ID's come from nuclei with low intensity.

[\[edit\]](#) Thomaschow 6 August 2012 (PDT)

Meeting:

What is a common problem that we all have to deal with?

- look at a library, want to have info on every member in the library.
- The power of having libraries, how to bring that power to microscopy.
- under a microscope, will tell you exactly what it is.

Make text more visual. Picture of FACS, only provide perhaps data/numbers as text.

Start out with a question you're trying to answer. Why conventional strategies don't necessarily work?

2 Parts:

1. What you're detecting.
2. What you're sorting.

Be more general than two-hybrid systems. How do you screen things? How to detect things under a microscope?

1. First slide: classes of problems that are to be addressed. Growth rate, fluorescence, protein interactions. What are you trying to screen for?

Most approaches do not provide whole information. A lot is lost when screening, because only the "winners" are addressed.

Abstract the idea of the barcode. Explain what problem we are solving:

Design miCode diagrams. Draw a flowchart ---> go from barcode to micode.

Diagrams for: gene 1 -> miCode 1.

gene 2 -> miCode 2.

Zoom in on these, show what is connected.

- Description of how phenotype is linked to genotype.

-Important to mention that microscopy can be automated.

[\[edit\]](#) **Thomaschow** 1 August 2012 (PDT)

[Notes on CellProfiler](#)

Moving meeting:

Refer to Luke's email about the moving schedule.

Thomaschow 30 July 2012 (PDT)

Meeting:

Presentation:

~6 slides

4. SLIDE 1. Invert the progress. Discuss end goal first, and describe what we did to achieve it.

1. Set up slides describing how to use miCodes (workflow).
2. Explain what miCodes can do that other techniques cannot. (What miCodes does that FACS cannot)
3. Stay consistent with graphics, display.
5. A sketch idea of a shirt design. (Barcodes?)

[\[edit\]](#) Thomaschow 17 July 2012 (PDT)

1. Met with Terry on imaging goals. Outlined in the following:

Population ID: 1. % of stains for organelle in whole image.

2. Try to measure portion of optimal library size.
3. Now, try to make pipelines practical. What are the functions of versions of the program.
4. VS -> collects # of cells positive for red, green, blue, colors for a given organelle. (library check).
 - a. ID individual organelles.
5. VC -> unique ID for every miCode.
 - a. ID individual cells.
 - b. ID organelles on a per-cell basis.
 - c. Automatically generate miCode.

TO DO:

1. False-Leucine zipper test -> images with stained peroxisome, compare to cytosolic background. Point user back to cells with interesting data. (Tag as interesting, direct to user).
 - a. Should we deal with cell clumps computationally or experimentally?
2. Identify whole cells, limit/tag further analyses to that single cell area.
3. Consider combinations of organelles (with same color, with different color).

[\[edit\]](#) Thomaschow 16 July 2012 (PDT)

Meeting:

1. Promoter library characterization (from the iGEM library). Try to characterize strengths based on each other, plot all together.

-Number of yeast promoters, some inducible some repressible, varying strengths. As part of our building scheme, need better guide to choosing promoters for various experiments.

1. Take existing Dueber promoter and characterize it (quantify the strength of the promoter).

2. Take a new promoter, characterize it and create a standard around it.

To do:

1. Take TDH3 promoter (strongest in registry/dueberlab), take 3 other promoters that are not characterized quantitatively and measure them based on TDH3.

Imaging:

1. Identify all cells by miCode (by Computer).

2. Identify all cells of interest with comp (interacting orthogonal protein interactions).

3. Have computer connect miCode with cells of interest.

Another way:

1. ID interesting cells by comp. (How partitioned is the background color close to the localization of the peroxisome?)

2. Send images of interesting cells to user.

Makes it easier. Story: Look to finding interesting cells only, send them to user for certain ID. Will work towards creating full library analysis.

Measure quality control -> Measure diversity of miCode library, statistics about overall image.

[\[edit\] Thomaschow 14 July 2012 \(PDT\)](#)

1. Picked yTC013 and yJD001 colonies to grow. Will try retransforming pTC007.

[\[edit\] Thomaschow 13 July 2012 \(PDT\)](#)

1. yTC012 did not grow, but yTC013 did.

2. Continued progress on CellProfiler.

[\[edit\] Thomaschow 12 July 2012 \(PDT\)](#)

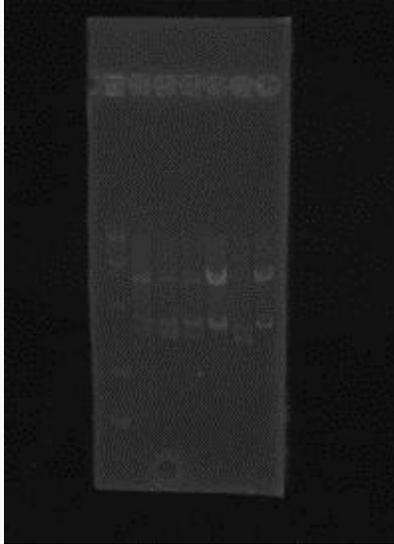
1. Integrated pTC007 and pTC008 into yJD001. Used Vincent's back dilution at 0.73 OD.

2. Made progress on Cell Profiler. Found that Fiji may be a candidate for processing. Will look up macro writing.

[edit]Thomaschow 11 July 2012 (PDT)

1. Test digest pTC007 and pTC008 with EcoRI and BamHI. Expect ~4700 and ~2500 for pTC008 and ~5500 and ~2600 for pTC007.

- pTC008 colony 1 turned out well. pTC007 showed faint extra bands, but will transform anyway.



2012-07-11 pTC007 pTC008 test digest EcoRI-BamHI.

2. Transformed into yeast, using a 2x dilution of Vincent's competent yJD001.

[edit]Thomaschow 10 July 2012 (PDT)

1. pTC007 and pTC008 grew, with few red colonies, so picked 3 each for culture.

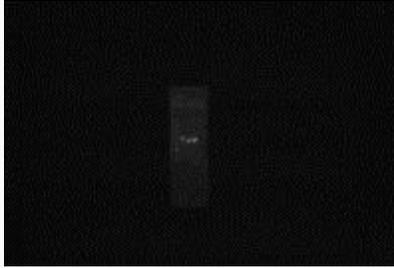
2. Miniprepmed.

[edit]Thomaschow 9 July 2012 (PDT)

1. Transform pTC007 and pTC008 into TG1 cells.

2. Miniprep additional colonies of pTC006, see if they work. Test digest with EcoRV and NdeI, to look for middle part of insert.

-Again, test digest revealed missing middle part for pTC006. Decided to drop the CAP1 plasmid, and continue with ABP1.



2012-07-09 NdeI-EcoRV pTC006 test digest. Expect ~1700, ~400, and ~300 bp's, but did not get such results.

3. iGEM wiki:

For reference:

Award	School	Region	Year	Link
Best Wiki Calgary		Americas	2011	http://2011.igem.org/Team:Calgary
Best Wiki HokkaidoU_Japan		Asia	2011	http://2011.igem.org/Team:HokkaidoU_Japan
Best Wiki Imperial College London		Europe	2011	http://2011.igem.org/Team:Imperial_College_London
Best Wiki Imperial College London		Europe	2010	http://2010.igem.org/Team:Imperial_College_London
Best Wiki Cambridge		Europe	2010	http://2010.igem.org/Team:Cambridge
Best Wiki Heidelberg		Europe	2009	http://2009.igem.org/Team:Heidelberg
Best Wiki TUDelft		Europe	2008	http://2008.igem.org/Team:TUDelft

Meeting:

Timeline of iGEM:

1. July 15th - team project description. Make as ambiguous as possible.

-iGEM wiki setup -> put up project description, learn code to develop structure for wiki page.

-wiki have done by next Monday, July 16th.

-Slovenia 2009 -> orthogonal coiled-coils.

2. Judging:

-will look at wiki first to get information for initial judging.

3. Sept 5th: Select top 3 tracks for project. Select team name. Have this by August 15th.

4. Sept 5th: Project abstract (100 to 150 words). Team name, project title, project abstract. Have this by August 15th.

5. Need two independent plans for gold medals.

6. Imaging: look at separate channels.

-Do deductive searching. Once you find an organelle, can you limit the next search to within cell?

7. Computation: need for small scale, practical method.

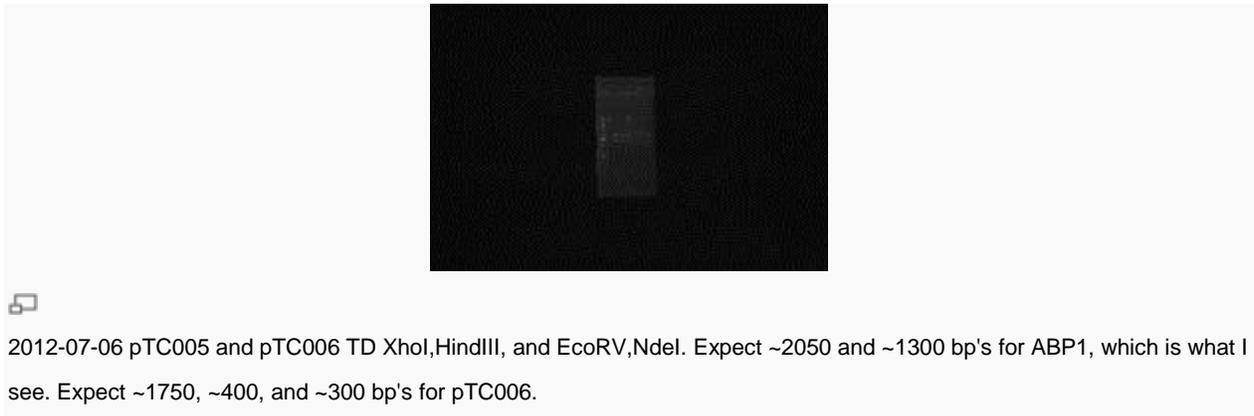
[edit] **Thomaschow 8 July 2012 (PDT)**

1. Sequencing confirmed; ABP1 GG part successfully cloned. Continue with cassette assembly.
2. Ran BsaI GG assembly on pTC005.

[edit] **Thomaschow 6 July 2012 (PDT)**

1. Miniprep DNA from colonies, test digested pTC005 with XhoI and HindIII, pTC006 with EcoRV and NdeI. These sites will cut at the suspected parts that are missing (middle of CAP1, and ABP1 part).

Bands of ABP1 look correct, will send for sequencing:

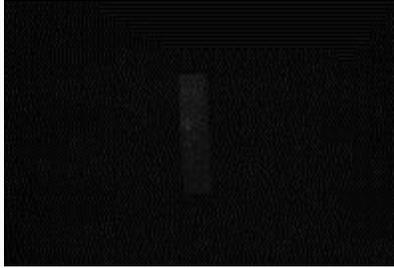


[edit] **Thomaschow 5 July 2012 (PDT)**

1. Sequencing did not match; it suggested that one of the 3 parts in the CAP1 PCR did not anneal properly.
2. Repicked 4 colonies of pTC005 and 3 of pTC006.
3. Redo PCR reaction.

[edit] **Thomaschow 3 July 2012 (PDT)**

1. Test digested colonies 2 and 3 of pTC006 with NcoI and SacI, to check if front part of the insert is present.



2012-07-03 pTC006 2 and 3 NcoI-SacI digest. Expect ~2300 and ~150 bp's.

2. Test digested colonies 2 and 3 of pTC006 with NcoI and NdeI, to check if front part of the insert is present.

Expect ~1900 and ~550 bp's which is what I saw. This may be a correct colony.



2012-07-03 pTC006 test digest NcoI - NdeI. Expect ~1900 and ~550 bp's.

3. Submitted for sequencing.

[edit] Thomaschow 2 July 2012 (PDT)

1. Sequencing of pTC006 and pTC005 did not match. pTC005 was missing the protein coding region, and pTC006 was missing the front end of the coding region. 4 base homologies were all different, however.

2. Test digested pTC006 and pTC005 with SacI and NcoI. Did not work, as expected.

3. Repicked pTC006 x2 colonies, grew in culture.

4. Meeting:

- potential application: miCode shRNA to knock down proteins along a signaling pathway in order to determine which proteins are necessary for pathway.

- protein dimerization around molecule of interest. Problem with this is it's hard to generate large library for the intention of identifying a biosensor.

- If cell population is higher, may need multiple data points associated with each cell if looking for phenotypes.

-Orthogonal protein-protein interactions in leucine zippers. Need a good way to test computational development of orthogonal sets. -> look up yeast 2 hybrid system.

applications: shRNA, protein dimerization, promoter strength testing, prion interactions, cancer Snare1 proteins.

Thomaschow 30 June 2012 (PDT)

1. Miniprep pTC006.
2. Sequencing of pTC005 failed, read short and annealed in a different region.
3. Sent sequencing with AW38F and AW39R for pTC005 and pTC006.
4. Test digest for both plasmids with BsaI did not work. Possibly did not run long enough.

[edit] Thomaschow 29 June 2012 (PDT)

1. Picked colonies from pTC005.
2. Sequence DNA.
3. Miniprep DNA.

[edit] Thomaschow 28 June 2012 (PDT)

1. Transform ABP1 GG part into TG1 cells on CAM plates. The pWCD0514 contains the CAMr sequence for Chloramphenicol resistance.

2. Troubleshoot PCR:

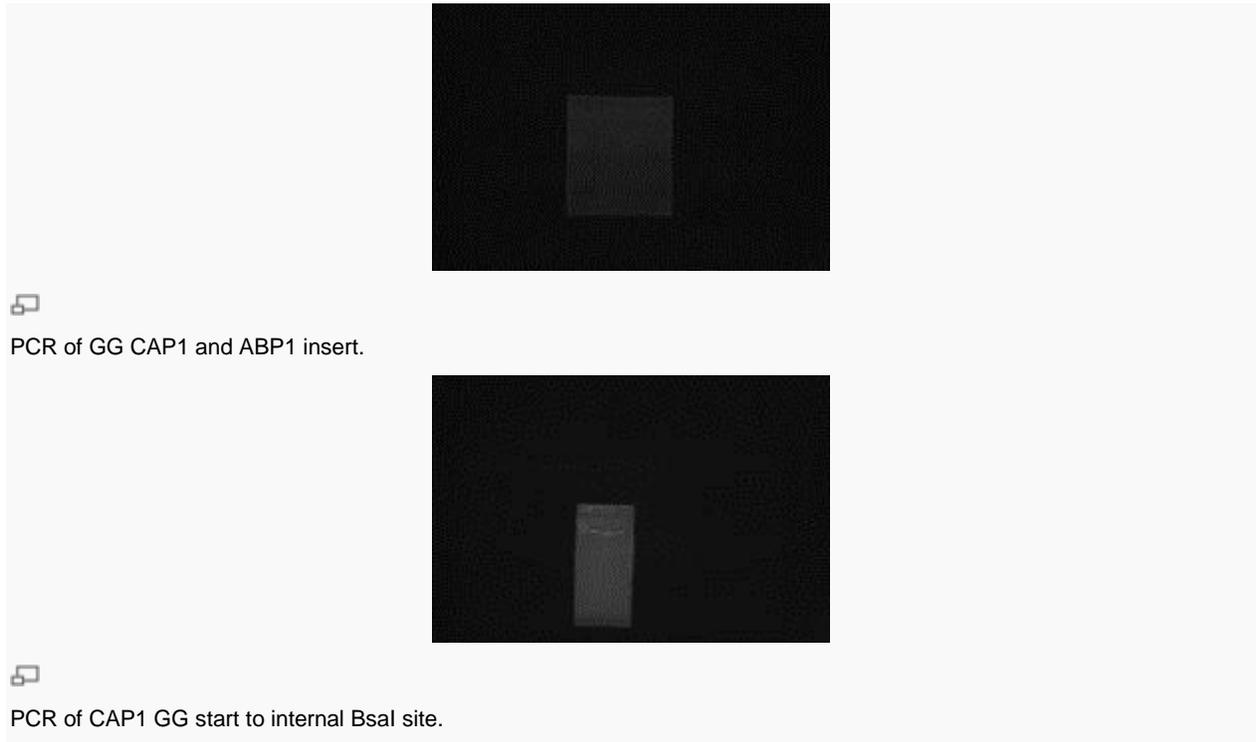
-Got no C1 product, but a ton of junk DNA.

3 Reactions in parallel:

1. Phusion at 50 with GC buffer.
2. Phusion at 50 with DMSO.

3. Expand at 45, using wcd1k45.

-PCR worked! 1 and 3 yielded good products. Expect ~500 bp's.



3. Transformed pTC005 and pTC006 onto LB-CAM plates.

[edit]Thomaschow 27 June 2012 (PDT)

1. Expected PCR products:

CAP1:

C1 -> ~500

C2 -> ~130

C3 -> ~160

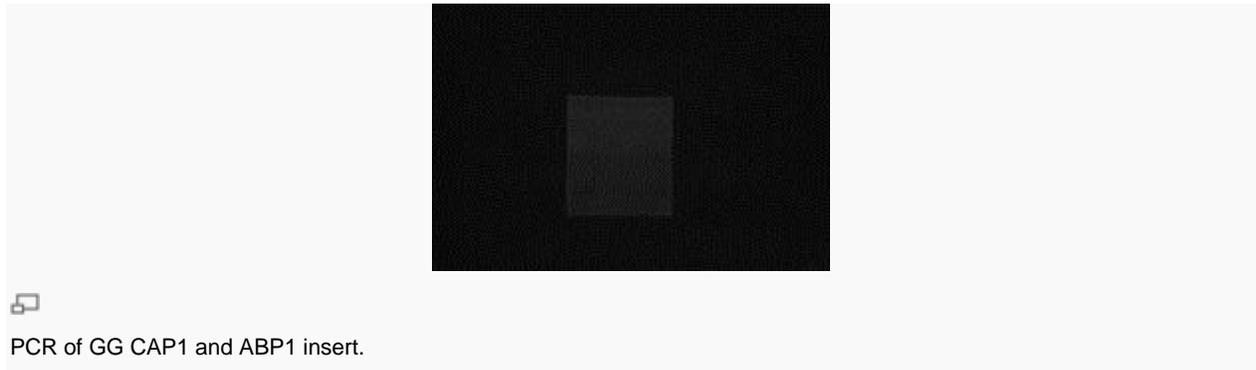
ABP1:

A1 -> ~360

A2 -> ~1380

A3 -> ~40

All but C1 worked. Redo PCR at 50 degrees annealing.



2. yTC008 grew to saturation, back-dilute to .2 OD, grow to midlog in parallel with saturated solution, then image.

3. BsmBI digested ABP1 insert, direct ligate into pWCD514.

4. PCR did not work at 50 degrees, too much junk DNA. Will troubleshoot tomorrow.

[edit] **Thomaschow** 26 June 2012 (PDT)

1. Checked cotransformations. yTC008 grew one colony, picked and seeded. Left others to grow more.

2. Redid yTC009 cotransformation, let grow for 2+ days.

3. Ordered oligos for golden gate assembly of new targeting sequences.

-The two sequence candidates for Actin are : CAP1 and ABP1. -Both sequences have a BsmBI and BsaI site, so constructed 6 oligos per sequence to effectively remove the internal restriction sites (2 per sequence).

For CAP1:

- AX55/AX60 -BsaI- AX59/AX62 -BsmBI- AX61/AX56. 3 PCR's.

For ABP1:

- AX57/AX64 -BsmBI- AX63/AX66 -BsaI- AX65/AX58. 3 PCR's.

-Completed these six PCR reactions, held overnight at 10 degrees.

[edit] Thomaschow 25 June 2012 (PDT)

1. Cotransformations yTC008 (yTC002 + pTC003), yTC009 (yTC002 + pMRY18), yTC010 (yTC004 + pTC001), yTC011 (yTC004 + pMRY18) did not grow (yet?). yTC008 was infested with mold. How terrifying.

2. Meeting:

- Aiming for four distinct organelles (of each basic shape). Need to visualize cotransformations to look for more possible distinctions.

-Imaging: Have statistical information on each organelle (lots of data collected for each organelle. Then use such data to process image, and can determine statistical significance of micocode to the actual genotype.) What percent certainty is this cell X?

-ImageJ: ellipsefit.

-Think of one unique experiment for which to use micodes.

-Statistics for intensity, size, shape, etc. Need to generate data for each instance of an organelle.

3. Golden gate strategies to create parts for expression.

[edit] Thomaschow 23 June 2012 (PDT)

1. Seeded yeast for midlog and saturation imaging. -Images turned out well, decided that Actin is much more distinct than Endosome and should be preferred over Endosome.

[edit] Thomaschow 22 June 2012 (PDT)

1. Seeded yeast for cotransformations yTC008 (yTC002 + pTC003), yTC009 (yTC002 + pMRY18), yTC010 (yTC004 + pTC001), yTC011 (yTC004 + pMRY18).

2. Learned about Will's cloning scheme, will need to design primers to PCR original targeting protein sequence, and a new protein sequence.

-Check for following internal restriction sites: BsmBI, BsaI, NotI.
-Make sure to add BsmBI and BsaI sites into the ends of the sequence.

[edit] Thomaschow 21 June 2012 (PDT)

Seeded yTC001 - 004 for yeast transformations. Also did more digest of pTC001, pTC003 for integrations.

1. Had meeting with CINVESTAV iGEM team for collaboration project.

Meeting:

CINVESTAV trying to manufacture n-butanol from two pathways in Escheridia Coli.

on input = O₂ concentration.

various light wavelengths of O₂ concentration

main challenge: regulation of system.

2 pathways - try 2 induction systems on both, see which works best.

Collaboration idea: Have them come up with with a way of visualizing butanol concentration across various strains of bacteria.

[\[edit\]Thomaschow 20 June 2012 \(PDT\)](#)

1. Seeded yTC001, yTC002, yTC003, yTC004, yTC006, and yTC007 to grow to midlog, then image.

-Accidentally used SD, should use YPD for faster growth.

2. Will also image above strains at saturation.

3. Need to cotransform to make yTC005 again.

[\[edit\]Thomaschow 19 June 2012 \(PDT\)](#)

1. Picked yTC001, yTC002, yTC003, yTC004. Will grow to saturation, image, then back dilute and grow to midlog, then image.

[\[edit\]Thomaschow 18 June 2012 \(PDT\)](#)

1. Checked yeast cotransformation colonies. yTC006 and yTC007 worked, while yTC005 yielded no colonies. Picked and imaged.

2. Robert reseeded Actin, ER, and VM mKate strains for more organelle cotransformations.

So far:

		mKate					
	Actin	VM	ER	NP	Nucleus	Nucleolus	Mito
Actin		Y					
VM	Y	Y					

venus	ER	Y	Y
	NP		
	Nucleus		
	Nucleolus		
	Mito		

Today, will need to transform to confirm the first three columns.

Meeting:

1. Image organelles through saturation.
2. Computer will have trouble visualizing cells in contact.
3. For automation, want to think about algorithms that provide a standard for determining organelle type (ie. x pixels,

[edit] **Thomaschow 15 June 2012 (PDT)**

1. Picked E. Coli colonies of pTC001, pTC002, pTC003, pTC004. Grow to saturation, then will miniprep.

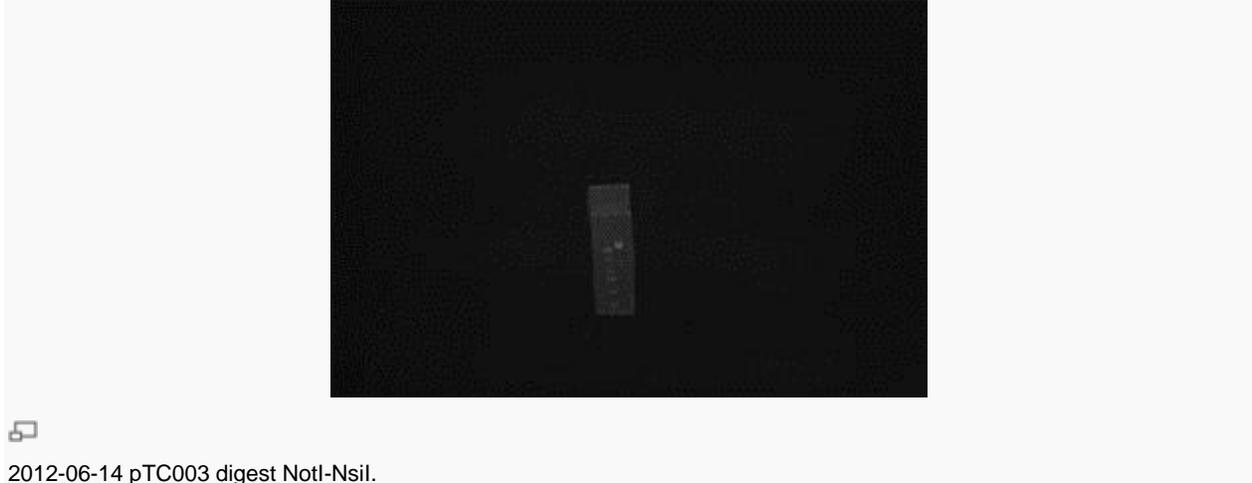
[edit] **Thomaschow 14 June 2012 (PDT)**

1. Restreaked yTC001, yTC002, yTC003, yTC004 colonies that effectively expressed fluorescence.
2. Will cotransform venus insert into mKate strain.
-Robert picked colonies from yTC002 and yTC004, grew up ON. This morning, back dilute and will grow to OD .8 in about 4 hours.
3. Digest venus plasmid with NotI and SacII.
4. Perform yeast transformation on venus insert into mKate strains for colocalization experiment.
-We are using the following strains:
mKate of Actin, Vacuolar membrane, and ER.
-We are transforming the following inserts into each of the strains:
venus of Actin, Vacuolar membrane, and ER.
This gives a total of 9 transformations. We will continue with the other organelles tomorrow.
5. Miniprep more venus plasmid for mixed organelle cotransformations.
6. Digest more pTC003 -> enough for three more transformations.

-24 uL DNA, 24 uL H2O, 6uL NEB, 3uL SacII, 3uL NotI. Total 60 uL.

-After Zymo, should have 30 uL for 2x DNA concentrate. - Digested with NsiI and NotI, because SacII ran out of stock. NsiI cuts into the Leu homology region, but according to Robert this should still work.

Ran on gel, expect ~6700 and ~2400 bp's, which is what I saw. The 2400 band was very faint.



2012-06-14 pTC003 digest NotI-NsiI.

[edit] [Thomaschow](#) 13 June 2012 (PDT)

[edit] [Thomaschow](#) 12 June 2012 (PDT)

1. Checked reverse sequencing of pTC001, pTC002, pTC003, pTC004.

siGEM091	11-Jun-12	pTC001	1	AA06	confirmed
siGEM092	11-Jun-12	pTC002	1	Z72	confirmed; read short
siGEM093	11-Jun-12	pTC003	1	AA06	confirmed
siGEM094	11-Jun-12	pTC004	3	Z72	confirmed; two deletions at 897 and 916

2. Back diluted yeast cultures to .2 OD, returned to 30 degree for 4 hours growth.

3. Image cells, this time uniform growth conditions across all strains. We are trying to normalize growth conditions across all strains, so to make the fluorescent signal consistent. With this, we will see which organelle targets produce the best signals.

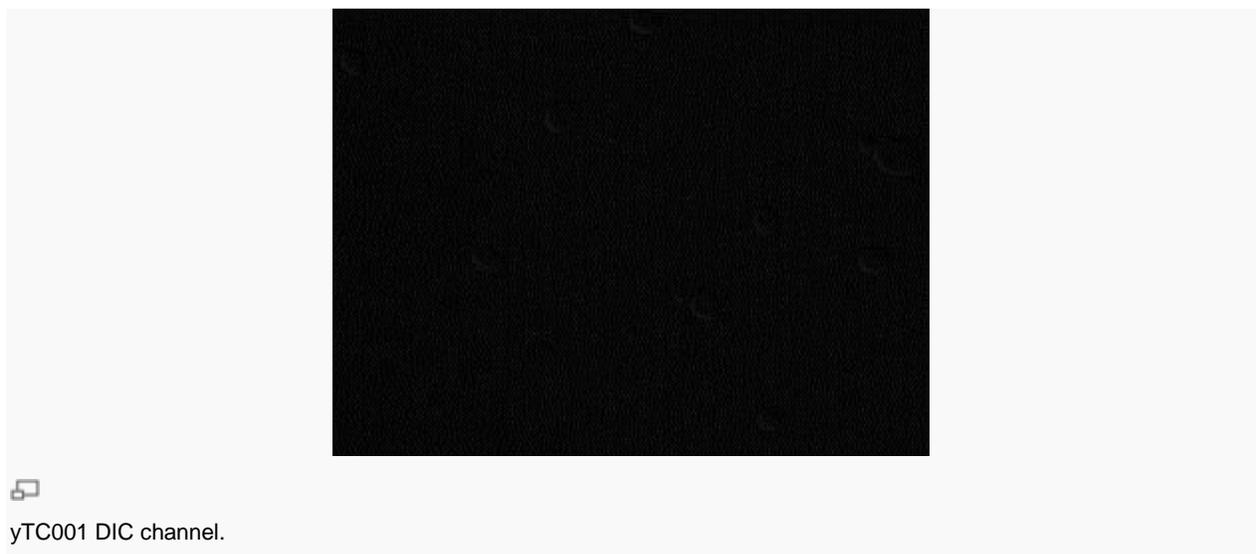
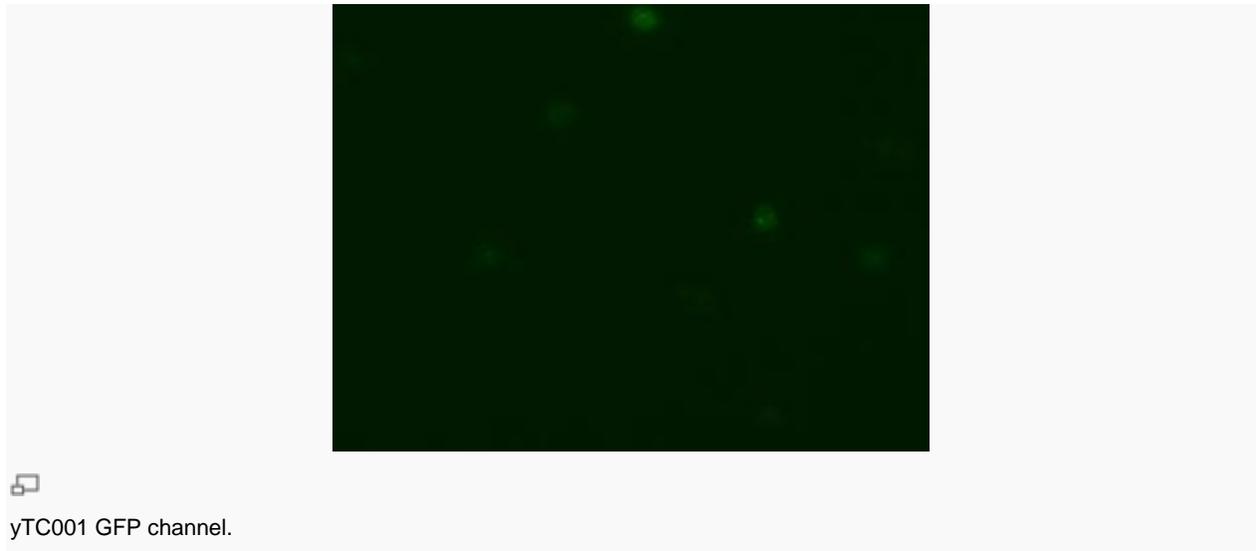
4. Made glycerol stock of the four yeast strains.

[edit] [Thomaschow](#) 11 June 2012 (PDT)

1. Forward sequencing of pTC003 and pTC004 confirmed. pTC003 colony 1 did not work. Need to use reverse primer for both plasmids, resend sequencing.

-Use Z72 reverse mKate probe for reverse sequencing.

2. Image yeast cells. -most looked good, with good localization, but low signal. Will need to adjust promoters.



3. Picked imaged colony, grow up in SD-Leu and SD-Ura.

Meeting:

Images:

-Cell-cell variability in signals

-Protein size is the major variable among organelle constructs

-Think in terms of pixels/pixel length. How many pixels in a cell? How would organelles differ in terms of # of pixels?

-Organelle candidates: 1. Nuclear periphery 2. Nucleolus 3. Vacuole 4. Cell periphery 5. Vacuolar membrane 6. Mitochondria.

- Product application:

1. Mammalian cell culture
2. How can our idea be used as a product?
- 3.

Next steps:

1. See what organelles can be combined.
2. Minimize cell-cell variation.
3. Growth rate changes? If this varies from a control, we don't want it. This may change phenotypes of the cells.
4. Have a fluorescent control that's consistent among cells (cytosolic GFP). Have a normal control, to test growth rates.
- 5.

[edit] **Thomaschow 8 June 2012 (PDT)**

1. Yeast transformations of pTC001, pTC002, pTC003, pTC004.

[edit] **Thomaschow 7 June 2012 (PDT)**

1. Reverse sequencing with O44 worked well. Spotted a few misreads (mKate has a mis-annotation), but otherwise seems correct.

- Diagnose previous reverse sequencing error: possibly tube mislabeling. Will run PCR on miniprep to confirm, using primers S16 and AA06 for pTC001, and S16 and AF32 for pTC002.

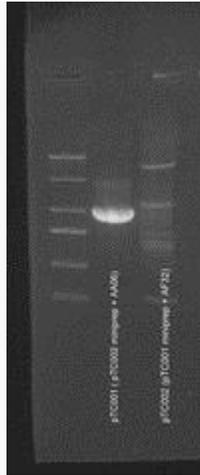
2. Electroporated cells containing pTC003 and pTC004 grew overnight. Negative control also yielded colonies, so picked 3 of each construct.

3. Run test PCR on pTC001 and pTC002, switching the plasmids (so if they were switched before, now the PCR should work properly, since switched back). I put the construct in "pTC002 miniprep" into pTC001 PCR tube, with S16 and AA06 F/R primers. I put the construct in "pTC001 miniprep" into pTC002 PCR tube, with S16 and AF32 F/R primers.

Again, results were confusing. It seems like my venus plasmid was indeed in the pTC002 miniprep tube, but mKate was not properly digested with AF32.

Should have expected bands ~1400 for both constructs.

Based on tests, conclude that tubes were switched. Will sequence with new reverse primers, to reach the rest of the organelle targeting sequence.



2012-06-06 FP test PCR on pTC001 and pTC002.

Interpretations of results:

-Tubes could have been mislabeled, but the PCR reaction for the second construct just happened to not work well, resulting in supercoiling.

4. Miniprepmed 2 more colonies of both pTC001 and pTC002.

5. Test digested pTC003 and pTC004 with XhoI/EcoRI and XbaI respectively. For pTC003, should expect bands ~5700, 2500, and 900. For pTC004, should expect ~7300, 1300, and 360.

-incubate for 30 minutes, using NEB 2.

-Accidentally pipetted 1 ul NEB2 into 50 uL of miniprepmed plasmid, but Will said should be ok (NEB is just salt).

6. Sequenced pTC001 and pTC002 with reverse primers Z14 and AF50 respectively. This should get up to the middle of the organelle. Again, I pipetted what I think was venus and mKate (which is opposite of the miniprep label).

[edit] **Thomaschow** 6 June 2012 (PDT)

1. Electroporated cells did not grow, not even the positive control. This must have been an issue in the cells, not the DNA constructs. Will redo electroporations with commercially competent TG1's (as opposed to self-grown).

2. Sequencing:

-there were interesting misreads at the beginning of the forward sequence with S16. One of the errors was a bug in my ApE program, which caused a shift of the sequence. Redownloaded version 2.36b, and it worked fine. The chromatogram also showed high noise around the 90th base, which I concluded as mis-

call rather than an insertion and point mutation. Other than that, forward sequences read fine (but only reached through half of the organelle reading frame).

-sequencing confirmed with forward primers S16 on both pTC001 and pTC002 constructs, but reverse primers AA06 and AF32 failed to sequence. Since these primers exist in the FP regions, will need to do a test digest to cut within the FP reading frame, to check if the fluorescent gene is there.

3. Plated electroporated cell redo of pTC003 and pTC004 constructs.

4. Conduct test digest of pTC001 and pTC002 with restriction enzymes NdeI, and DraIII, respectively.

-For pTC001, should expect 2 bands ~6000 and ~2600 bp's if venus is present, and 1 band if venus is not present.

-For pTC002, should expect 2 bands ~7400 and ~1100 bp's if mKate is present, and 1 band if mKate is not present.

Sequencing results were baffling. 2 bands, both above 6000 bp's for both constructs. Grabbed wrong tubes? My constructs are not supposed to be this long.



2012-06-06 FP test digest on pTC001 and pTC002.

possible errors:

- tube switch
- re-ligation
- incomplete digestion
- supercoiling
- restriction enzymes might not be that strong

troubleshoot:

- increase digestion time (from 15 to 45 minutes)
- use different restriction enzymes (NcoI for pTC001 and AlwNI for pTC002)

- For pTC001, should expect 3 bands ~7500, 680, and 520 if venus is present, and 1 band if venus is not present. - For pTC002, should expect 3 bands ~3300, 2700, and 2500 if mKate is present, and 2 bands if mKate is not present.

5. Test digest 2 results were still confusing. pTC001 had 2 bands ~6000 bp's and ~3000 bp's, but pTC002 seemed to work, with 2 visible bands ~3000 and <3000 (should have 2 bands ~2700 and ~2500, but they could be overlapping). However, the smaller band was dimmer, despite being possibly 2 overlapping bands.



6. Re-sequence pTC001 and pTC002 with reverse primer O44 for both.

7. Picked more colonies of pTC001 and pTC002.

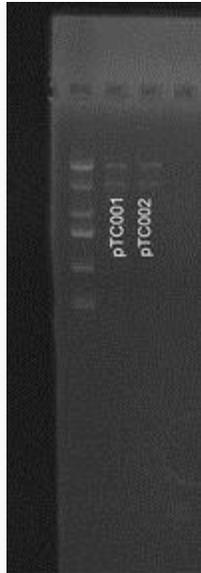
Will also recommended finding other reverse primers to sequence with (at least for pTC002).

[edit] **Thomaschow** 5 June 2012 (PDT)

1. Test digest electrocomp cells plasmids pTC001 and pTC002 with EcoRI and BamHI. Expect ~5700 and ~2900 bp's for pTC001. Expect ~5600 and ~2900 bp's for pTC002.

2. Run test digests on gel. Remember, if looking for a difference in band length (not cutting pattern), it is helpful to include the negative control (parent vector).

Test digest worked well.



2012-06-05 Endosome plasmid test digest. Saw ~6000 and 3000 for both, which were expected.

3. Sequence the electrocomp plasmids. -For sequencing, will use:

pTC001: forward primer (S16) and reverse primer (AA06). S16 binds upstream of gene-linker-FP in the promoter region, while AA06 binds within the mKate.
pTC002: forward primer (S16) and reverse primer (AF32). S16 binds upstream of gene-linker-FP in the promoter region, while AF32 binds within the mKate.

4. Electroperated for Actin targeting vector and negative control (pTC003 and pTC004).

5. Yeast transformation of 2 of Celia's plasmids and 1 of Austin's.

[edit] **Thomaschow** 4 June 2012 (PDT)

Transformations on electrocomp cells worked well, with good ratios (plates had some satellite colonies, but were refrigerated on Saturday). All we have to do is increase transformation efficiency, since Gibsons worked well.

1. Grow TG1 to midlog. 25ml x2.

2. Single digest vector backbone (mKate and venus).

3. Perform Gibson on genes that have not been inserted yet.

4. Pick colonies from electrocomp, miniprep, test digest, sequence. Did not sequence the heat shock transformation on Friday.

-For sequencing, will use forward primer (S16) and reverse primer (O44). S16 binds upstream of gene-linker-FP in the promoter region, while O44 binds downstream of FP.

• For gold, we should improve or characterize a part. • We should go through the parts registry and see what we could maybe incorporate into our design. • Maybe include PGAL inducible promoter? Maybe homing endonucleases? • We need to be very clear and standardized about how we collect our fluorescent images. bronze- wiki, submit one part silver: characterize function of biobrick part gold: improve function of another biobrick part, help another iGEM team, or human practices (steer away from human practices) optimize fluorophores for yeast look at yeast parts in the registry and check characterization (pGal promoter, homing endonuclease dsDNA breaks for high efficiency entry into the genome) special prizes: best biobrick advance, best of tracks: foundational or informational barcoding technology for specific focus or notice that registry doesn't have good parts or for targeting in yeast to improve the registry, commercial and non-commercial options for this, don't reinvent something that already exists jobs: look for software, keep tabs on what is due when, familiarize yourself with yeast parts in the registry, knowing in general what is on there to build off of it standardize fluorescent microscopy, detail about how the images are taken Take fluorophores from registry, optimize for yeast.

Software development Commercial platforms to use? If we design our own, why? Familiarize self with yeast parts on the registry

Methods for microscopy -> how are these images going to be taken? Design a specific set of guidelines for fluorescence imaging.

[edit] **Thomaschow** 1 June 2012 (PDT)

Colonies did not grow well. Got nothing for Endosome-venus, but one colony for Endosome-mKate. Others had low colony count, but only on mKate plates. The negative control for mKate also had a colony.

Troubleshooting:

Will suggest electroporation, to increase transformation efficiency.

Also, he suggested to PCR the vector to linearize for better Gibson Assembly (prevent re-ligation).

We will use mKate and venus plasmids from the NC, my endosome (faint), and Celia's vacuole (bright).

1. Electroporated 6 cell vector samples (mentioned above). 2. PCR vectors

We will use primers:

Venus: AU60 F, AD59 R
m-Kate: AU62 F, AD59 R

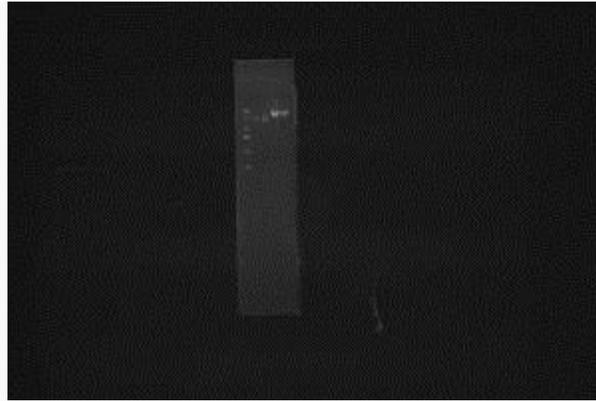
The forward primer will PCR the BglII restriction site, but the reverse starts before the site. This way, no

re-ligation occurs because one will have a blunt end and the other will have a sticky end. Gibson efficiency increased.

Plated the electroporated cells.

PCR of mKate and venus vectors didn't work, so we will redo four PCR reactions, with the following changes:

mKate vector, 35 cycles (instead of 30), at 55 degrees. mKate vector, 35 cycles (instead of 30), at 65 degrees. venus vector, 35 cycles (instead of 30), at 55 degrees. venus vector, 35 cycles (instead of 30), at 65 degrees.



2012-06-01 venus mKate vector PCR products. Expect ~7000 bp's, but both were less than 6000.

PCR of plasmid did not work well.

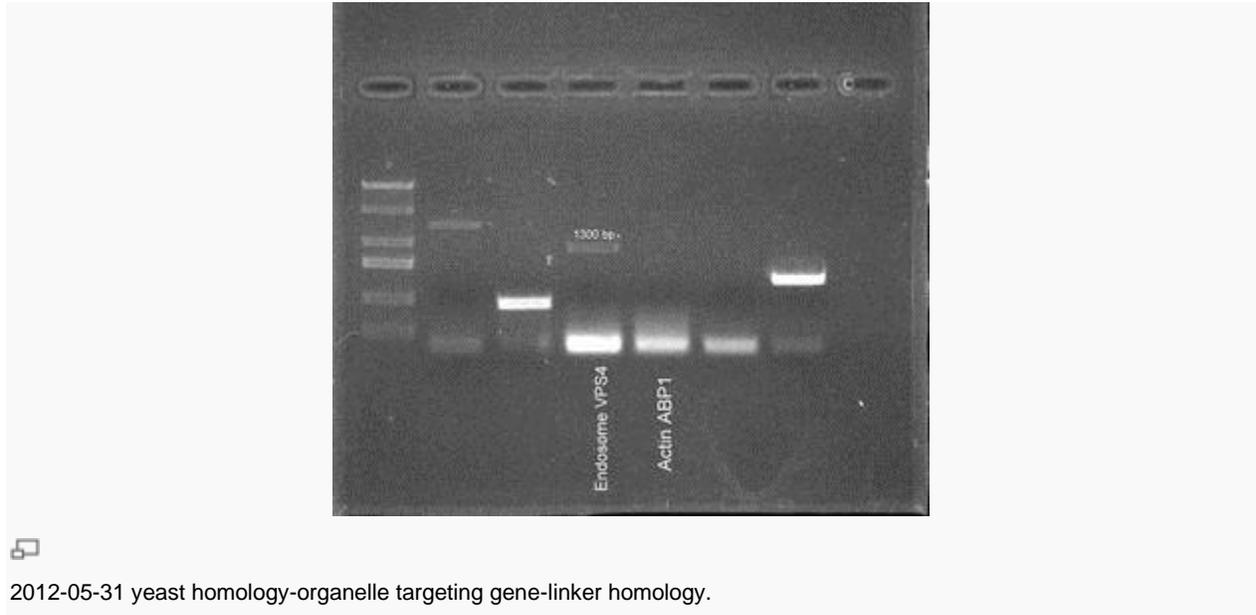
Thomaschow 31 May 2012 (PDT)

1. Checked sequencing of linker-GFP plasmid. Forward and reverse primers matched. The reverse primer read slightly short. 2. PCR of organelle targeting gene. My PCR information is as follows:

```
Template: YJD001 yeast genomic DNA.  
Primers: Endosome -> VSP4 AV09.iGEM0031  
                    AV10.iGEM0032  
          Actin    -> ABP1 AV11.iGEM0033  
                    AV12.iGEM0034
```

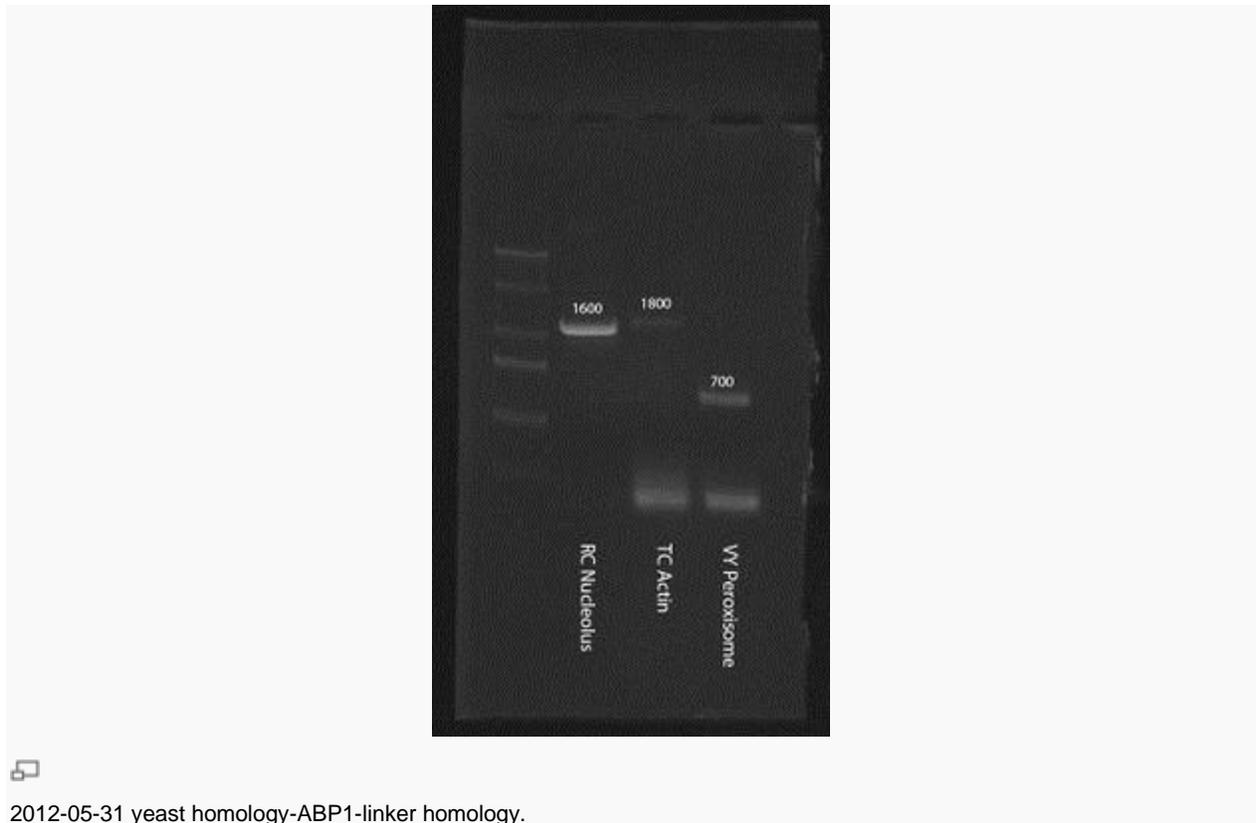
NOTE: We forgot to screen for internal restriction sites in the targeting genes. Must remember to do so before ordering primers.

PCR turned out not too good. Saw primer dimers in all bands, run at 65 degrees. VSP4 showed bands, but ABP1 did not. Redo PCR with ABP1, this time at 55 degrees.



2012-05-31 yeast homology-organelle targeting gene-linker homology.

PCR rerun of Actin - ABP1 went well, still with primer dimers though.



2012-05-31 yeast homology-ABP1-linker homology.

3. Single digestion of FP vector.

4. Gel purify of vector digest.

5. Gibson Assembly of vector with organelle Will pointed out some potential issues with Gibson on a singly cut plasmid. - Re-ligation events could occur due to sticky ends, causing high background. - Consider competition between exonuclease and ligase. The speed of each may determine whether re-ligation is an issue. - However, re-ligation may not be a problem because Gibson is done at a much higher temperature of 50 degrees, rather than room temp. This would favor annealing of strands with longer homology than the restriction site, thus preventing fast re-ligation and giving time for exonuclease to chew back.

Add 2.5 ul vector digested with BgIII and 2.5 ul organelle target sequence. Heated on 50 degrees for around 10 minutes, to minimize re-ligation events.

Added 15 ul of mastermix (not commercial), as described in the protocol.

Heat at 50 degrees for 50 minutes.

Checked VSP4 and ABP1 for restriction sites. VSP4 contains one BgIII site. ABP1 contains one XbaI site.

6. Transformed by heat shock into TG1 bacteria, plated 2 plates per organelle (mKate and venus). Kept negative controls.

[edit] **Thomaschow** 30 May 2012 (PDT)

1. Pick colonies (venus GFP insert).

2. Miniprep.

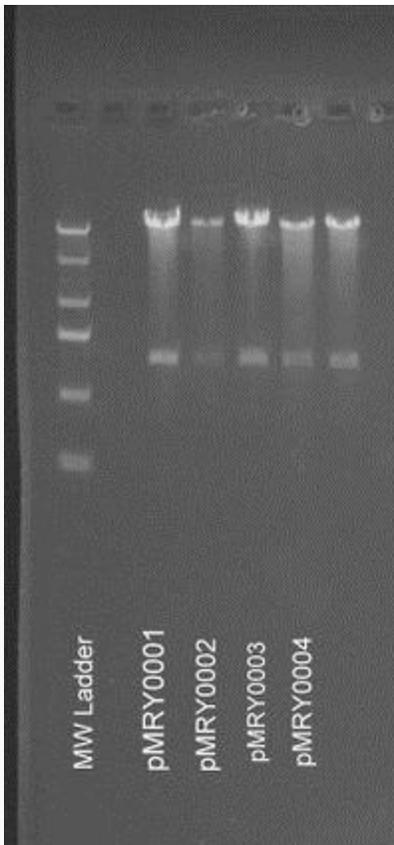
3. Test digest.

4. Sequencing.

1. Got 5 colonies. Did not use negative control, so picked all.

2. Miniprepped all 5 colonies. NOTE: during miniprep, make sure to use diluted A4 buffer (80 mL 100% ethanol to a new A4 bottle). Otherwise, DNA will be washed completely through.

3. Performed test digest with BgIII/XhoI. The two fragments should be ~6600, and ~770 bp's, which is what we saw in all 5 plasmid digests.



2012-05-30 pWCD421-linker-venus test digest with BglII and XhoI.

4. Sequenced the pWCD421-linker-Venus.

Sequencing Primers

Z71 (20bp) TCACGCCCAAGAAATCAGGC (forward)

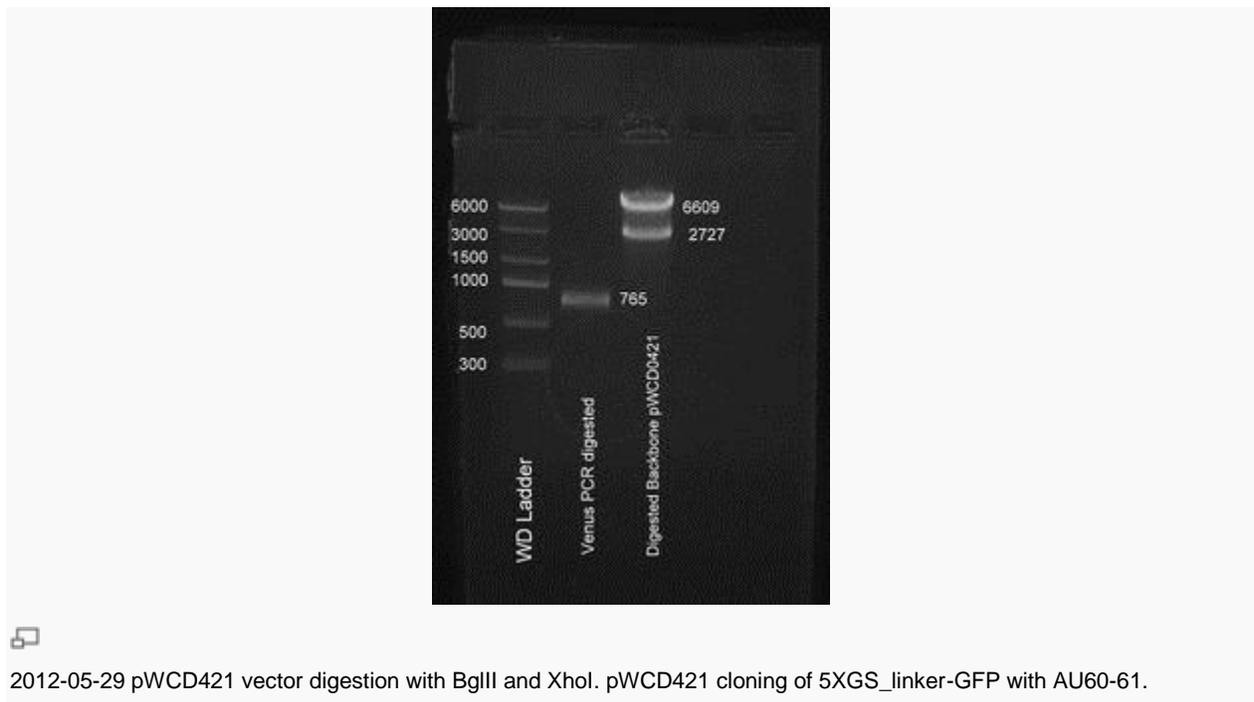
O44 (18bp) gttttaaaacctaag (reverse)

[edit] **Thomaschow** 29 May 2012 (PDT)

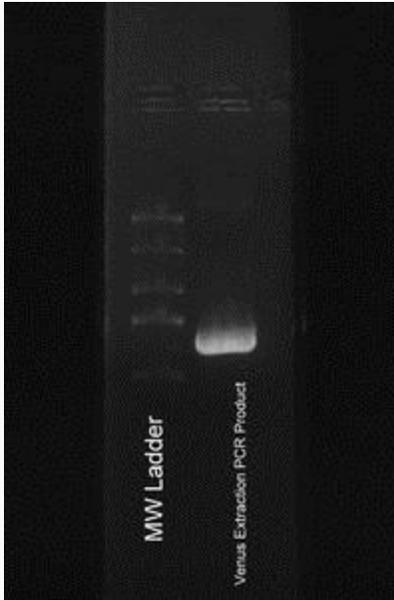
FP vector construction:

1. Gel purified 1. Clone destination vector.
2. Pick colonies.
3. Miniprep, test digest, sequence.
4. PCR genes -> gibson assembly.
5. Pick colonies -> miniprep, test digest, sequence.

Performed Venus backbone digestion of pWCD0421 plasmid vector. Expected bands ~2700 and ~6600 bp's.



Created 5XGS linker -- GFP insert, ~770 bp's.



2012-05-29 pWCD421 cloning of 5XGS_linker-GFP with AU60-61.

By end of week, should have 28 vectors to integrate into yeast.

Final product:

--Yeast homology-----promoter--BglIII---gene----linker---FP gene---BamHI-stop-XhoI--Yeast homology

Two ways to use the miCode:

1. Predetermined assembly -> scientist will clone the miCode one by one into their specific genotype cell. Works well with many promoters of few variants.

2. Randomized assembly -> one pot reaction, where X miCodes combine with X genotypes. Works well with few promoters of many variants.

To do randomized assembly, ideally want:

gene --- DNA barcode --- miCode. So that we can do high throughput sequencing on the gene and barcode only.

The issue is how to assign DNA barcodes. We can use Golden Gate in one pot reaction, but to do this

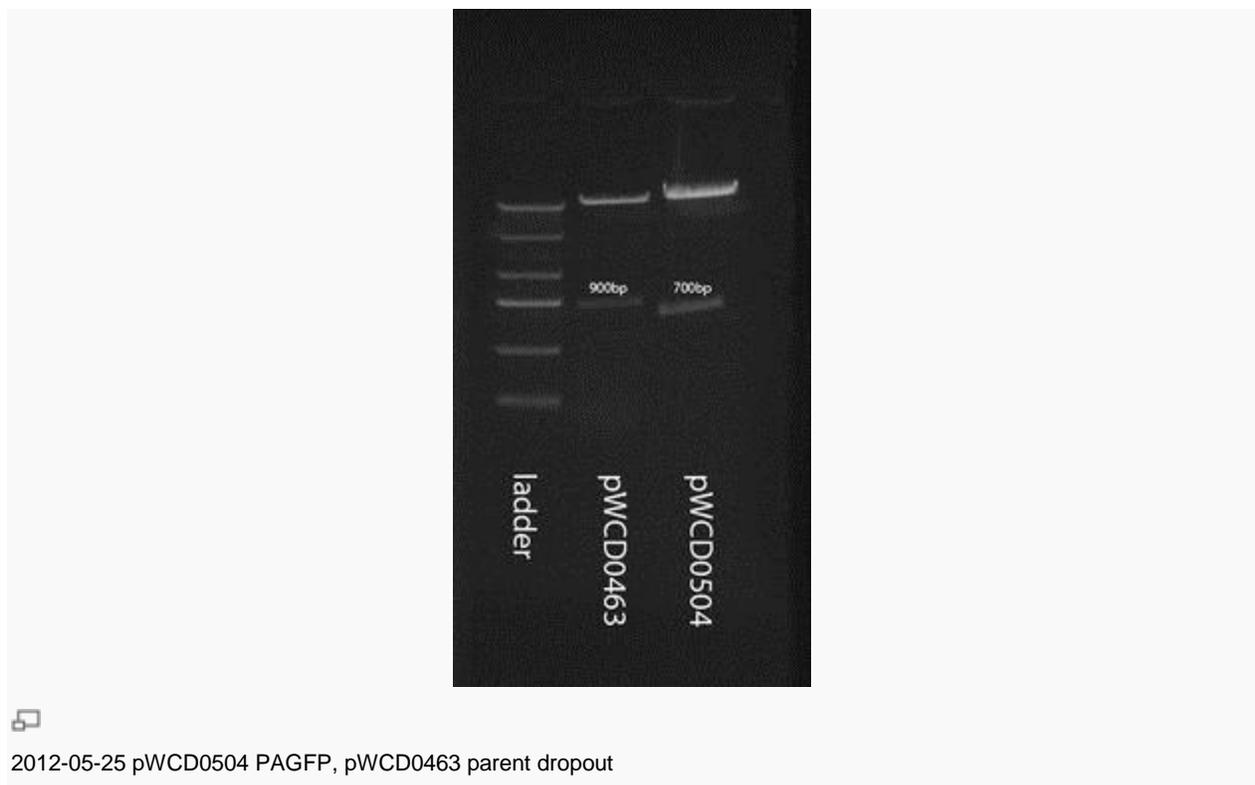
requires that we break the association between the DNA barcode and the miCode, which may lead to random assortment of barcodes and miCodes.

[edit]Thomaschow 25 May 2012 (PDT)

Attended cousin's graduation, so did not come to lab.

1. Constructed primers for Gibson Assembly of target gene and linker-GFP constructs.
2. Oligos arrived for linker-XFP cloning of pWCD0421 and pWCD0480, so those were performed.
3. Oligos for Gibson Assembly ordered.

Test digest of PAGFP in pWCD0504 with BglII/XhoI and parent pWCD0463. PAGFP should be ~720 bp's, and parent dropout ~900 bp's.



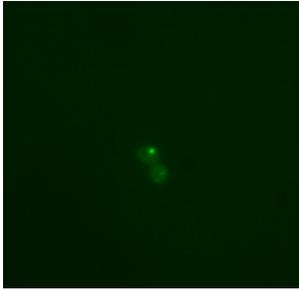
[edit]Thomaschow 24 May 2012 (PDT)

1. Determined optimal ORF/protein for tagging, using the yeast genome database formed by members of UCSF.

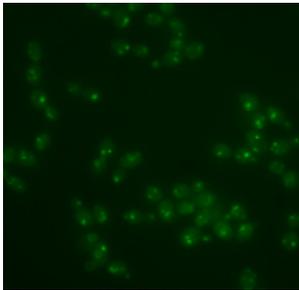
Selected 2 genes for tagging, one serving as the colocalization reference (this gene was listed as a standard on the website). Colocalization performed using GFP and TAP.

Endosome:

SNF7 (colocalization reference)

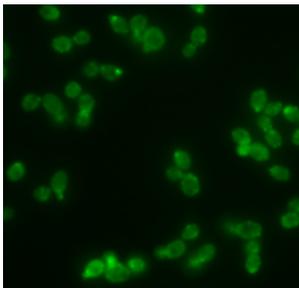


DID2

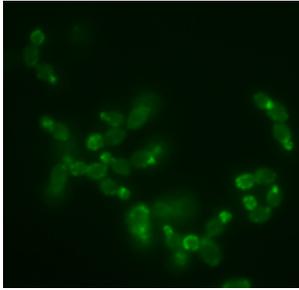


Actin:

SAC6 (colocalization reference)



ABP1



Vector construction scheme:

Target vector (with promoter)

Gene of interest (above)

Fluorescent tag

```
Fluorescent tag_____
Gene_____
Target Vector_____promoter___
```

Use the Gibson Assembly to combine the three components.

Design primers for Gibson:

```
Forward: promoter homology (20 bp) - bglII - gene homology (annealing)
AGAAGAAAACAAAAACAAA-AGATCT- gene homology
Reverse: gene end homology (annealing)- scar -linker homology (20 bps)
[rev compliment]
Gene homology (end)- GGATCT- ggttctggtagtggcagcgg
Ordering reverse: cgcctgccactaccagaacc - AGATCC- revcomp gene homology
**In reverse primer, make sure to remove the stop codon at the end of the
gene in your gene homology region
```

PCR'ed FP gene

[edit]Thomaschow 23 May 2012 (PDT)

1. Assigned lab benches, acquired materials for lab work.
2. Found site <http://yeastgfp.yeastgenome.org> that lists active proteins that are localized in various areas for yeast, that have successfully been tagged.
3. Performed ligation step with previous vector and insert.

[edit]Thomaschow 22 May 2012 (PDT)

1. Continue transformation protocol, watched PCR
2. Used Ape to design primer for PAGFP insertion into target vector.
3. Picked yeast colony from yesterday, allow to grow.
4. Digested vector plasmid, and PAGFP plasmid.
5. Saved double digestion (target vector and insert, with BglII and XhoI) for ligation tomorrow.

Ideal organelle targeting qualities:

1. Small/short sequence.
2. Specific, stays localized.
3. Shown to work?

Targeting Info:

full protein sequence

portion required for targeting

reference

length of sequence

shown to work with FP? Picture

Comments about specificity

terminus requirement?

[edit]Thomaschow 21 May 2012 (PDT)

1. Learned to use wiki, made account to Dueber Wiki.
2. Got familiarized with lab and iGEM members.
3. Created document for plasmids recording.
4. Designing Plasmids/Gene Strains

BglBrick/BioBrick

Gibson Assembly

Golden Gate

Polymerase Cycling Assembly

DNA Assembler Technology

Modifying strains: Yeast Genomic Integrations with Homing Endonucleases

PMID: 21876185

5. Learned how to insert plasmid into yeast, for transformation.

6. Brainstormed methods of tagging fluorescence to organelles.