Fluorescence In Situ Sequencing (FISSEQ)

Introduction

Why do we need analytic tools for synthetic projects? The tools for synthetic biology have grown incredibly powerful: DNA synthesis, genome engineering, synthetic cells, directed evolution, cell-free systems, metabolic engineering, and nanomaterial science. However, these tools only cover the second half of the “read/write” cycle. In this class, we will discuss the rationale for developing measurement technologies (“read”) to complement these engineering tools (“write”), so that we can understand the effects of our bioengineering efforts and make new products that resemble real biological systems.

We will review various approaches to molecular measurements, including DNA and RNA sequencing, proteomics, and 3D structural morphometry. We will focus predominantly on in situ detection of single molecules (in situ is Latin for “in place,” referring to detection of molecules inside cells). Finally, we will discuss applications of these technologies to fibroblast wound healing, understanding how the brain works, and to developing new organoids to further our understanding of biological development and create new biomedical interventions to advance human health.

Readings

Background Reading:


Additional Reviews of FISSEQ and Single-Cell Sequencing


Additional Theory


Homework

This is probably the most difficult assignment of the entire course. We discussed with Núria and we tough that it was very difficult to do the lab work. We made an approximation to the materials and by far, it was the most expensive lab assignment and a high difficulty.

Also even having the privilege access to a great research center like the prbb. The guys at the Beach Lab thought it was really impossible to do it at the Lab were almost there is no biology Lab material.

The longest protocol of HTGAA!!!!!!!!!!!!

Lab Homework Assignment: Create an in situ sequencing library inside a polyacrylamide hydrogel, and detect the sequencing amplicons using fluorescent sequencing by hybridization.

Materials:

- 3×1", 1 mm Thick Gold Seal Microscope Slides (EMS 63710-05, 1 gross)
- PTFE Printed Slides 1 Oval 24.4×16.7mm (EMS 63416-32, 72)
- CultureWell Chambered Coverglass Inserts with 6 mm diameter (EMS 70461-2R2)
- 200 Proof Ethanol
- Nuclease-free/Ultrapure H2O (e.g. Millipore)
- 1X PBS pH ~7.4 (ideally DNase-free)
- 1M Tris-HCl Buffer stock pH ~7.4
- 2X SSC Buffer stock pH ~7.4
- Glacial acetic acid
- GE Healthcare Life Sciences PlusOne Bind-Silane (17-1330-01)
- GE Healthcare Life Sciences PlusOne Repel-Silane ES (17-1332-01)
- 40% Acrylamide/Bis Solution 19:1 (Bio-Rad 1610144)
- TEMED (Bio-Rad 1610800)
- Ammonium Persulfate (APS) (Bio-Rad 1610700, 10g bottle)
- T4 DNA Ligase (NEB M0202S)
- 25 mM dNTP Solution Mix (Enzymatics N2050F)
- Phi29 DNA Polymerase (Enzymatics P7020-LC-L)
DNA Oligonucleotides (IDT):
Template Species #1:
/5phos/TCACGGACCTGCACCTCAACCACTCCTCAACACTCCCGCA
AGTGCTCAGACTCACTCGAGTGACCACCGACGGTGGGTCACTCGAGA

Splint Ligation/RCA Primer #1:
/5Acryd/CGCGCAGGTCCGTGATCTCGAGTGACCAC*G
Sequencing Primer #1: /5dye/CGTGGTCACTCGAGATCACGGACCTGCGCG (choose dyes based on microscope configuration)

Template Species #2:
/5phos/TAGACTGGGCACTCACATTCAACCCAACACTCCTCAACACTCCCGCA
ATGAAGCAG

Splint Ligation/RCA Primer #2: /5Acryd/GAGATGCCAATCTACTGCTTCATTCGAC*G
Sequencing Primer #1: /5dye/CGTCGAATGAAGCAGTAGACTGGGCATCTC (chose dyes based on microscope configuration)

Equipment:
- Pipettes & Tips (20, 200, 1000 uL)
- Binder clips (any size)
- Razor blade
- Clean glass or plastic beakers (large enough to submerge slides)
- PCR machine or heat block
- Fluorescence microscope, ideally with >=40X objective and at least 2 channels of fluorescence detection (e.g. Cy3 and Cy5)
- Chemical hood
- Optional: 30 deg C incubator
- Optional: Vacuum line for aspiration

Instructions:

Note: “Washing” the sample just involves aspirating any fluid currently on the sample (gently using a pipette, or using a vacuum line), then gently pipetting the wash buffer or next reagent onto the sample.

Prepare the glass surfaces:

The BindSilane treatment covalently attaches groups to the glass that will cross-link with the polyacrylamide matrix, so that the gel will be well attached to the glass surface. The RepelSilane treatment forms a water repellant film on the other glass surface so that the gel does not stick. Creating a sandwich with a gel between these two surfaces allows you to pour very thin polyacrylamide gels (~50 um thickness).

1. Wash the glass slides and PTFE printed slides thoroughly with Ultrapure H2O and then ethanol until slides are completely clean with no visible residue.
2. Mix BindSilane Reagent (scale recipe to fill beaker enough to cover a glass slide completely):
   - 8 mL Ethanol
   - 200 uL glacial acetic acid
- 1.8 mL Ultrapure H2O
- 5 μL BindSilane
  Note: Do BindSilane treatment inside a chemical hood!
3. Dip glass slides into the BindSilane Reagent and incubate for 10 seconds, then remove the slide and let air dry.
4. Wash glass slides thoroughly with ethanol and let air dry.
5. Pipet RepelSilane over the exposed oval glass surface of the PTFE printed slide and incubate for 10 seconds, then let air dry.
  Note: Do RepelSilane treatment inside a chemical hood!
6. Wash PTFE slides thoroughly with ethanol and let air dry.

Pour the sequencing gel:

Here we will pour a thin polyacrylamide gel, embedding the DNA sequencing templates inside the gel.

1. Mix 1 mL of gel and bring to room temperature
   - 850 μL Ultrapure H2O
   - 100 μL 40% Acrylamide/Bis Solution 19:1 (final 4%)
   - 40 μL 1 M Tris-HCl pH
2. Prepare 10% APS solution and 10% TEMED solution on ice
3. Hybridize the sequencing templates with the splint ligation probes
   - Add equal moles of each sequencing template and splint ligation probes in 2X SSC (see the next step for molar concentration)
   - Heat to 90 deg C for 30 seconds, then gradually cool to room temperature at 0.1 deg C/sec in a PCR machine or by moving the tube from the heat block to room temperature and incubating for 30 minutes
4. Add 1 μL of pre-hybridized sequencing template mix to the gel mix such that the final concentration of all DNA complexes (sets of sequencing template + splint ligation probe) are at 0.16 nM, which gives on average 1 sequencing amplicon in each 10 μm^3 volume of the gel (e.g. 1 mL / 10 μm^3 molecules in 1 mL)
  Note: If the density is too high or too low, try again adding a different amount of DNA complexes.
5. Mix thoroughly by pipetting, being careful not to introduce bubbles (oxygen inhibits polymerization).
6. Add 5 μL 10% TEMED and mix thoroughly by pipetting, being careful not to introduce bubbles (oxygen inhibits polymerization).
7. Add 5 μL 10% APS and mix thoroughly by pipetting, being careful not to introduce bubbles (oxygen inhibits polymerization).
8. Quickly pipet 10 μL of gel mixture onto the BindSilane glass slide.
9. Carefully place a RepelSilane-treated PTFE slide on top of the gel droplet, sandwiching the gel between the two surfaces with the PTFE layer acting as a thin spacer.
10. Use 1+ binder clips to secure the glass slides together
11. Incubate for >20 minutes at room temperature, or until the gel has polymerized fully.
    Note: It is helpful to pour a bunch of these so you can test whether the gel has set up on an extra slide.
12. Carefully remove the PTFE slide without stretching or deforming the gel too much. The gel should remain attached to the BindSilane-treated glass slide.
13. Take a CultureWell Chambered Coverglass insert, and using a razor blade cut across the insert to connect the two wells into a single, large oval area, which should be larger than the oval of the PTFE slide. Position the CultureWell over the gel and press down to create a tight seal with the glass slide surrounding the gel. If the gel extends into the area covered by the CultureWell Insert, use a razor blade to scrape away excess gel, leaving only an area of gel that can fit completely within the CultureWell Insert. Be sure to keep both the glass slide and CultureWell Insert very clean (e.g. no fingerprints, minimal handling) to create a leak-free seal.

14. Wash the gel twice for 1 minute each with 1X PBS, making sure the CultureWell Insert is not leaking. **Use DNA Ligase to circularize the two DNA species and use rolling circle amplification to generate an in situ sequencing amplicon:**

The DNA splint is modified on the 5' end with an Acrydite, which covalently tethers it into the gel, while the 3' end is modified with phosphorothioate bonds to prevent Phi29 from digesting it using Phi29's 3'→5' exonuclease activity. The linear DNA template is complementary to the DNA splint on both ends, so that it looks like a circle. T4 DNA Ligase will seal the nick in the DNA template, and then the DNA splint acts as a primer for phi29 amplification.

1. Prepare Splint Ligation Mix by adding in order on ice (Recipe for 200 uL reaction volume, scale as necessary):
   - 20 uL 10X Ligase Buffer
   - 175 uL Ultrapure H2O
   - 5 uL T4 DNA Ligase

2. Wash the sample once for 2 minutes with 1X T4 DNA Ligase Buffer in water.

3. Add Splint Ligation Mix and incubate at room temperature for 1 hour.

4. Wash the sample twice for 2 minutes each with 1X PBS.

5. Prepare the Rolling Circle Amplification Mix (RCA, Recipe for 200 uL reaction volume, scale as necessary):
   - 20 uL 10X Phi29 Buffer
   - 177 uL Ultrapure H2O
   - 2 uL 25 mM dNTP Mix
   - 1 uL Phi29 enzyme

6. Wash the sample once for 2 minutes with 1X Phi29 Polymerase Buffer in water.

7. Add RCA Mix and incubate at 30 deg C (room temperature is OK) 4 hours to overnight (put the slide inside a plastic bag or pipette tip box and seal with plastic wrap to minimize evaporation).

8. Wash the sample twice for 2 minutes each with 1X PBS.

**Use sequencing by hybridization to determine the identity of each amplicon inside the gel:**

Each RCA amplicon inside the gel is one of two “species.” Hybridize the fluorescent probes to the sample, which will light up each amplicon species in one of the two colors for microscopy. Note, we call this “sequencing by hybridization” because the process of DNA hybridization reveals the sequence of the template! In other words, at room temperature the fluorescent probe will only hybridize to other DNA sequences that are very similar, so if the
fluorescent probe binds to the RCA amplicon, that tells us a lot about the sequence of the amplicon.

1. Prepare each fluorescent probe at 1 mM concentration in 2X SSC.
2. Add to sample and incubate at room temperature for 20 minutes.
3. Wash the sample five times for 4 minutes each with 1X PBS.
4. Image on a fluorescence microscope. Each RCA amplicon appears as a fluorescent dot between 100-1000 nm in diameter in one of the two colors!

Congratulations: You have identified single molecules in situ, inside a hydrogel!

**Computational Homework Assignment:** Analyze a FISSEQ dataset and find some *in situ* sequences.

Computational Homework was also incredible Adolfo and Francisco both engineers had plenty of troubles to work with matlab. Actually, none of them had an actual version of the software and the process gave us many errors at different levels. The last day Francisco obtained a MATLAB and they want to give it a try. Let’s see if there is time for the assignment!!!!

During the last days of the course there were severals mails asking for the assignment in general and for the computational part. For me with my background was difficult without the help of the Lab colleagues. A don’t know anything about Python,Fiji etc… but seem really interesting

No Matlab but let’s try to install the rest and see if some college pass me the software.

First install canopy express for FREE
I downloaded all the files:

I still did not get the matlab 2015. So I cannot continue I probably I would need help since I never used matlab in my life.

Requirements:

- Computer (best with 16 GB RAM)
- MATLAB
- Python 2.7
- Fiji ImageJ Distribution
- Bio-Formats plug-ins for Fiji/ImageJ
- Bowtie 1.0
- R and RStudio

Instructions:


1. Download a free academic version of Canopy Python 2.7 and follow the installation instructions.
2. Download and unzip the files from 2014 FISSEQ Nature Protocols
3. Download and unzip the RefSeq-to-Gene ID Conversion Table]
4. Build the reference index in Bowtie (see the Bowtie instruction manual):
   $ bowtie-build -c -f human.rna.fna refseq_human
5. Start MATLAB and add the downloaded folders to the search path:
   » addpath('~/fisseq', '~/fisseq/bfmatlab')
6. Define the input and output directories for Image Registration, then run the Image Registration script. Set the number of blocks per axis for local registration (default = 10); set the fraction overlap between neighboring blocks (default = 0.1); and adjust the alignment precision, where 10 will register images to 1/10 of a pixel (default = 1).
   » input_dir='decon_images/'
register_FISSEQ_images(input_dir,output_dir,10,0.1,1)

7. **Question:** What happens when you use different values for the parameters? How does it affect the image registration quality? Open the results in Fiji and take a look! Note, you may have to adjust the contrast in Fiji to get a good look at the images.

8. Start python and run the script to generate base calls to the file read_data_*.csfasta. The maximum number of missing base calls allowed per read is 6 by default. (* denotes an automatically generated time stamp.)

   ```python
   $ python
   » import FISSEQ
   » FISSEQ.ImageData('registered_images', '.', 6)
   » quit()
   ```

9. **Question:** Take a look at the reads in the resulting .csfasta file. How do they look? What happens to the number of reads if you change the value for maximum number of missing base calls ('6' in the command line).

10. Align reads to refseq_human using Bowtie 1.0, and write mapped reads to bowtie_output.txt. Note: Use the exact name of read_data_*.csfasta!

    ```bash
    $ bowtie -C -n 3 -l 15 -e 240 -a -p 12 -m 20 -chunkmbs 200 -f -best -strata -refidx refseq_human read_data_*.csfasta bowtie_output.txt
    ```

11. Spatially cluster the Bowtie reads to annotate clusters using gene2refseq, and write to results.tsv. The default kernel size of 3 performs a 3 × 3 dilation before clustering.

    ```bash
    $ python
    » import FISSEQ
    » G = FISSEQ.ImageData('registered_images', None, 6)
    » FISSEQ.AlignmentData('bowtie_output.txt', 3, G, 'results.tsv', 'human.rna.fna', 'gene2refseq', '9606')
    » quit()
    ```

12. **Question:** Take a look at the output. What happens if you change the size of the kernel to something less than 3? To something much greater than 3?

13. Open the FISSEQ RStudio project file (Menu → File → Open project...).

14. Find the HISTORY tab on the upper right console window, and double-click on individual commands in order to re-execute the previous R session, and learn how to: import and filter data using a specific criterion (i.e., cluster size); plot a distribution of reads by a specific criterion (i.e., RNA classes and strands); convert a table of reads into a table of gene expression level; correlate gene expression from different images; and find statistically enriched genes in different regions.

15. **Task:** Are there any correlations between the features of FISSEQ clusters? E.g., is cluster size correlated with cluster quality?

16. **Task:** Find some clusters of different size and quality, and then look at the first image in Fiji and see if you can see the FISSEQ amplicon associated with that cluster. (Note: X/Y is inverted in the clustering file.)
Design Homework Assignment: Think back to your experience so far with HTGAA. Were there any experiments where in situ data of RNA, DNA, protein, or other cellular features would be helpful in understanding the engineering process? You should try to answer the following questions:

- What are some reasons in situ data could be better than bulk data for this experiment? Try to think of cases where a bulk measurement would cause you to miss some insight.
- What kinds of molecules would you like to detect? E.g. what species of RNA? How would you go about targeting those molecules?
- What factors would limit your ability to detect the things you are interested in? There are probably lots of these! For example, if you are interested in RNA expression in e. coli, each e. coli is only big enough for a few FISSEQ amplicons, so at most you could only detect 2-3 RNA molecules!! Try to think of strategies to overcome these limitations. Try to be as detailed as possible and think creatively! These are the kinds of questions we ask every day and that come up as we talk to other scientists who want to use FISSEQ. These questions drive our technology development process!

In situ data can give you more information about the environment factors or factors related to cell tissue behavior or interactions. The problem with the techniques that are toxic and kill the cells is that you cannot study many of the “a life” or natural situations. It could be useful in tissue formation for example bone or specializes tissue formation to understand that everything is going ok, interactions between cells and the environment conditions. The technic could allow you to see and mark which mRNAs are intervening at each moment or process. SO see the parts of the transcription mechanisms that are active at different moment so getting clues about when and what elements.