Calibration curve for data analysis

1. Aim of the experiment

Determine a calibration curve for our aptamer-thrombin binding experiment in order to convert relative K_D (RFU) into absolute K_D (M) of the formation of [Cy3/Cy5-labelled aptamer + thrombin] complex

a. Hypothesis / expected results

We expect to observe a linear relation between the relative fluorescence and the concentration.

b. Team / TAs / schedule

iGEM student	TA	Scheduled TA help time
Felix	Ivan	
Matteo	Ivan	

c. Consumables / lab equipments

		Reference	Supplier	Amount required
	Native human a-thrombin, Biotin (storage buffer, 0.02M HEPES, pH 7.4, 0.15M NaCl), Catalog nb RP43103		ThermoFisher Scientific	Thrombin 13 mg/L (1:200 of 2.6g/L aliquots) 0.25ul in 49.75ul of PBS
	PBS		Sigma-Aldrich	~60ul
	Neutravidin 500[ug/ml]		ThermoFisher Scientific Prod 22832 Lot LH149056	~30ul
	Tris-HCl	[2]		1ml for 20ml buffer
	NaCl	[2]		2ml for 20ml buffer
	$MgCl_2$	[2]		0.2ml for 20ml buffer
	Cy3 labeled primer, 2umol		IDT	~30ul
	Cy5 labeled primer, 2 umol		IDT	~30ul
	Spotted MITOMI chips		Ivan's chips made on 10.07.17	2

Experimental design by: Matteo Pirson, 2017/07/15
Proofread by: Ivan Istomin, 2017/07/16 Comments: hypothesis and the goals of experiment are well outlined. The procedure requires changes in the routine of how to flow DNA dilutions.

d. Detailed description / protocol

Neutravidin surface chemistry

1. Set pressure for control layer at 15 psi, flow layer at 3 psi
2. Fill in the control lines with dH_2O one at a time, check that all lines are working. Close chamber valves (neck valves).
1. Flow 2 [mg/mL] biotin-BSA with the general outlet open until BSA approximately reaches the outlet. Close the outlet and allow any air inside the chip to escape. Then flow the BSA for 20 minutes (15 min) in order to passivate the glass surface.
2. After this point, each time a new reagent is to be flowed through the chip, make sure **no air enters the chip**. Close the general inlet and open the waste and reagent lines. Allow the reagent to flow through the waste line for 30 sec to let air escape. Then close the waste line and open the general inlet and allow the reagent to flow.
3. Wash chip with PBS for 5 minutes
4. Flow 500 [µg/mL] Neutravidin for 20 minutes in order to bind to biotin, buttons up.
5. Wash chip with PBS for 5 minutes, buttons up
6. Flow 2 [mg/mL] biotin-BSA for 20 minutes in order to block Neutravidin sites outside button area, buttons down
7. Wash chip with PBS for 5 minutes, buttons down

Chip now has neutravidin sites blocked everywhere by biotin but in the colored button area.

Calibration (Do this part under the fluorescent microscope)

1. Flow thrombin (13 mg/L, 20 min.) having the button down for 2 minutes and the rest of the time with buttons up.
2. Stop the flow by closing the inlet.
3. Scan the empty chip before flowing any fluorescent solution. Fix the exposure time to 100 ms.
4. Flow DNA at the lowest dilution through the waste and then through the chip during 5 min., then image at 100 ms (exposure time). While imaging, flow the second dilution through the waste. After scanning the first dilution, start flowing the second dilution in the chip (5 min.). Iterate this procedure for all 6 dilutions.

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Notes:

- Prepare a waste line tube with a becher (many waste)
- Make sure there is **no air** !
- For DNA-dilutions, take around 6 data points in the range [20; 1000] nM. The DNA is diluted in ABS (or with PBS).
- **Do the calibration curve for Cy3 and Cy5 on different MITOMI chips.**

Imaging on fluorescent microscope

1. Use the NIS program
2. Align the chip on the microscope with the grid in the software. Make sure that every chip cell is in the field of view when selected from the grid in the software. Set the upper left chip cell as the reference point
3. Choose the appropriate laser/channel for imaging
4. Choose the exposure time in order to maximize the signal:noise ratio, not to saturate the camera, and not to photobleach the fluorophores.
5. Choose a filename
6. Press “run now”. The program should take an image of each cell in the chip.
7. When the image is created, select it and export it to a new folder using the “export ND document” option, under “export” in the “file” menu
8. Select “mono image” and the name, then click “export”
9. Open ImageJ. Go to “plugins”, “stitching”, “grid”. Choose the grid size, no overlap, , make sure “display fusion” is selected, and select the folder where you saved the image from the microscope. Choose a file name, but it must end in “xy{iii}”
10. Once the stitched image is generated, scale it down by pressing ctrl-E.

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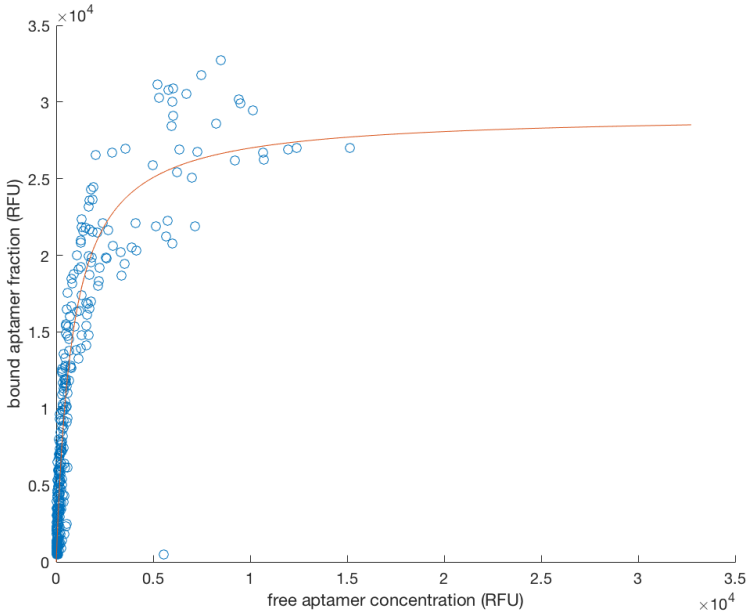
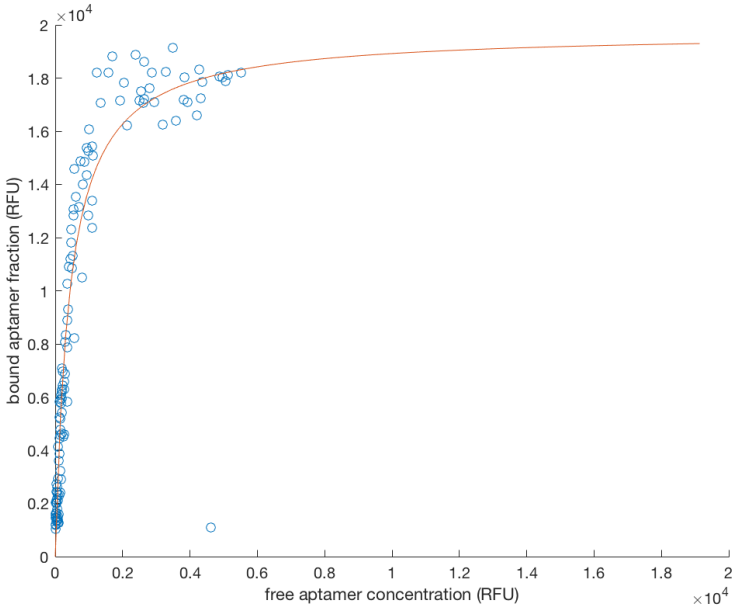
e. Results

Biotinylated-Thrombin – Cy3 labelled aptamer



Biotinylated-Thrombin – Cy5 labelled aptamer



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2. Conclusion

KDs were pretty accurately determined and we could conclude that the sandwich assay was successful ; only remains to be known whether there is some background interaction between the two aptamers in the absence of thrombin.

References

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2. Oligonucleotide Inhibitors of Human Thrombin that Bind Distinct Epitopes, Tasset Diane M., Kubik Mark F., Steiner Walter, Journal of Molecular Biology 272, 1997, p.688-698

3. Maerkl, S. J. & Quake, S. R. A Systems Approach to Measuring the Binding Energy Landscapes of Transcription Factors. *Science (80-.).* **315,** 233–237 (2007).

Appendix

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