5-2024

Light-Induced Phase Separation Purification for Monoclonal Antibodies

Xuyang Chen
xuyangc@clemson.edu

LIGHT-INDUCED PHASE SEPARATION PURIFICATION FOR MONOCLONAL ANTIBODIES

A Thesis Presented

To the Graduate School of

Clemson University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Engineering

Professional Communication

By

Xuyang Chen

May 2024

Accepted by:

Dr. Marc Birtwistle, Committee Chair

Dr. Scott Husson

Dr. Eric Davis

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure

ABSTRACT

Monoclonal antibodies are becoming an increasingly important subject in the pharmaceutical industry. They have been applied for treating COVID-19, breast cancer, leukemia, asthma and diagnostics, among many other uses, and tremendous advances have been made. However, challenges still exist in the production of monoclonal antibodies.

Intrinsically disordered protein regions (IDRs) have emerged as one of the fundamental biological process adjustors. Phase transitions driven by intrinsically disordered protein regions play an important part in the development of IDRs. Here, we explore the possibility of using a light-activated IDR system that drives liquid-liquid phase separation within living cells to purify monoclonal antibodies.

INTRODUCTION

Monoclonal antibodies are proteins made from recombinant Chinese hamster ovary (CHO) cells. The FDA has approved many monoclonal antibodies for large-scale production. Since the first discovery of monoclonal antibodies in 1978 by Stuart Schlossman, the FDA has approved over a hundred monoclonal antibody treatments by 2021 (Mullard, 2021). The pharmaceutical industry has established a functioning production process for commercial monoclonal antibodies. The standard process involves obtaining CHO cells from a cell bank, seeding the CHO cells into a culture container, inoculating the CHO cells into larger tanks, culturing the CHO cells, and then centrifuging or filtering the solution to harvest the product (Fahrner et al., 2001) . These are the typical steps to produce monoclonal antibodies.

Once monoclonal antibodies have been produced, purifying them from the other undesirable byproducts and host cell remains is necessary. Protein A chromatography has proven to fit monoclonal antibody purification and has been integrated into the platform industrial process. Protein A is a surface protein originally found on the cell wall of a bacteria called *Staphylococcus aureus*. It has a strong affinity to antibodies as it specifically binds to them (Graille et al., 2000). When the harvested solution flows through the protein A column, the monoclonal antibodies will bind to the protein A ligands in the column and stay in the column while other components continue to flow through. Protein A has excellent affinity to monoclonal antibodies, so the antibody recovery rate is around 70-90 percent (Fahrner et al., 2001). However, protein A chromatography does not remove all the impurities from the antibodies. Antibody aggregates,

often antibody dimers (Fahrner et al., 2001), will not be removed. In addition, there will be leached protein A, virus particles, and other host cell proteins and DNA along with the antibodies. This product will not be accepted because protein A has a relatively high immunogenicity.

Then, the problem becomes removing the protein A, the antibody aggregates, and other process impurities from the solution. The industrial process typically runs the solution through one or more ion-exchange chromatography and hydrophobic interaction chromatography steps. Simply by changing the solution pH, we can change the antibody's binding affinity, allowing us to separate antibodies from protein A, aggregate and remaining host cell impurities. Lastly, we need an additional viral clearance step to ascertain the safety and compatibility of the monoclonal antibody for human administration (Liu et al., 2010).

However, the purification process, especially Protein A chromatography, is expensive. Protein A resin costs $8,000–$15,000/L (McNulty et al., 2021), while the resin binding capacity is 30 miligrams of monoclonal antibody per mililiter (CYTIVA, 2020). With cyclical elution and regeneration steps to recover the antibody product and cleanse the protein A resin, the protein A ligand has a limited lifespan of 50-200 cycles (McNulty et al., 2021). On top of being an expensive step in production, the purification process also accounts for up to 90% of the production time, which takes about 6 weeks (Renshaw, 2024).

In this paper, we offer the option to use light-induced phase separation to isolate monoclonal antibodies from other undesirable byproducts. This proposed method promises expedited process time and convert monoclonal purification into a continuous process. Figure 1 shows the alternative purification steps.

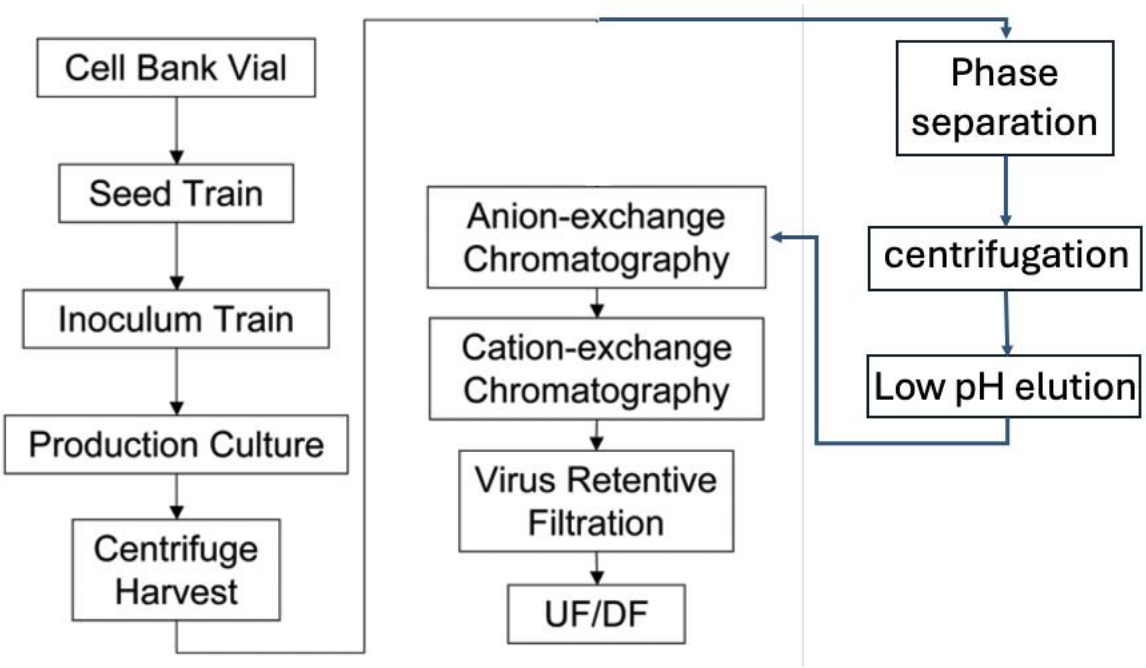


Figure 1. How phase separation would fit into the modern-day commercial production of monoclonal antibodies. Instead of protein A chromatography, we propose employing phase separation to recover monoclonal antibodies efficiently (Kelley, 2009).

Shin et al. (2018) studied a protein construct (optoFus) with an intrinsically disordered protein and the photolyase homology region (PHR) of Arabidopsis thaliana Cry2. This light-sensitive protein dimerizes with itself upon shining blue light. The phase separation described in this paper is highly sensitive to blue light exposure and appears reversible when the cells are kept in the dark.

We hypothesize that linking protein A to the optoFus construct allows the construct to bind with monoclonal antibodies. Then, we can use phase separation to bring the monoclonal antibodies into the protein-rich phase, which can later be processed by centrifugation (Figure 2). This technique will speed up the purification process, and provide a continuous, larger-scale purification that matches the production scale. This method could revolutionize the monoclonal antibody industry with a faster purification and scale-free purification process.

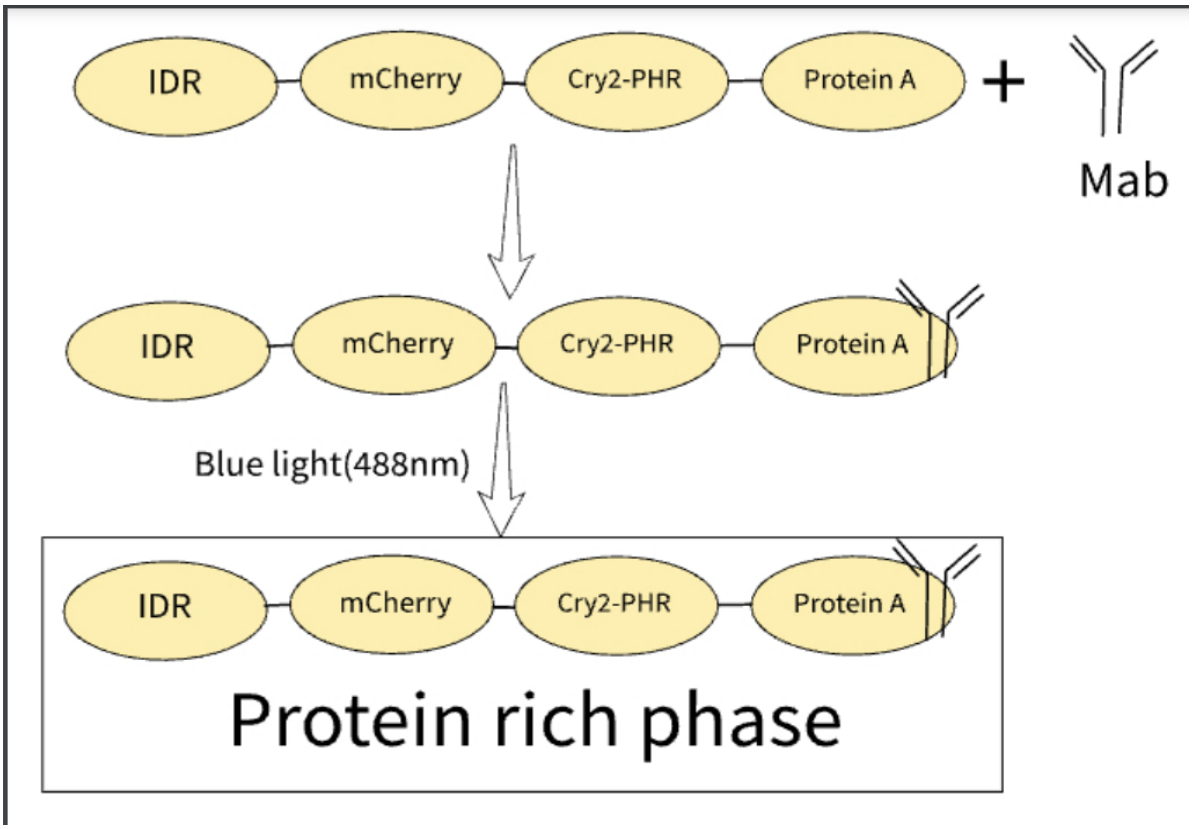


Figure 2. Proposed phase separation mechanism. Monoclonal antibody first binds to protein A due to its high affinity at neutral pH. Then, upon shining blue light, Cry2PHR dimerizes with itself to induce liquid-liquid phase separation.

However, the absence of experimental data about the fusion of protein A with the optoFus construct presents a notable limitation. The potential loss of antibody-binding affinity or phase separation induction capabilities within the fusion protein is a significant concern. Furthermore, since no data are available for using light to purify monoclonal antibodies, it remains to be seen whether the monoclonal antibodies will maintain their function after this process. Consequently, the efficacy of monoclonal antibodies following this proposed purification process remains unknown and suggests further investigation.

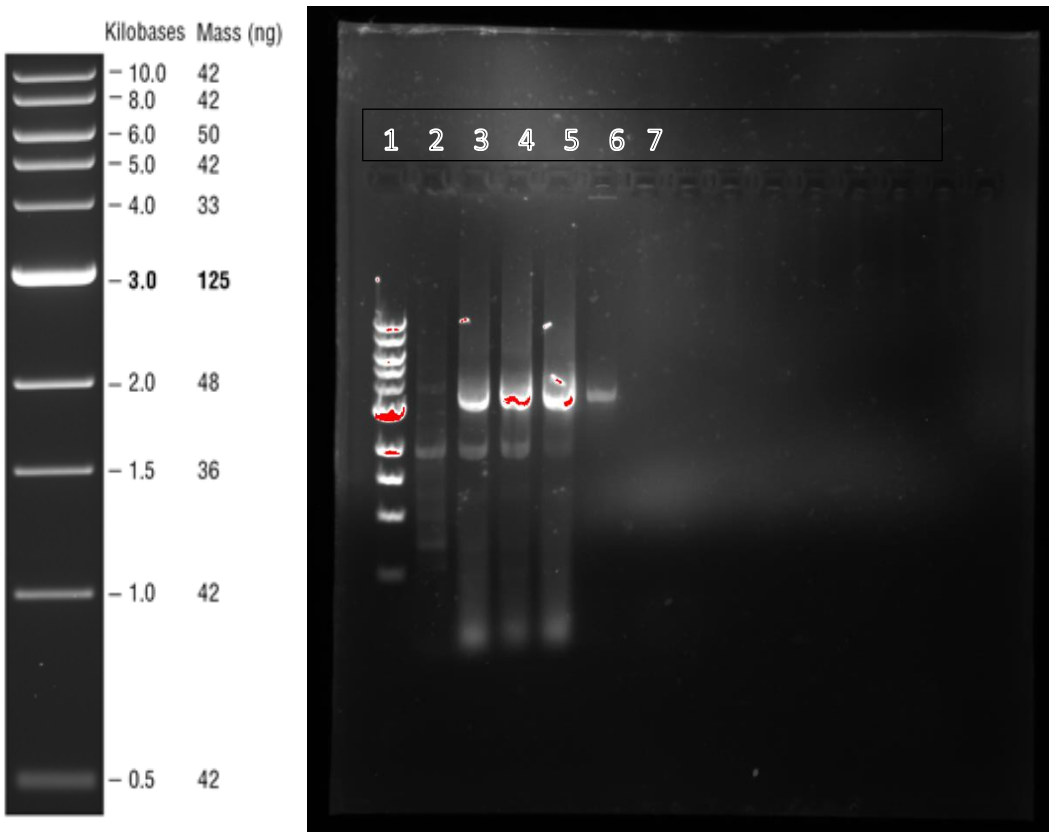
In this study, we planned to design a new plasmid, with protein A (Szczesny et al., 2018) and FusN-mCherry-Cry2 (Shin et al., 2017) transgenes, transfect HEK293T cells with the new plasmid, and observe red fluorescence within HEK293T cells. Should the phase separation process continue to be unaffected by the presence of protein A, then red fluorescent liquid droplets would be found within HEK293T cells after blue light exposure. This investigative approach allows us to explore the potential impact of protein A on the phase separation

phenomenon. It would then become clear whether Cry2PHR will maintain its phase separation capability with protein A present.

Results

Polymerase chain reaction and double digestion

After performing Miniprep to obtain optoFus and protein A plasmid DNA, we performed gradient PCR to amplify the linearized pReceiver plasmid DNA. By doing gel electrophoresis (figure 3), we confirmed that the PCR product was the pReceiver plasmid.



(a) (b)

Figure 3. (a) NEB 1kb ladder. (b) After gel electrophoresis, the gel is observed under UV light. Column 1 is loaded with the NEB 1kb ladder. Column 2-7 were loaded with corresponding primers and PCR productions. Column 2 has no clear bands. Columns 3, 4, 5 and 6 have clear bands. Column 7 has no bands.

As we can see, in columns 3, 4, 5 and 6, we have clear bands between the 4kb and 3kb ladder bands. Based on the plasmid size of 3551bp, we can confirm that this PCR product is the pReceiver plasmid DNA.

Next, we performed double digestion on the pKK-TEV-protein A plasmid to obtain only the protein A gene of interest. Based on the protein A plasmid map (Szczesny et al., 2018) provided by Addgene, we selected two optimal restriction enzymes that are fit to cut the protein A gene out of the original plasmid, which are NheI-hf (NheI-HF, 2024) and XhoI (New England Biolabs, 2024). Furthermore, there are available sites on the pReceiver backbone and the optoFus construct so that it would be easy to assemble the fragments later. Figure 4 below shows the map of protein A plasmid.

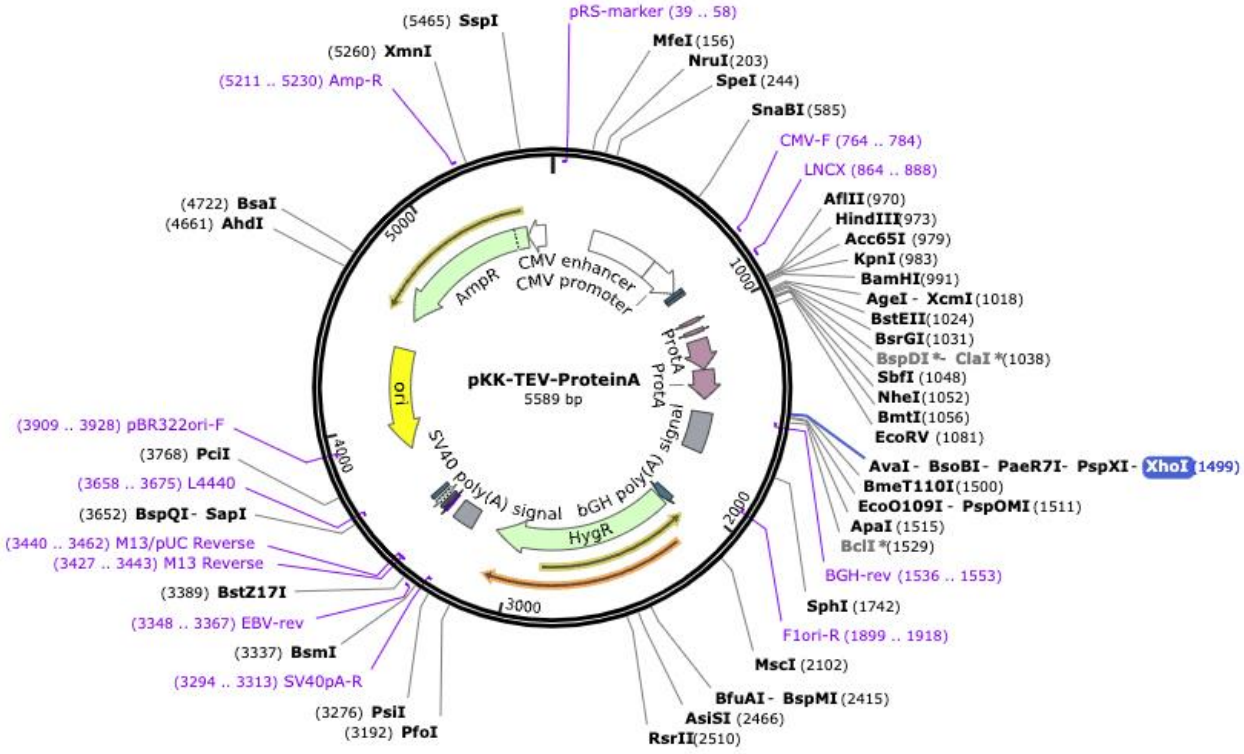
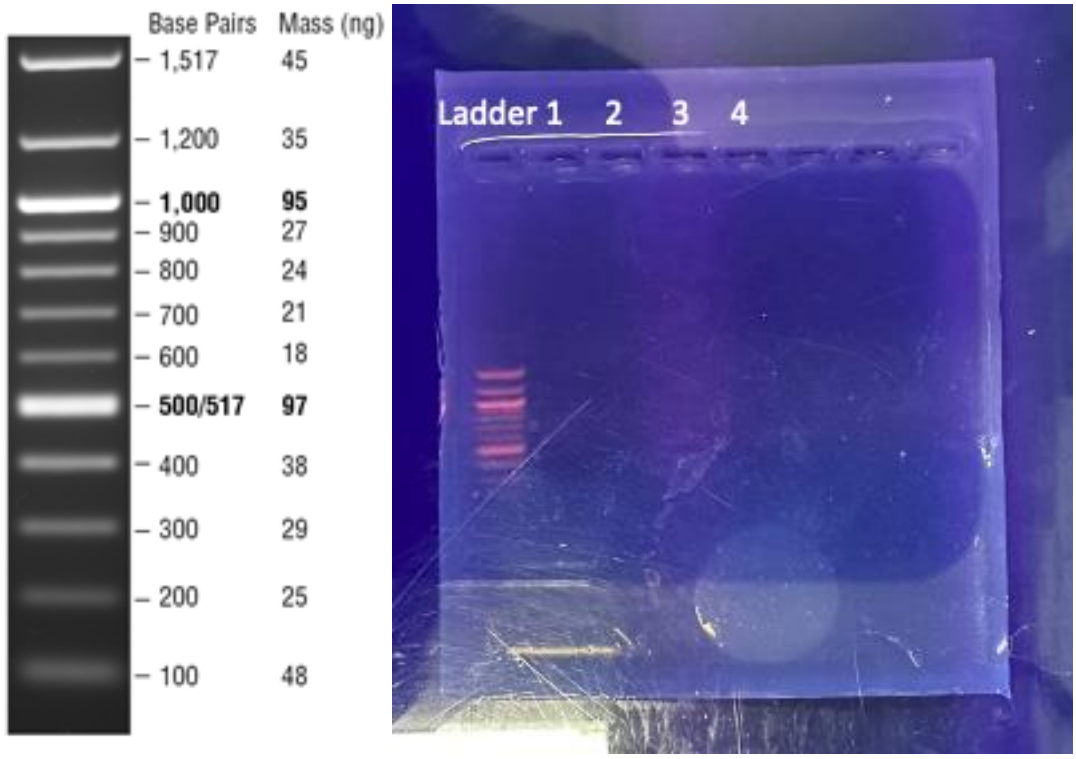


Figure 4. pKK-TEV-protein A plasmid map view. NheI cutting site is at 1052bp. XhoI cutting site is at 1499bp.

However, we did not get any positive results from the gel electrophoresis results of our double-digestion PCR product. In Figure 5, we can see clearly that the 100bp ladder is in column 1, yet there are no bands in the other columns. Our target protein A fragment band should appear at the 500bp band.

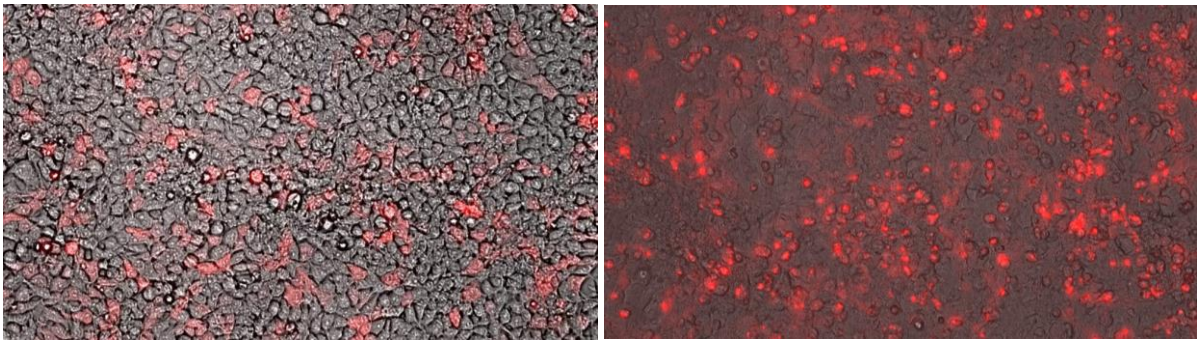


(a) (b)

Figure 5. (a) NEB 100bp ladder (b) After gel electrophoresis, the gel is observed under UV light. The left column is loaded with the NEB 100bp ladder. Columns 1, 2, 3 and 4 are loaded with our double digestion PCR product.

OptoFus transfection in HEK293T cells and fluorescence imaging

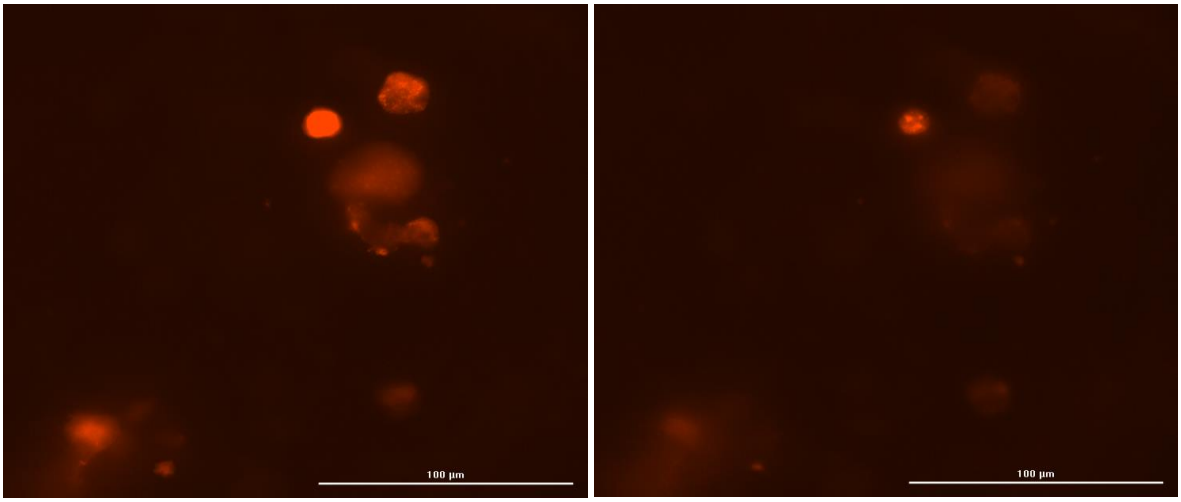
We aim to establish an imaging protocol by transfecting HEK293T cells with only the optoFus construct. Then, by observing the mCherry red fluorescence under a microscope, we will determine whether phase separation has occurred. However, the results were not conclusive. In Figure 6 (a-b), we can see the transfected HEK293T cells with red fluorescence. The transfection rate is about 40%, meaning the optoFus construct has been expressed in many cells. HWith the 20x objective in the microscope, we could see that red puncta were present in some cells. Because we didn't provide any blue light exposure, it's hard to say that those puncta are phase-separated droplets inside living cells. We used the citation 5 from BioTek to expose the transfected HEK293T cells to blue light in a completely dark environment to prove that the puncta are from light-induced phase separation.



(a) (b)

Figure 6. (a) transfected HEK293T cells, (b) transfected HEK293T cells in a dark environment. Images were taken with ECHO microscope.

Results were still inconclusive when we observed the transfected HEK293T cells in the cytation 5 with the 60x objective. In Figure 7 (a), there are puncta inside the cells even before turning on the blue light filter. Keeping the cells in a completely dark environment does not affect the red puncta. In Figure 7 (b), no apparent liquid droplets were found after exposure to blue light for 5 min.



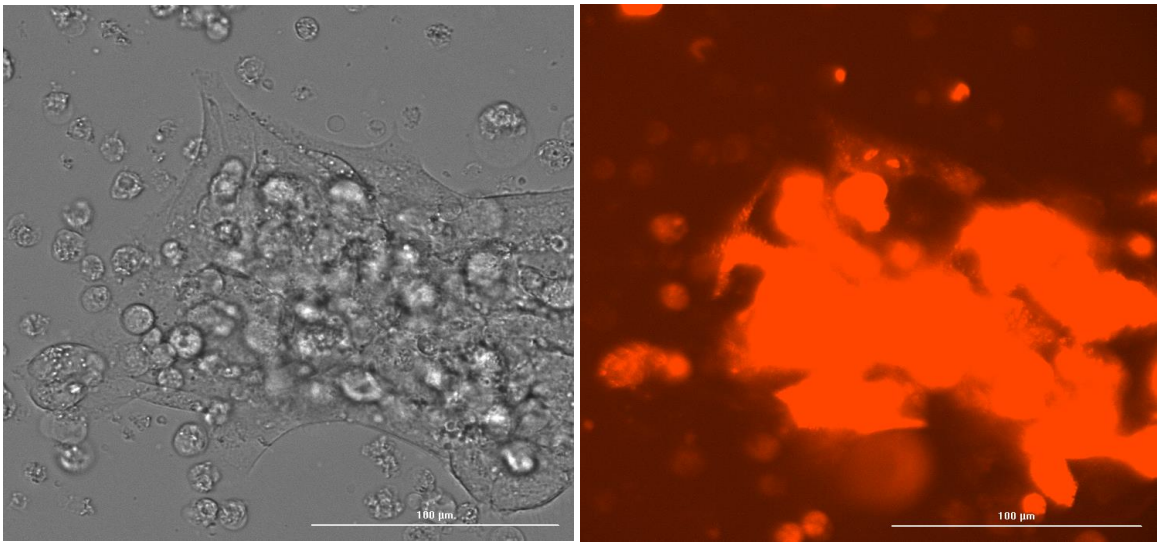
(a) (b)

Figure 7 (a) Transfected HEK293T cells before exposure to blue light. Kept in cytation 5 for 5 min. (b) Transfected HEK293T cells after 5 min of exposure to blue light. Light intensity was lowered by 0.1 uW to watch for droplets.

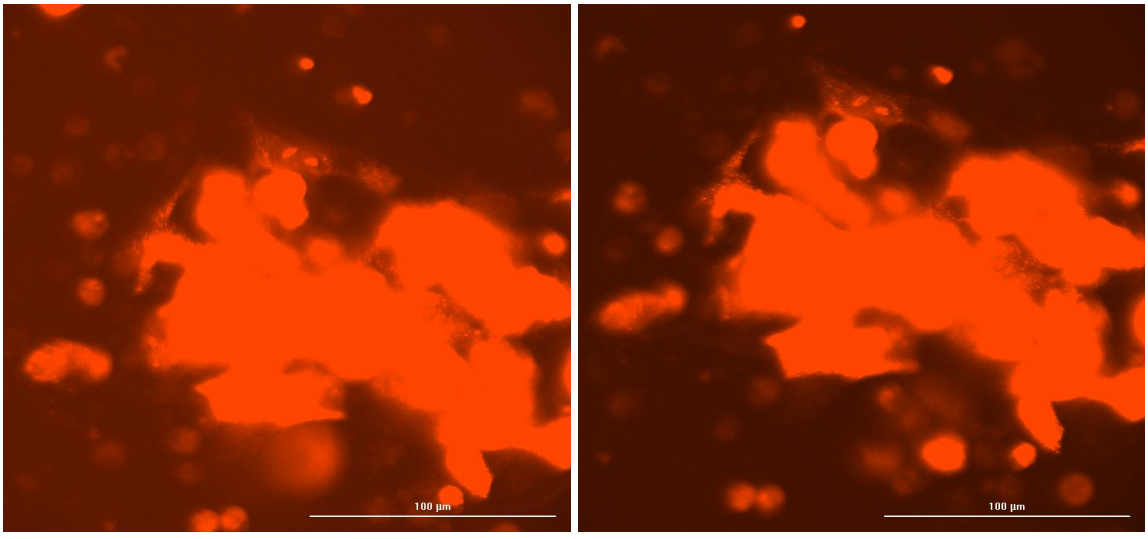
Several attempts were made to acquire clearer images, but none were successful. Figure 8 (a) shows the cell's layout in transmitted light. In figures 8(b), (c) and (d), there are no apparent differences. Because the cells stacked on each other, we could not acquire a clear image of the red fluorescence layout before blue light exposure. We can only conclude that there are puncta in transfected HEK293T cells. However, no evidence supports that these puncta came from light-induced phase separation as these puncta appear unaffected by either blue light exposure or a

completely dark environment. The phase separation function of the optoFus construct and reversibility are well-researched by Yongdae Shin et al (Shin et al., 2017). Fifteen seconds of blue light exposure can cause apparent phase separation. The reason the optoFus construct here is unaffected by blue light is unknown.

However, as shown in figure 8, transfected HEK293T cells experience cell death within 48h of first observing red fluorescence. Possible explanation is that too much lipofectamine 300 reagent was used during transfection and it can be toxic to HEK293T cells in high concentration. Another explanation is that oversaturated optofus construct is toxic to cells when irreversible droplets are formed. Transfected cell death requires further research.



(a) (b)



(c) (d)

Figure 8 (a) Transfected HEK293T cells in transient light. (b) Transfected HEK293T cells in TEXASRED filter before exposure to blue light. Cells were kept in cytation 5 for 15 min. (c) Transfected HEK293T cells in TEXASRED filter after 5 min of exposure to blue light. (d) Transfected HEK293T cells in TEXASRED filter after 5 min of exposure to blue light. Then, cells were kept in cytation 5 for 20 min.

Discussion

In this study, we explored the possibility of using IDR-driven phase separation to purify monoclonal antibodies. We designed a plasmid incorporating the protein A and optoFus gene of interest onto the pReceiver backbone. We successfully linearized the backbone plasmid for cloning and acquired 100 µl of the PCR product of the pReceiver backbone plasmid. We obtained no clear bands for all four columns of protein A double digestion PCR product. We suspect that the protein A plasmid is contaminated with RNA as our nanodrop spectrophotometer UV absorbance ratio (260 nm/280 nm) for the plasmidis above 2.0 (figure 8). However, we could not assemble the whole plasmid due to technical difficulties. We conducted more mini preps to obtain ample plasmid DNA for subsequent PCR amplification and Gibson assembly. Regrettably, this yielded negative results. We consistently acquired plasmid DNA with low DNA yield (figure 9), which impeded our progress in downstream experimental procedures.

Initially, we proposed that the glycerol stocks containing the frozen bacteria with our desired plasmids have been contaminated. Consequently, we performed streak plating procedures to isolate the desired bacterial strains and selected colonies from the plates to prepare for miniprep. Despite these efforts, the nanodrop spectrophotometer, illustrated in Figure 10, yielded unsatisfactory results as the nucleic acid concentration is very low, constantly below 50 ng/µl.

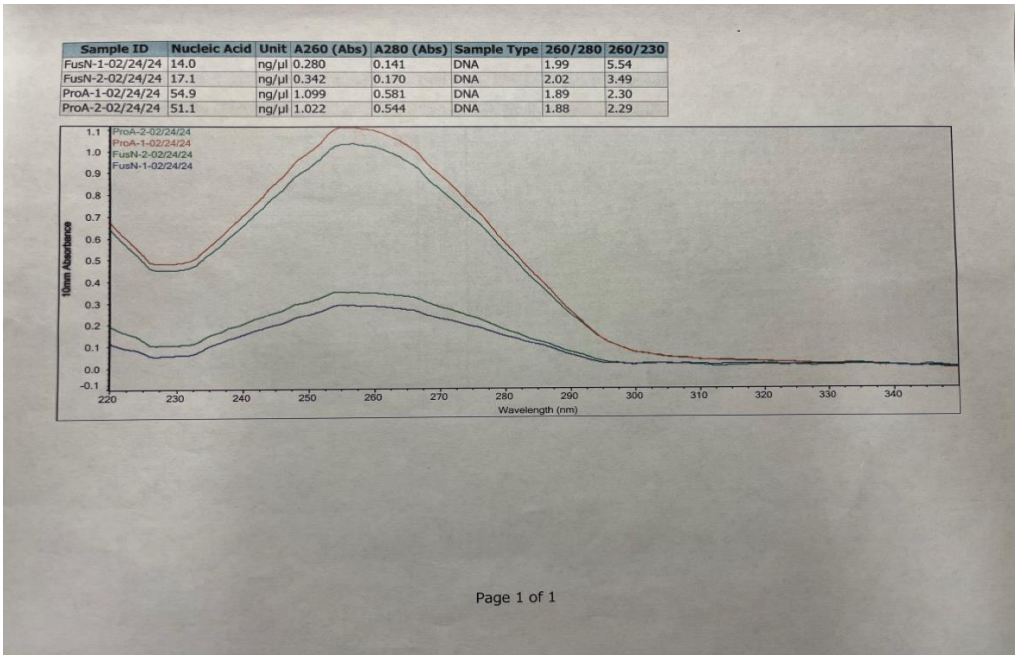
protein a plasmid 11.03	496.6	9.932	4.668	2.13	2.25

Figure 9. Protein A plasmid nanodrop spectrophotometer result

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
4	(Reblank)	Odyssey	2/10/2024 2:52 PM	-45.2	ng/µl	-0.905	-0.700	1.29	0.93	DNA	50.00
5	Fus-Cry2-4-02/14/24	Odyssey	2/14/2024 10:56 AM	15.1	ng/µl	0.302	0.193	1.56	2.66	DNA	50.00
6	Fus-Cry2-4-02/14/24	Odyssey	2/14/2024 10:57 AM	22.6	ng/µl	0.452	0.290	1.56	1.15	DNA	50.00
7	Fus-Cry2-4-02/14/24	Odyssey	2/14/2024 10:58 AM	21.4	ng/µl	0.427	0.282	1.52	1.42	DNA	50.00
8	Fus-Cry2-4-02/14/24	Odyssey	2/14/2024 2:51 PM	424.1	ng/µl	8.483	3.994	2.12	2.42	DNA	50.00
9	proteinA-2-02/14/24	Odyssey	2/14/2024 2:53 PM	-4.5	ng/µl	-0.091	-0.027	3.40	0.46	DNA	50.00
10	Fus-Cry2-4-2-02/15/24	Odyssey	2/15/2024 11:31 AM	21.8	ng/µl	0.435	0.226	1.93	1.44	DNA	50.00
11	Fus-Cry2-4-2-02/15/24	Odyssey	2/15/2024 11:33 AM	20.2	ng/µl	0.404	0.203	1.99	1.27	DNA	50.00

Figure 10. optoFus and protein A plasmid nanodrop spectrophotometer results

The protocol we developed for observing red fluorescent liquid droplets inside living cells remains inconclusive as we cannot say that the puncta that appear on the image came from light-induced phase separation. The puncta are not affected by blue light exposure or a completely dark environment, in contrast to the conclusion By Yongdae Shin et al (Shin et al., 2017). The possible explanation is that while the plasmid was being expressed in HEK293T cells, certain small molecules interfered with the optoFus protein and caused it to lose its function to dimerize with itself. The other explanation is that the optoFus construct is overexpressed in HEK293T cells, causing oversaturation. The



Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	Sample Type	260/280	260/230
FusN-1-02/24/24	14.0	ng/µl	0.280	0.141	DNA	1.99	5.54
FusN-2-02/24/24	17.1	ng/µl	0.342	0.170	DNA	2.02	3.49
ProA-1-02/24/24	54.9	ng/µl	1.099	0.581	DNA	1.89	2.30
ProA-2-02/24/24	51.1	ng/µl	1.022	0.544	DNA	1.88	2.29

Figure 11. optoFus and protein A plasmid nanodrop spectrophotometer results after streak plating

oversaturated optoFus construct dimerized with itself without any foreign disturbance and caused irreversible phase separation. The latter is the more plausible explanation. Therefore, our next step would be to adjust the transfection DNA amount to determine the appropriate amount of optoFus expression given a DNA gradient of 250 ng per well because we don't need to have a high transfection rate to observe phase separation on a large scale. We only need it in a single cell to confirm light-induced phase separation inside living HEK293T cells and establish the correct observation protocol.

However, due to the same technical difficulty discussed above, we could not continue with optoFus imaging. So, the next step for the project would be to transfect HEK293T cells with gradient DNA concentration to observe for phase-separated liquid droplets inside living cells. Subsequently, we can continue transfecting HEK293T cells with the optimal DNA amount to

establish the imaging protocol for observing phase separation. Only then can we use the same protocol to observe the fusion protein with protein A present for phase separation.

Methods

Bacterial overnight culture

Bacterial cultures were cultured in 3ml LB media supplemented with 3ul Ampicillin at 30°C with ambient air for 40h in a FALCON 14mL polypropylene Round-Bottom tube. Bacteria were kept in glycerol stocks in the -80°C refrigerator. LB media was made from Fisher BioReagents BP1421-500 Tryptone, VWR J850-500G Yeast extract, and Fisher BioReagents BP358-1Sodium Chloride and nuclease-free water. Ampicillin was made by Xiaoming Lu from Birtwistle lab, Clemson University. pKK-TEV-Protein A was a gift from Andrzej Dziembowski (Addgene plasmid # 105788 ; http://n2t.net/addgene:105788 ; RRID:Addgene_105788). pHR-FUSN-mCh-Cry2WT was a gift from Clifford Brangwynne (Addgene plasmid # 101223 ; http://n2t.net/addgene:101223 ; RRID:Addgene_101223) (Szczesny et al., 2018).

Miniprep

We followed the Promega PureYield plasmid miniprep system (promega, 2024). Except for every centrifugation step with less than 1 minute duration, we used 1 minute instead. We centrifuge for 6 minutes instead of 3 minutes at step 4.

Cell culture

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (gibco) in T75 CELLSTAR cell culture flasks at 37°C, with 5% CO_2 in a humidified Thermo Scientific Heracell 150i CO_2 incubator.

Transfection

We strictly follow the *lipofectamine 3000 reagent protocol* on a 6-well Falcon plate. We seeded HEK293T cells into a Falcon Multiwell 6-well plate at the concentration of 1500000 cells per well and then waited 24-48 hours for the cells to reach 70 percent confluency. pHR-FUSN-mCh-Cry2WT was a gift from Clifford Brangwynne (Addgene plasmid # 101223 ; http://n2t.net/addgene:101223 ; RRID:Addgene_101223) (Shin et al., 2017).

Live cell imaging

Live cell imaging was completed using the BioTek Cytation 5 cell imaging multimode reader. We observed cells using a 60x objective. The blue light filter is an EGFP filter cube, EX 469 nm, EM 525 nm. When observing cells, turning off the LED light creates a complete dark environment inside the cytation 5.

References

Fahrner, R. L., Knudsen, H. L., Basey, C. D., Galan, W., Feuerhelm, D., Vanderlaan, M., & Blank, G. S. (2001). Industrial Purification of Pharmaceutical Antibodies: Development, Operation, and Validation of Chromatography Processes. *Biotechnology and Genetic Engineering Reviews*, *18*(1), 301–327. https://doi.org/10.1080/02648725.2001.10648017

Graille, M., Stura, E. A., Corper, A. L., Sutton, B. J., Taussig, M. J., Charbonnier, J.-B., & Silverman, G. J. (2000). Crystal structure of a *Staphylococcus aureus* protein A domain complexed with the Fab fragment of a human IgM antibody: Structural basis for recognition of B-cell receptors and superantigen activity. *Proceedings of the National Academy of Sciences*, *97*(10), 5399–5404. https://doi.org/10.1073/pnas.97.10.5399

Kelley, B. (2009). Industrialization of mAb production technology: The bioprocessing industry at a crossroads. *mAbs*, *1*(5), 443–452. https://doi.org/10.4161/mabs.1.5.9448

Liu, H. F., Ma, J., Winter, C., & Bayer, R. (2010). Recovery and purification process development for monoclonal antibody production. *mAbs*, *2*(5), 480–499. https://doi.org/10.4161/mabs.2.5.12645

McNulty, M. J., Berliner, A. J., Negulescu, P. G., McKee, L., Hart, O., Yates, K., Arkin, A. P., Nandi, S., & McDonald, K. A. (2021). Evaluating the Cost of Pharmaceutical Purification for a

Long-Duration Space Exploration Medical Foundry. *Frontiers in Microbiology*, *12*, 700863. https://doi.org/10.3389/fmicb.2021.700863

Mullard, A. (2021). FDA approves 100th monoclonal antibody product. *Nature Reviews Drug Discovery*, *20*(7), 491–495. https://doi.org/10.1038/d41573-021-00079-7

Shin, Y., Berry, J., Pannucci, N., Haataja, M. P., Toettcher, J. E., & Brangwynne, C. P. (2017). Spatiotemporal Control of Intracellular Phase Transitions Using Light-Activated optoDroplets. *Cell*, *168*(1–2), 159-171.e14. https://doi.org/10.1016/j.cell.2016.11.054

Szczesny, R. J., Kowalska, K., Klosowska-Kosicka, K., Chlebowski, A., Owczarek, E. P., Warkocki, Z., Kulinski, T. M., Adamska, D., Affek, K., Jedroszkowiak, A., Kotrys, A. V., Tomecki, R., Krawczyk, P. S., Borowski, L. S., & Dziembowski, A. (2018). Versatile approach for functional analysis of human proteins and efficient stable cell line generation using FLP-mediated recombination system. *PLOS ONE*, *13*(3), e0194887. https://doi.org/10.1371/journal.pone.0194887