

Biologically-inspired selective filters

by

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B.S., Harvey Mudd College, 2013

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Physics
2019

This thesis entitled:
Biologically-inspired selective filters
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Biologically-inspired selective filters

Thesis directed by Assistant Professor Loren Hough

Placeholder text for abstract.

Dedication

Nice dedication goes here.

Acknowledgements

Loren Hough Meredith Betterton Matt Glaser Noel Clark Franck Vernery
Kathryn Wall Eric Verbeke Nate Crossette Katie Rainey Scott Tilden Sophie Reskin Elena
Arroyo Taylor Moon Lu ??? Andrea Egan Nick Bax
Allison Holt Nabanita Das Jeffre Allen
Mike Stefferson Stephanie Bryant Sadhana Sharma Christopher Bowman Danielle Konetski
Benjamin Fairbanks Joseph Dragavon

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Chapter 1

Bound diffusion measurements

Following up on the principles of the bound-diffusion theory, we wanted to see whether we could measure bound diffusion in a biomaterial inspired by the nuclear pore complex. In order to do that, we made Nup-filled hydrogels and measured the diffusion of transport factors and inert proteins within them. We measured the diffusion in a few different ways: monitoring the concentration profile and total accumulation as the proteins diffused into the cell, and using fluorescence recovery after photobleaching (FRAP).

Fluorescence recovery after photobleaching (FRAP) relies on the redistribution of fluorophores after patterned photobleaching in order to determine their diffusion constant. After a small portion of the hydrogel is bleached, the bleached spot gradually exchanges with the non-bleached fluorophores outside, and the average fluorescence intensity within the bleached spot recovers. The recovery lifetime can be used to determine the fluorophore's diffusion constant, and the final recovered intensity as compared to the intensity outside the bleach spot can be used to determine the mobile fraction of fluorophore.

1.1 Experimental parameters

Our reaction-diffusion model of selectivity is controlled by a relatively small number of parameters. Ideally, we would like to vary all of these experimentally in order to verify the model's predictions. In reality, most are highly difficult to alter in a well-controlled way. Of the model's parameters, the contour length L_C of the tethered Nups is the simplest to control. Varying this

contour length will vary the bound diffusion coefficient D_B according to Eqn. ???. This section discusses L_C and the other experimental parameters used in our hydrogel nuclear pore mimics.

1.1.1 Nup contour length and valency

The most straightforward way to vary D_B is to change the contour length of the Nups that are anchored into the hydrogel. To that end, we compared hydrogels containing the constructs FSFG concat-1 and FSFG concat-2 (Sec. ??, Appendix F). These Nup fragments have $L_C = 50$ nm and 100 nm, respectively. Given the parameters described below, the bound diffusion constant should increase by roughly 40% from FSFG concat-1 to FSFG concat-2.

In addition to differing lengths, the FSFG concat-1 and concat-2 differ in their number of binding sites, with six and twelve respectively. In order to control for the change in binding valency, we tested FSFG concat-2 hydrogels with the same molar concentration of FG repeats as the FSFG concat-1 gels as well as testing gels with the same molar concentration of Nups.

1.1.2 Binding affinity of NTF2 and FSFG

Although bound diffusion is the key parameter from our reaction-diffusion model of selectivity, the kinetic parameters of off-rate k_{off} , on rate k_{on} , and dissociation constant $K_D = k_{\text{off}}/k_{\text{on}}$ are also important. These parameters are surprisingly difficult to measure, yielding values between 10 nM and 10 μM depending on the experimental conditions. We estimated the dissociation constant for NTF2 and FSFG concat-1 using isothermal titration calorimetry (ITC). The heat of injection was recorded as FSFG was titrated into a stock of NTF2. While the resulting titration curves had low signal-to-noise and did not reach saturation, they clearly indicated binding (Fig. 1.1). Simple fits are likely inaccurate, given the high degree of multivalent binding, but may provide an order-of-magnitude estimate of the affinity. Several ITC curves agree on a dissociation constant of $K_D \approx 200\mu\text{M}$. This is roughly compatible with the millimolar per-FSFG constant measured by the Rout lab with NMR and ITC [1]. Similarly weak values were predicted through NMR, simulation, and stopped-flow anisotropy [2].

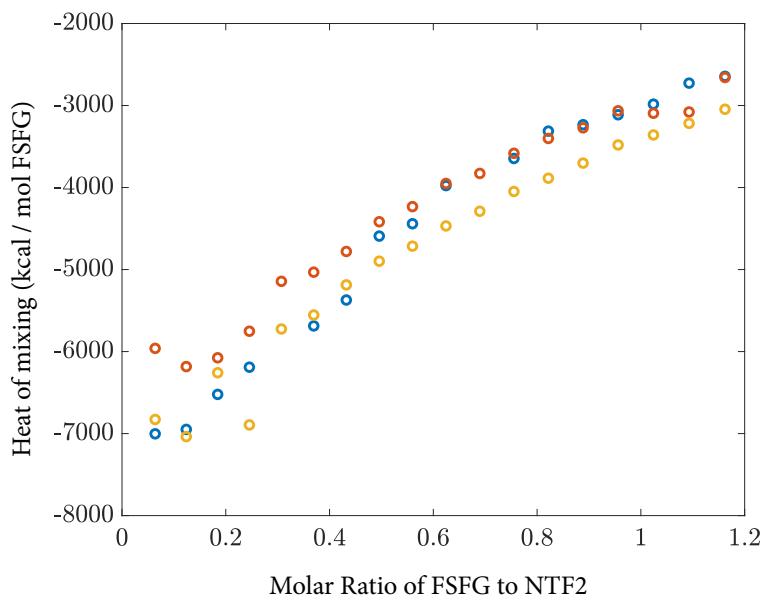


Figure 1.1: Isothermal calorimetry titration curve: heat of injection vs. molar ratio of FSFG to NTF2. Fits are questionable but multiple runs suggest $K_D \approx 200 \mu\text{M}$.

Due to the twin difficulties of varying K_D in a controlled way and accurately measuring its value, we did not attempt to experimentally alter this parameter. Ideally, a transport factor - Nup pair with $K_D \approx 1\mu\text{M}$ would have been used to maximize selectivity. For the purposes of determining the bound diffusion constant, the only value that is necessary to measure is the ratio K_D/N_T , which can be estimated using the partitioning of transport factors and inert proteins into the FSFG hydrogels (Sec. 1.3.2). We investigated using the ubiquitin-associated (UBA) domain of the mRNA exporter Mex67 as a Nup-binding domain in GFP fusion proteins. In principle, varying numbers of this small (<10 kDa) domain could be added to GFP to explore the effect of varying binding affinity and valency in transport factors. The constructs GFP-UBA, GFP-UBAx2, and GFP-UBAx3 were created by Eric Verbeke, but did not express and/or bind well.

1.1.3 Quantifying concentration of tethered Nups

Another potentially-tunable parameter of the bound-diffusion model is N_T , the total concentration of tethered Nups. It is straightforward to control the Nup concentration in the hydrogel precursor solution by resuspending a known mass of lyophilized protein. Nup concentrations up to ~ 50 mg/mL can be resuspended. However, it is much more difficult to determine how much protein was tethered to the hydrogel upon crosslinking.

BCA protein quantitation assays were used to place upper bounds on N_T . Two methods were attempted: incubating the hydrogel itself in the working reagent, and soaking the hydrogel in a known volume of buffer and testing the concentration of FSFG released. When applying the first method, the hydrogel was first soaked to remove excess precursor solution and thoroughly rinsed. The gel was placed into a 96-well plate and buffer added until the appropriate sample volume was reached. A standard BCA protocol was then followed. Upon incubation with the working reagent, the hydrogels turned purple, as expected. Standard absorption measurements and processing yielded an estimate of 0.5 mg/mL tethered FSFG-concat 1; this should be taken as an approximate value only. The second method, that of soaking hydrogels and measuring the FSFG released, placed a similarly-low upper bound on tethered FSFG concentration. Hydrogels

made with 5 μL of precursor solution were soaked in 45 μL buffer to equilibrate. The buffer was then measured to have a concentration of 1.0 mg/mL FSFG, implying a tethered FSFG concentration of < 1 mg/mL.

The concentration of Nups within the pore may reach 100 mg/mL [?]. The low concentration of tethered Nups that we were able to achieve is therefore a major barrier to selectivity. It is likely that the disordered nature of FSFG makes the labeled end less accessible to the hydrogel scaffold than would be the case for an ordered protein. In an effort to overcome this limitation, we tested other linkers and conjugation methods. We conjugated the FSFG-cys to PEG-diacrylate of varying lengths (700 Da and 10 kDa), to multi-armed PEG-diacrylate, and to maleimide-PEG-acrylate. While labeling was verified using Ellman's reagent (Appendix D), there was no noticeable difference in transport factor partitioning into these hydrogels.

1.1.4 Free diffusion constant

The final tunable parameter from the binding-diffusion model is the diffusion constant of the transport factor when it is not bound to a Nup, the free diffusion constant D_F . Decreasing D_F is predicted to increase a material's selectivity while decreasing the absolute flux of transport factor (Figs. ??, ??). The free diffusion is predominantly determined by the protein's size and the viscosity of the solution, according to the Stokes-Einstein equation $D = k_B T / 6\pi\eta R$, where $k_B T$ is the thermal energy, η the solution viscosity, and R the particle radius. The solution viscosity could potentially be increased using a viscous additive such as glycerol; however, these attempts appeared to interfere with the binding of NTF2 and FSFG.

The diffusion of the non-binding 30 kDa protein mCherry was used as a proxy for the free diffusion of similarly-sized NTF2 within the FSFG hydrogels.

1.2 Experimental procedures

The nuclear pore mimics in this dataset were designed with the lessons of Chapter ?? in mind. They consist of hydrogels with the largest average pore size and simplest geometry: a single

microliter-scale droplet. While this geometry does not allow for direct measurements of selectivity, the in-gel diffusion constants of both NTF2 and mCherry can be determined and bound diffusion calculated. Two types of experiments were carried out: influx experiments, in which the entry of fluorescent proteins into the hydrogels was monitored, and FRAP experiments, in which a portion of an equilibrated hydrogel was bleached and the recovery observed. Both experiments provide a measurement of the proteins' diffusion constants. These experiments were performed at CU's BioFrontiers Advanced Light Microscopy Core Facility. Thanks go to Joseph Dragavon for much assistance with the microscopy.

1.2.1 Hydrogel preparation

Detailed precursor solution recipes are given in Appendix E. For the dataset analyzed in this chapter, all nuclear pore mimic hydrogels were 6% acrylamide and contained 2 mM LAP. The precursor was made with potassium transport buffer (PTB) and contained either FSFG concat-1 bis or FSFG concat-2 bis. Lyophilized FSFG-bis was resuspended in PTB, allowed to sit at room temperature for at least 20 minutes, and added to the precursor solution. After the precursor solution was thoroughly mixed, it was degassed in a vacuum desiccator for 10 minutes and immediately pipetted into a disassembled 400- μm -thick PDMS gasket chamber (Sec. ??). Drops between 0.5 and 2 μL were carefully pipetted onto the plastic slide and the chamber assembled around the drops. Typically, each chamber measured a few centimeters on a side and contained a control gel with no Nups as well as one or more Nup-filled gels. The chamber was then illuminated as uniformly as possible with 365 nm light at approximately 200 mW/cm² with a ThorLabs M365 LP1 LED. Condensation around the gels indicated that they had crosslinked. The chamber was immediately rinsed with at least 100 μL of PTB, filled with fresh PTB, and sealed with a PDMS slab and clingwrap. The gels were left to soak at 4°C for at least 12 hours so that any remaining precursor solution and protein could leave the gel.

1.2.2 Influx of transport factor and inert protein

After soaking in buffer, the buffer solution was removed by pipette or wicking with a Kimwipe and a fluorescent reservoir solution added. Aspirating the chamber was avoided if possible, as it sometimes disturbed the seal between the gel and the chamber. The reservoir solution contained 20 μM each freshly-thawed NTF2-fluorescein (NTF2-F) and mCherry in PTB. The chamber was resealed after adding the solution. If no influx experiment was planned, the gels were then allowed to equilibrate for 24 hours before FRAP was performed.

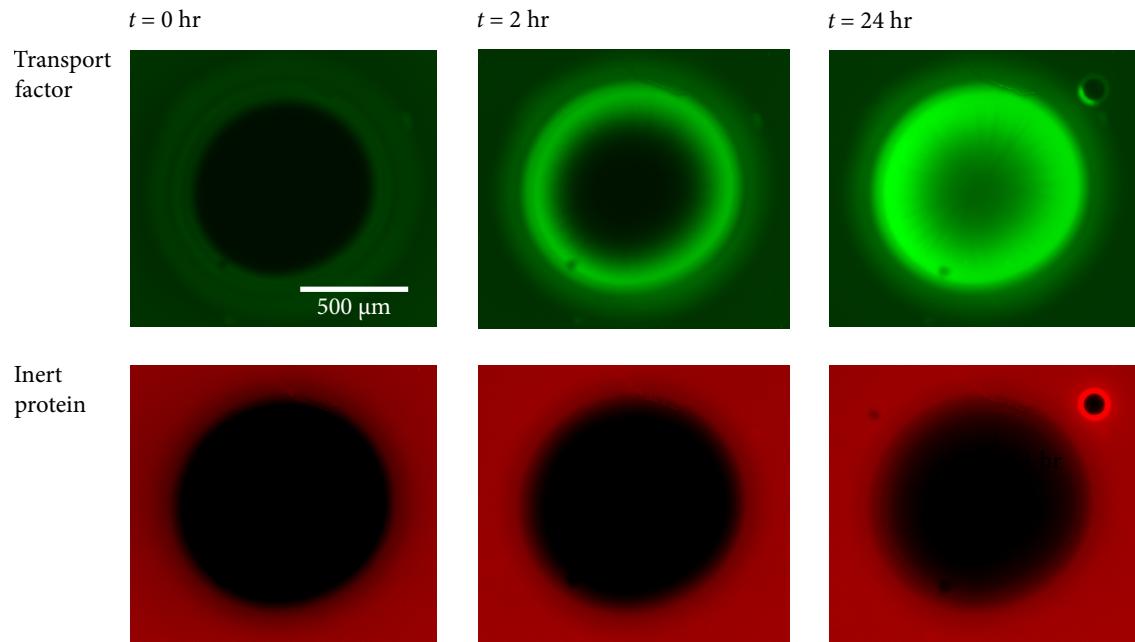
When influx experiments were run, they began as soon as possible after loading the reservoir solution. Videos were recorded at 4x magnification on an Olympus IX-81 widefield microscope using FITC (excitation 464-500 nm / emission 516-556 nm) and TRITC (excitation 532-544 nm / emission 573-613 nm) filter cubes. Exposure times were typically 30-100 ms with 3-8 dB gain. Videos consisted of 120 frames at one frame per minute. Minimal photobleaching took place over the course of these experiments. After the influx experiment, the chamber was usually stored for equilibration 4°C, protected from light, and a FRAP experiment performed the following day.

Influx experiments on hydrogels containing FSFG show binding of NTF2-F to mCherry (Fig. 1.2). A bright wavefront of NTF2-F slowly progresses into the gel, while mCherry remains largely excluded. As expected, mCherry equilibrates more rapidly than does NTF2-F, due to the lack of binding. Influx experiments were analyzed as described in Sec. 1.4.

1.2.3 Fluorescence recovery after photobleaching

FRAP is typically performed using a confocal microscope, but we were able to bleach the hydrogels using an Olympus IX-81 widefield microscope. The widefield was preferable because the greater depth of field allowed for thicker hydrogel samples, which were easier to fabricate and manipulate. As described above, the hydrogel was first allowed to equilibrate with 20 μM NTF2-F and mCherry in PTB. A reference image was taken at 4x magnification in both fluorescence channels. A circular region of the hydrogel around 300 μm (?? check this) in radius was then

Figure 1.2: Influx image series. A hydrogel containing a nominal 5 mg/mL FSFG concat-2 was challenged with 20 μ M NTF2-F and mCherry. The hydrogel shows selective entry of NTF2-F and has largely equilibrated within 24 hours.



photobleached by taking a five-second exposure at 40x magnification using a DAPI (excitation 352-402 nm / emission 417-477 nm) filter cube. Following the bleach, the 4x objective was rapidly returned and a time series recorded. Typical series consisted of 15-30 frames taken as rapidly as possible (5-10 s per frame), followed by 30-60 frames taken at a slower rate (1-2 minutes per frame). Total experiment time was 1-4 hours. Typical exposure times were 10 ms for NTF2-F and 40 ms for mCherry, both with a gain of 3 dB.

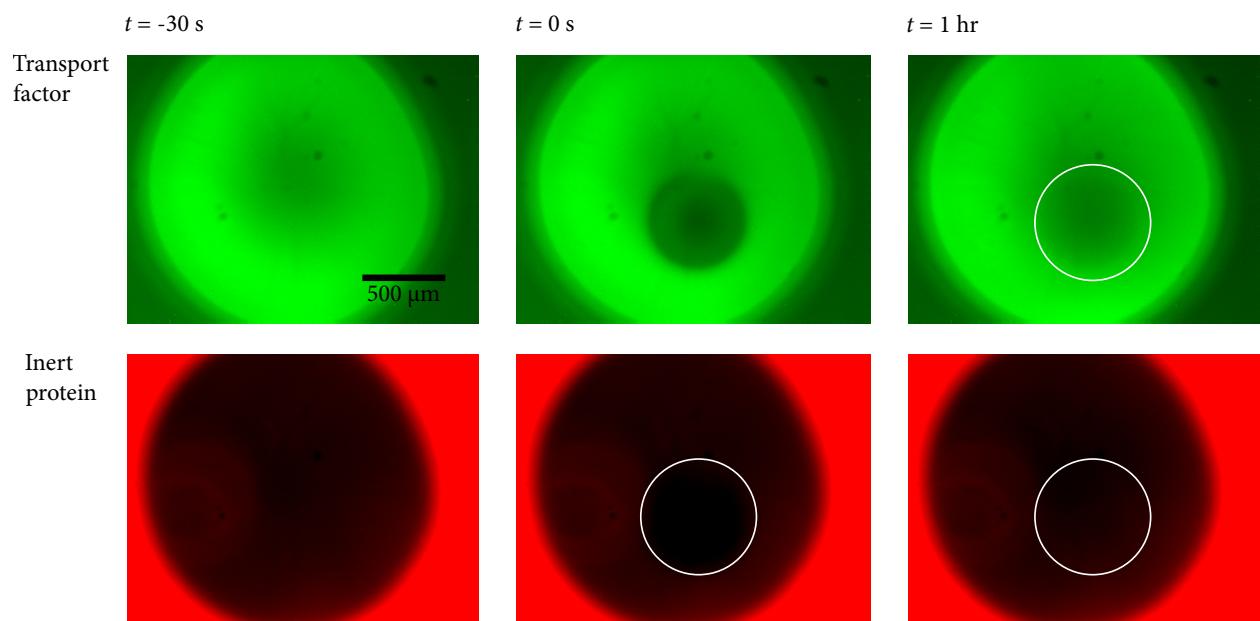
In order to increase throughput, up to three FRAP experiments were run concurrently by sequentially bleaching and then imaging several locations within a single chamber. This led to a maximum delay time of approximately 35 s between the end of the bleach segment and the beginning of the time series. Delay times were recorded and taken into account in the data processing. Figure 1.3 shows a representative series of images pre- and post-FRAP for both NTF2-F and mCherry.

Despite allowing 24 hours for equilibration, the fluorescence intensity across the hydrogel was not always uniform at the beginning of a FRAP experiment. This could be due to slow diffusion into the hydrogels, or to inhomogeneous crosslinking. The center of a hydrogel is more likely to be tightly crosslinked than the edges, as swelling is most inhibited at the center. Gels which were too inhomogenous to display a clear bleach spot were discarded, but many nonuniform hydrogels were used in the final dataset, with the lack of equilibrium addressed in the data analysis (Sec. 1.5). Smaller hydrogels ($0.5 \mu\text{L}$ of precursor solution) equilibrated more readily, at the cost of increasing the effect of fluorescent protein exchange with the reservoir, since the bleach spot then covered a significant fraction of the hydrogel. This effect was also taken into account during data analysis.

1.3 Steady-state hydrogel properties

Both the influx and FRAP experiments rely on steady-state properties of the hydrogel as well as time-dependent ones. These properties include the partition coefficients of transport factors and inert proteins, as well as some dependent on the geometry of the hydrogel.

Figure 1.3: FRAP image series. NTF2-FITC (top, green) and mCherry (bottom, red) bleaching and recovery shown separately. Hydrogel contained...



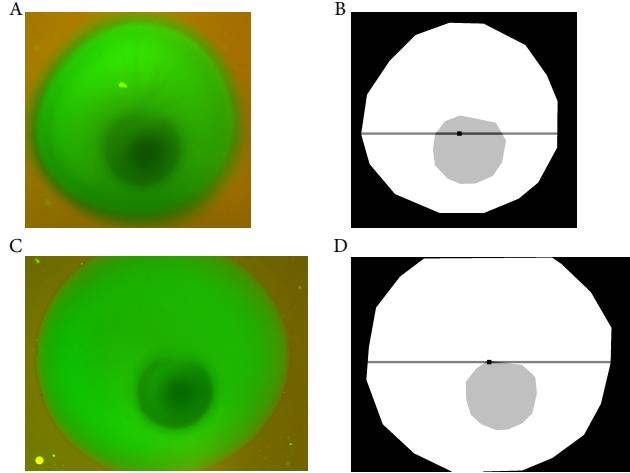


Figure 1.4: Sample hydrogel mask with radius and center calculations. (A) An equilibrated hydrogel containing FSFG concat-1, immediately post-bleach. (B) The corresponding hydrogel mask (white), bleach spot mask (light gray), calculated gel diameter, and calculated gel center.

1.3.1 Gel dimension estimates

Although the hydrogels were not perfectly circular, all analysis treated them as circular or nearly so. In total, the analysis made use of a gel's radius, center, perimeter, and area. I began by manually defining two masks: one that covered the entire gel, and one that covered only the bleach spot (Fig. 1.4). The gel area was calculated by summing all of the pixels in the gel mask and, where necessary, making use of the $1.58 \mu\text{m}$ per pixel scale of the Olympus 4x objective. The perimeter was calculated using Matlab's `bwperim` function, which takes a binary mask and returns a mask whose only nonzero entries are that mask's perimeter. Summing over these pixels and scaling provides the gel perimeter. It should be noted that in some cases the full area of the gel was not within the field of view. In these cases, sometimes the partial area in the field of view was used as the area estimate, and sometimes I embedded the gel image in a larger frame and estimated the remaining area when drawing the mask. Following sections indicate which method was used and the mathematical reasoning. However, all perimeter calculations were performed with the estimated full area.

The hydrogel radius was estimated taking the diameter to be the widest row of the gel mask. The widest row also set the y-coordinate of the gel center, with the x-coordinate calculated to be midway along the non-zero values for that row. As seen in Fig. 1.4, this is a quick and relatively crude method, but in almost all cases it works reasonably well. An advantage of this method is

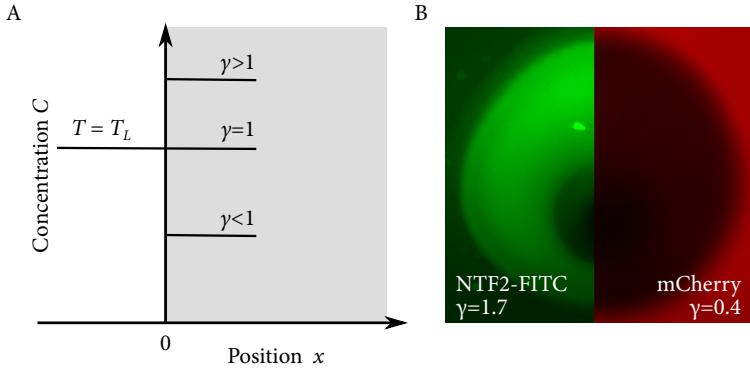


Figure 1.5: Partitioning of NTF2 and mCherry into FSFG hydrogel. (A) Partition coefficient γ depends on whether the hydrogel excludes or binds protein. (B) Equilibrated FSFG concat-1 hydrogel (nominally 10 mg/mL) showing partitioning of $T_L = 20 \mu\text{M}$ each NTF2-FITC and mCherry. Contrast adjusted for ease of viewing.

that it works even for hydrogels which do not fit in the vertical field of view.

1.3.2 Partition coefficients and fraction of time spent bound

The concentration of NTF2 and mCherry in a flow chamber's reservoir can be directly controlled, but the partitioning of a protein into the hydrogel depends on the degree to which the presence of the gel sterically excludes the protein, as well as on binding interactions between the gel and protein. When a transport-factor-sized inert protein is present to control for the steric effects, information about the transport factor's dissociation constant and fraction of time spent bound can be extracted from knowledge of the partition coefficient γ . In particular, p_B , the fraction of time spent bound, is necessary in order to calculate the bound diffusion coefficient.

As shown in Fig. 1.5A, the partition coefficient is the ratio of a protein's concentration in a well-equilibrated hydrogel to that in the surrounding reservoir. This quantity is calculated by dividing the average intensity of the gel by that of the reservoir within the field of view (Fig. 1.5B). If the gel is not fully equilibrated, the partition coefficient can be estimated using a line scan through the reservoir and gel, though this will likely underestimate the true value.

When the system is in chemical equilibrium, the concentration of free transport factor (T), free Nup (N), and transport factor - Nup complex (C) is related to the dissociation constant K_D by $K_D = NT/C \approx N_T T/C$ in the linear approximation $N \approx N_T$. The total tethered Nup concentration, both free and bound, is the constant N_T . The fraction of transport factors that are

bound is then given by

$$p_B = \frac{C}{C+T} = \frac{C}{C + \frac{CK_D}{N_T}} = \frac{1}{1 + \frac{K_D}{N_T}} \quad (1.1)$$

To relate this expression to measurable quantities, write the protein concentrations within the hydrogel in terms of their partition coefficients. The concentration c_0 of the inert protein and the transport factor is equal in the reservoir. If γ_T is the partition coefficient of the transport factor and γ_I that of the inert protein, then the transport factor concentrations can be expressed as

$$T = \gamma_I c_0 \quad (1.2)$$

$$C = T_T - T = \gamma_T c_0 - \gamma_I c_0 \quad (1.3)$$

The total transport factor concentration within the gel is $T_T = T + C$ and is a constant. Therefore, within the gel, the chemical equilibrium condition can be expressed as

$$\frac{K_D}{N_T} = \frac{T}{C} = \frac{\gamma_I c_0}{\gamma_T c_0 - \gamma_I c_0} = \frac{\gamma_I}{\gamma_T - \gamma_I} \quad (1.4)$$

Combining Eqns. 1.1 and 1.4, the bound probability can be expressed in terms of the partition coefficients as

$$p_B = \frac{1}{1 + \frac{K_D}{N_T}} = \frac{1}{1 + \frac{\gamma_I}{\gamma_T - \gamma_I}} = 1 - \frac{\gamma_I}{\gamma_T} \quad (1.5)$$

1.3.3 Bound-diffusion calculation

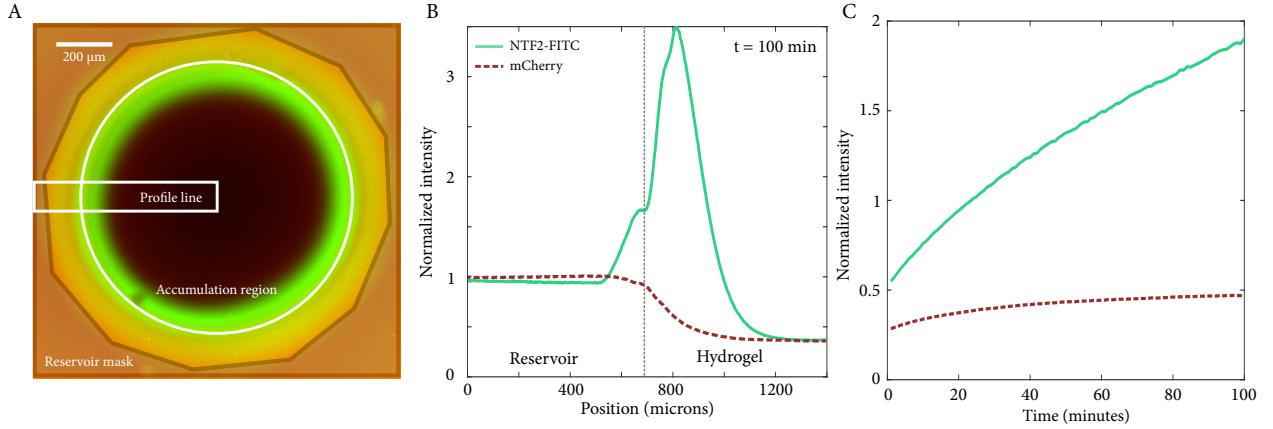
Once the observed diffusion constants for NTF2 and mCherry have been calculated, along with the fraction of time spent bound p_B , the bound diffusion is given straightforwardly by the weighted average

$$D_{\text{obs,TF}} = p_B D_B + (1 - p_B) D_F \quad (1.6)$$

This result assumes Fickian diffusion. In reality the diffusion will be slightly anomalous due to binding and the presence of the hydrogel. However, the binding is highly transient and the hydrogel relatively permeable to proteins of the size of NTF2 and mCherry (Sec. ??).

Taking the free diffusion coefficient of the transport factor to be approximately equal to the observed diffusion of the inert protein ($D_F = D_{\text{obs,I}}$), the bound diffusion coefficient of the transport

Figure 1.6: Intensity profiles and total accumulation within hydrogel as determined from influx time series. (A) Image of an FSFG concat-2 hydrogel with a nominal Nup concentration of 10 mg/mL. Reservoir contains 20 μ M NTF2-F and mCherry in PTB. Reservoir mask is shown (red polygon) as well as accumulation area and rectangle over which the profile averaging was performed. (B) Normalized intensity profiles as calculated using the box in (A). (C) Normalized average intensity within hydrogel.



factor is

$$D_B = \frac{D_{\text{obs,TF}} - (1 - p_B)D_{\text{obs,I}}}{p_B} \quad (1.7)$$

Note that neither the dissociation constant or the total Nup concentration need to be measured independently in order to calculate the bound diffusion constant.

1.4 Influx analysis

After recording a time series of fluorescent protein influx into a hydrogel, diffusion constants can be estimated using either a plot of the intensity profile across the gel or a plot of total accumulation within the gel. Figure 1.6 shows an example of each plot. The intensity within the gel is normalized to the average intensity of the reservoir, as calculated using the mask shown in Fig 1.6A. Therefore, each profile trace begins at $I = 1$ within the reservoir and either dips or rises to the partition coefficient value within the gel. As the gels are not equilibrated, much of the gel interior shows only background fluorescence. Likewise, the average intensity within the hydrogel trends towards the partition coefficient but does not reach it on the time scale of an average experiment.

Analysis of the accumulation and profile curves used the diffusion-equation solutions described

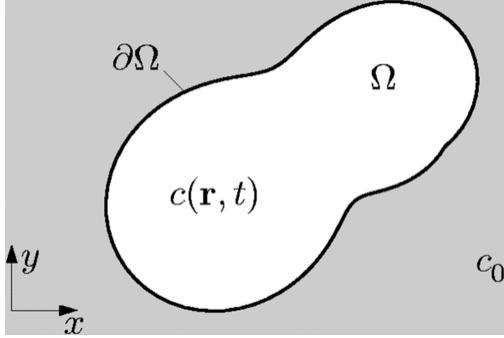


Figure 1.7: Geometry used to solve diffusion equation in [3]. The reservoir concentration c_0 fixed the concentration at the edge of the region Ω . The concentration $c(\mathbf{r}, t)$ is determined within Ω as a function of position and time.

by Mortensen, Okkels, and Bruus [3]. This method assumes that the edge of a nearly-circular two-dimensional region is held at a fixed concentration while the interior equilibrates (Fig. 1.7). Such boundary conditions correspond to an infinite fluorophore reservoir. Given that our reservoirs are 10-20 times larger than the hydrogel volume, this is a reasonable assumption. In a slight modification, we allow for an arbitrary partition coefficient γ by taking the fixed boundary concentration c_0 to be γT_0 , where T_0 is the fluorescent protein intensity in the reservoir. The assumption of near-circularity is also a good one.

The two most troublesome restrictions of the Mortensen equations are Fickian diffusion and the homogeneity of the hydrogel. As discussed in Sec. ??, the presence of the hydrogel meshwork and binding make diffusion within the hydrogels slightly anomalous. Additionally, despite the improvements to the fabrication protocol, the hydrogels likely contain some degree of nonuniformity in their density, with the centers most likely being slightly denser than the edges due to differential swelling. The effects of swelling and non-uniform thickness are greatest at the gel edge. Unfortunately, the points at the edge of the gel and earliest in the experiment are the most important to the following fits. As a result, the diffusion constants extracted using the profile and accumulation plots are not reliable. The analysis is presented below nonetheless, as a similar approach was taken in analyzing the FRAP experiments.

1.4.1 Profile analysis

Mortensen *et al* first define a characteristic timescale for equilibration $\tau = (\mathcal{A}/\mathcal{P})^2(\pi/4D)$ where \mathcal{A} and \mathcal{P} are the area and perimeter, respectively, of Ω . For a circle $\mathcal{A}/\mathcal{P} = a/2$, but this

ratio was numerically calculated for the hydrogels (Sec. 1.3.1). For times $t \ll \tau$, the concentration profile as a function of the distance r from the gel center can be approximated as

$$c(r, t) = c_0 \operatorname{erfc} \left(\frac{r}{\sqrt{4Dt}} \right) \quad (1.8)$$

where D is the diffusion constant.

The typical duration of an influx experiment was approximately τ , making the short-time approximation questionable throughout most of the time series. We attempted to fit the intensity profile (Fig. 1.8) and obtained fits with fairly low error but with unreliable fit parameters. In addition to the issues of timescale, it was often difficult to determine where the gel edge was located, as well as the partition coefficient. Given these problems, it is not surprising that fits at successive timepoints yielded incompatible values of diffusion constant for both mCherry and NTF2. A similar problem prevented the use of diffusion constants obtained from intensity fits at a fixed position over time (Fig. 1.9).

A more exact solution for $c(r, t)$ can be written if the gel is assumed to be circular. Mortensen *et al* quantify the error introduced by small deviations from circularity and find it to be small. Assuming a circular gel, the concentration profile at an arbitrary time t is given by

$$\frac{c(r, t)}{c_0} = 1 - 2 \sum_{n=0}^{\infty} \frac{J_0(\alpha_n r)}{\alpha_n a J_1(\alpha_n a)} \exp(-\alpha_n D t) \quad (1.9)$$

where a is the gel radius and $\alpha_n a$ is the n th zero of the Bessel function of the first kind J_0 .

I fit the intensity profiles to Eqn. 1.9 using different numbers of terms N . For the mCherry curves, the resulting fit parameters tended to converge as N increased, and the RMSE stabilized. However, neither was true for the NTF2 curves. Increasing values of N led to steadily decreasing values of diffusion constant. This may be due to the effect of binding on NTF2 diffusion.

1.4.2 Accumulation analysis

The total accumulation within the hydrogel can be modeled by integrating the concentration profile over the entire gel. When this is done, the averaged intensity within the hydrogel $N(t)$ is

Figure 1.8: Fits to Eqn. 1.8 near the beginning of the influx experiment for (A) mCherry and (B) NTF2-Alexa488 intensity profiles. Hydrogel nominally contains 10 mg/mL FSFG-PEGDA 700 kDa. The entire intensity profile is shown in the top panel with the portion used for fitting highlighted in red. Approximate locations of the gel edge, background fluorescence level, and partition coefficient are also shown.

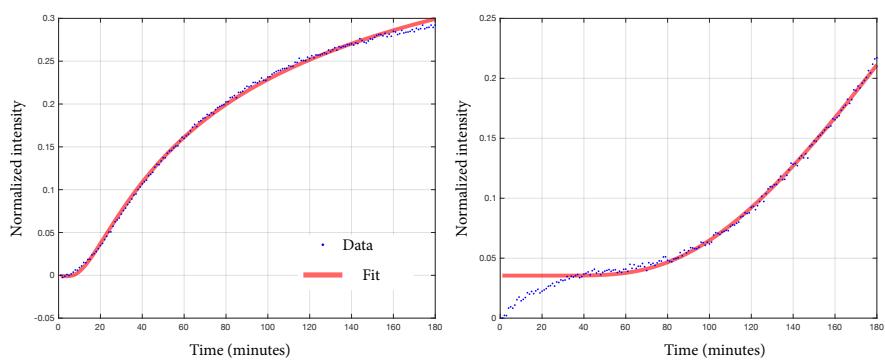
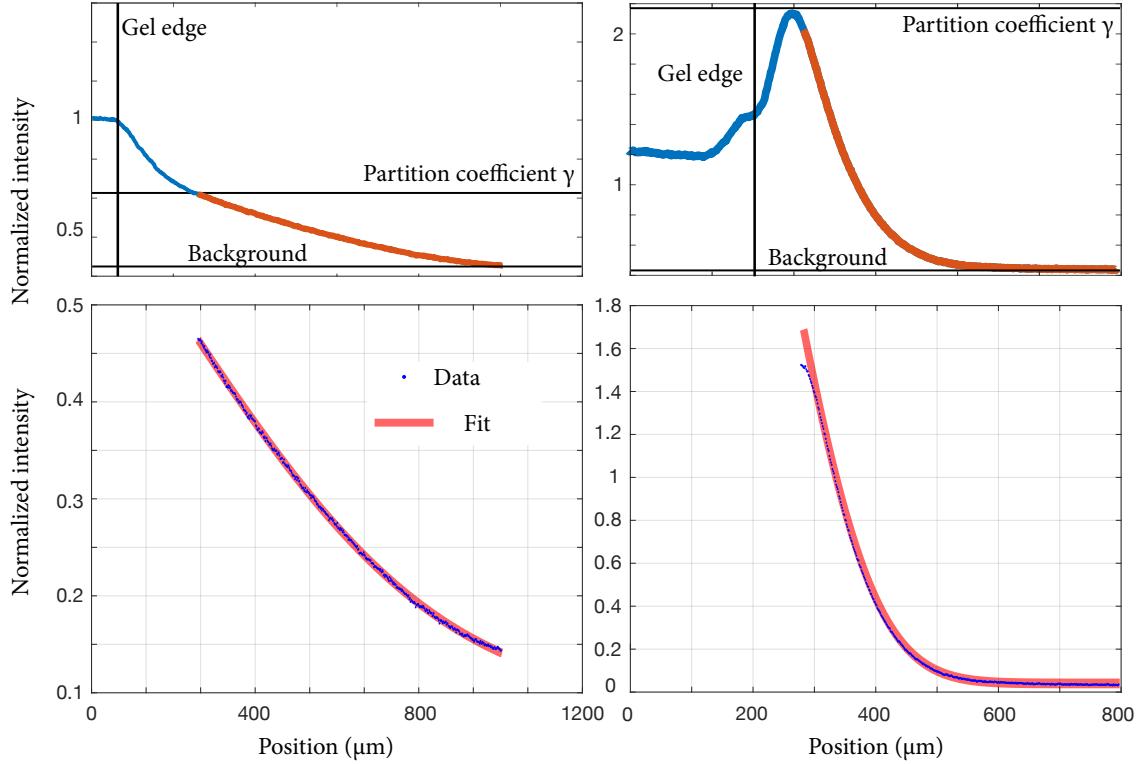


Figure 1.9: Fits to Eqn. 1.8 at a fixed point near the gel edge for (A) mCherry and (B) NTF2-Alexa488 intensity profiles. The short-time approximation likely only holds for times $t \lesssim 20$ minutes. Hydrogel nominally contains 10 mg/mL FSFG-PEGDA 700 kDa.

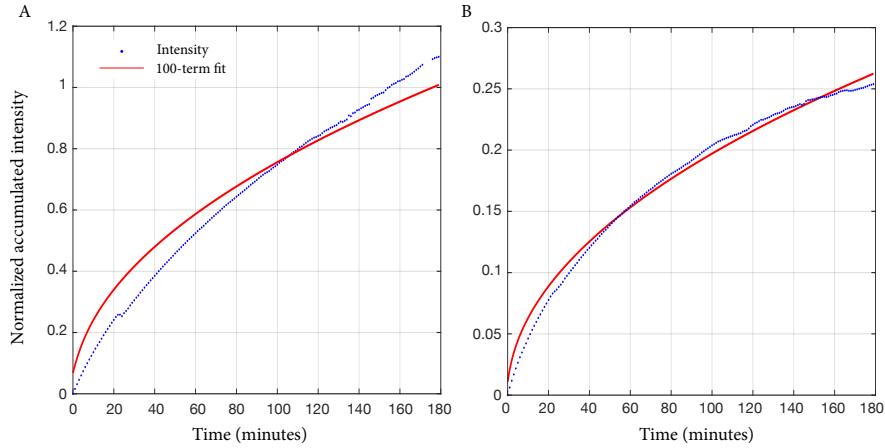


Figure 1.10: Accumulation fits to Eqn. 1.10 for (A) mCherry and (B) NTF2-Alexa488. Hydrogel nominally contains 10 mg/mL FSFG-PEGDA 700 kDa.

found to be

$$\frac{N(t)}{N_0} = 1 - \sum_{n=0}^{\infty} \frac{4}{(\alpha_n a)^2} \exp\left(-\frac{(\alpha_n a)^2 \pi t}{16\tau}\right) \quad (1.10)$$

where N_0 is the equilibrium value, here equal to the partition coefficient γ once the intensity has been normalized to that of the reservoir.

Fits to the first 100 terms of Eqn. 1.10 are shown in Fig. 1.10 for mCherry and NTF2. These fits had systematic error, likely for the same reasons as the profile fits.

While the profile and accumulation data do contain information on the diffusion constants of NTF2 and mCherry, I was unable to reliably extract that information using the influx experiments. The model presented above is a remarkably good match to the experimental setup except for the presence of binding and the unpredictable gel-edge effects. The FRAP experiments overcome the problem of edge effects by making use of equilibrated regions deep within the hydrogel.

1.5 FRAP analysis

1.5.1 Accounting for photobleaching

Small but noticeable amounts of photobleaching occur over the course of the FRAP experiment. In order to correct for photobleaching, the intensity of the bleached spot must be normalized to that of the entire gel, including the bleached region. The normalized intensity used to fit the

recovery curves is given by

$$N(t) = \frac{c_b(t)}{c_g(t)} \quad (1.11)$$

where the average intensity of the bleach spot is $c_b(t) = C_b(t)/A_b$. The total intensity within the bleach spot is $C_b(t)$ and A_b is the area of the spot as shown in Fig. 1.4. The average intensity of the entire gel $c_g(t)$ is defined similarly. Using this normalization removes the effects of photobleaching, as verified by simulating recovery data with various photobleaching rates.

1.5.2 No-exchange solution to diffusion equation

The simplest realistic model of fluorescence recovery assumes that the hydrogel is perfectly uniform and equilibrated, that the presence of the gel is unimportant, and that there is no exchange of fluorescent proteins between the hydrogel and the reservoir during the experiment. Upon making those assumptions, the recovery curve can be fit using a sum of two Bessel functions as described in [4,5]. The model used in [5] distinguishes between binding affinity regimes; NTF2-FSFG binding is sufficiently transient that it falls into the effective diffusion regime, allowing standard diffusion equations to be used without modification to calculate an effective diffusion constant as described in Sec. 1.3.3. The solution to fluorescence recovery with effective diffusion is given by

$$N(t) = A \exp(-\tau_D/2t) (I_0(\tau_D/2t) + I_1(\tau_D/2t)) + C \quad (1.12)$$

where the diffusion lifetime τ_D is related to the diffusion constant by $D = w^2/\tau_D$ if w is the bleach spot radius.

The amplitude A and offset C are related to the bleach depth (given by C) and final recovered value (given by $A + C$). Typical bleach depths were 5-10% of the initial intensity and tended to be smaller for mCherry than for NTF2. In principle, the final recovered value reflects the immobile fraction of fluorophore. If there is an immobile fraction, the bleached region will not recover to its initial value. Given the weak binding between NTF2 and FSFG, we do not expect an appreciable immobile fraction of either NTF2 or mCherry. For the most part, the hydrogels appear

to recover to their initial value. A few do not, for reasons that are unclear even after accounting for photobleaching.

The no-exchange model fits well to the larger hydrogels and those that are well-equilibrated. Many of the large gels, however, were not fully equilibrated even after a 24 hour incubation with the reservoir solution. The concentration within these gels will change over the course of the experiment as they continue to equilibrate. Smaller hydrogels were quicker to uniformly equilibrate; however, these gels are small enough that exchange of fluorescent proteins with the reservoir is important over the timescale of FRAP recovery. Both of these problems can be accounted for using a more thorough solution to the diffusion equation.

1.5.3 Fourier transform solution to diffusion equation

To account for exchange of fluorescent proteins with the reservoir, as well as for incomplete equilibration, some of the assumptions of the previous section must be relaxed. In particular, the assumption of radial symmetry must be abandoned. In order to incorporate angular as well as radial dependence, the solution must be written as a sum over Bessel function and cosine modes. While such a solution is cumbersome, it allows for an accurate description of the concentration throughout the hydrogel immediately post-bleach. Additionally, the boundary condition can be defined so as to allow exchange between the hydrogel and reservoir.

This analysis begins by numerically calculating the two-dimensional polar Fourier transform of the initial concentration distribution within the gel, decomposing it into a finite number of modes weighted by their contribution to the image. These mode coefficients are then used to create the equation to which the experimentally-measured recovery curve is fit. The fit parameters include the observed diffusion coefficients for NTF2 and mCherry, allowing the bound diffusion constant to be determined as described in Sec. 1.3.3.

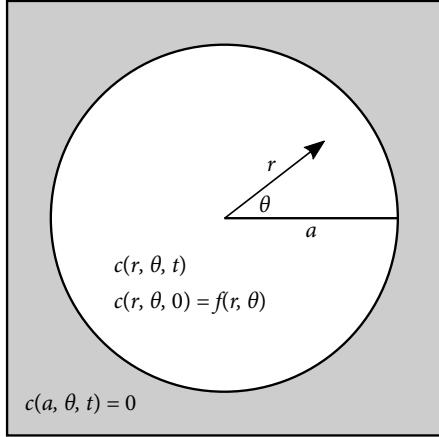


Figure 1.11: Geometry for diffusion-equation solution Eqn. ???. A circle of radius a is centered at the origin. The boundary $r = a$ is held at concentration $c = 0$ and the interior has initial concentration profile $f(r, \theta)$.

1.5.3.1 Analytic series solution

The problem is that of diffusion in a circular region of radius a , centered at the origin, whose boundary is held at a fixed concentration. The region $r < a$ has an arbitrary initial concentration distribution $f(r, \theta)$ which consists of the first post-bleach image (Fig. 1.11). To find the concentration $c(r, \theta, t)$ for $r < a$, we used the Green's function integrals described in [?], as heat transfer obeys the same differential equation as diffusion of particles. Green's functions describe a system's response to an instantaneous point source. An initial distribution can be built by integrating the Green's function over the region of interest. The correct Green's function must be identified for a particular geometry and set of boundary conditions, but a formal solution is straightforward to write once they have been obtained.

As this solution is two-dimensional, two sums must be taken over the basis functions. Bessel functions J_ν of the first kind and integer order ν are the radial basis, and cosines are the angular basis. The time dependence is carried by decaying exponentials whose time constant is related to the diffusion coefficient D as well as to the zeros of the Bessel functions. The terms are summed over the Bessel orders as well as over the zeros of each Bessel function. For our situation, the full

solution to the diffusion equation within the hydrogel is given by [?]

$$c(r, \theta, t) = \sum_{\nu=-\infty}^{\infty} \sum_{\alpha=0}^{\infty} \frac{\exp(-D\alpha^2 t) J_{\nu}(\alpha r)}{(J'_{\nu}(\alpha a))^2} \times b_{\nu, \alpha} \int_0^{2\pi} \int_0^a \cos(\nu(\theta - \theta')) J_{\nu}(\alpha r') f(r', \theta') r' dr' d\theta' \quad (1.13)$$

where, as in the previous section, αa are the zeros of J_{ν} , and $b_{\nu, \alpha}$ are normalization constants defined in Sec. 1.5.3.2. Note that the sums run over all positive and negative integer Bessel orders and over all zeros of each Bessel function.

This solution applies to a circle whose boundary is held at $c = 0$, so it must be shifted by an offset in order to match the experimental concentration at the boundary. Note that the relevant boundary concentration is just within the hydrogel, not the concentration of the reservoir. The reservoir is assumed to be infinite, in order to maintain the hydrogel edge at its equilibrium concentration, but the concentration of the reservoir itself is irrelevant.

The integral above is difficult to use as written because it contains both primed and unprimed coordinates in the angular term, $\cos(\nu(\theta - \theta'))$. The unprimed coordinate can be removed from the integral using the identity

$$\cos(\phi_1 - \phi_2) = \cos \phi_1 \cos \phi_2 + \sin \phi_1 \sin \phi_2$$

The mode coefficients $C_{\nu, \alpha}$ and $S_{\nu, \alpha}$ can then be defined as

$$c(r, \theta, t) = \sum_{\nu=-\infty}^{\infty} \sum_{\alpha=0}^{\infty} \frac{\exp(-D\alpha^2 t) J_{\nu}(\alpha r)}{(J'_{\nu}(\alpha a))^2} (C_{\nu, \alpha} \cos(\nu \theta) + S_{\nu, \alpha} \sin(\nu \theta)) \quad (1.14)$$

with

$$C_{n, \alpha} = b_{\nu, \alpha} \int_0^{2\pi} \int_0^a \cos(\nu \theta') J_{\nu}(\alpha r') f(r', \theta') r' dr' d\theta' \quad (1.15)$$

$$S_{n, \alpha} = b_{\nu, \alpha} \int_0^{2\pi} \int_0^a \sin(\nu \theta') J_{\nu}(\alpha r') f(r', \theta') r' dr' d\theta' \quad (1.16)$$

The problem is reduced to determining the mode coefficients by evaluating the integrals.

1.5.3.2 Calculating mode coefficients

Before mode coefficients can be calculated using Eqns. 1.15 and 1.16, the normalization constants $b_{\nu,\alpha}$ must be determined. To find an expression for them, begin with Eqn. 1.14 at $t = 0$, when $c(r, \theta, 0) = f(r, \theta)$.

$$f(r, \theta) = \sum_{\nu=-\infty}^{\infty} \sum_{\alpha=0}^{\infty} \frac{J_{\nu}(\alpha r)}{(J'_{\nu}(\alpha a))^2} (C_{\nu,\alpha} \cos(\nu\theta) + S_{\nu,\alpha} \sin(\nu\theta)) \quad (1.17)$$

This expression for $f(r, \theta)$ can be substituted into Eqns. 1.15 and 1.16. Using the cosine coefficients as an example,

$$\begin{aligned} C_{n,\alpha} &= b_{\nu,\alpha} \int_0^{2\pi} \int_0^a \cos(\nu\theta') J_{\nu}(\alpha r') \times \\ &\quad \left[\sum_{\mu=-\infty}^{\infty} \sum_{\beta=0}^{\infty} \frac{J_{\mu}(\beta r')}{(J'_{\mu}(\beta a))^2} (C_{\mu,\beta} \cos(\mu\theta') + S_{\mu,\beta} \sin(\mu\theta')) \right] r' dr' d\theta' \\ C_{n,\alpha} &= b_{\nu,\alpha} \sum_{\mu=-\infty}^{\infty} \sum_{\beta=0}^{\infty} \int_0^{2\pi} \int_0^a \cos(\nu\theta') \frac{J_{\nu}(\alpha r') J_{\mu}(\beta r')}{(J'_{\mu}(\beta a))^2} \times \\ &\quad (C_{\mu,\beta} \cos(\mu\theta') + S_{\mu,\beta} \sin(\mu\theta')) r' dr' d\theta' \\ C_{n,\alpha} &= b_{\nu,\alpha} \int_0^{2\pi} \int_0^a \frac{J_{\nu}^2(\alpha r')}{(J'_{\nu}(\alpha a))^2} (C_{\nu,\alpha} \cos^2(\nu\theta') + S_{\nu,\alpha} \cos(\nu\theta') \sin(\nu\theta')) r' dr' d\theta' \end{aligned}$$

The integral and sum have been switched and the orthogonality of the basis functions used to collapse the sums. The integral of the $S_{\nu,\alpha}$ term vanishes, as it is an odd function ($\cos(\nu\theta') \sin(\nu\theta')$) integrated over all θ' . Similarly, the $C_{\nu,\alpha}$ term drops from the sine coefficient integral. The remaining term gives

$$C_{n,\alpha} = b_{\nu,\alpha} \int_0^{2\pi} \int_0^a \frac{J_{\nu}^2(\alpha r')}{(J'_{\nu}(\alpha a))^2} C_{\nu,\alpha} \cos^2(\nu\theta') r' dr' d\theta'$$

or finally

$$\frac{1}{b_{\nu,\alpha}} = \int_0^{2\pi} \int_0^a \frac{J_{\nu}^2(\alpha r') \cos^2(\nu\theta')}{(J'_{\nu}(\alpha a))^2} r' dr' d\theta' \quad (1.18)$$

So long as the integral is truly over the entire circle $r < a$, this constant is the same for both $C_{\nu,\alpha}$ and $S_{\nu,\alpha}$. In that case, Eqn. 1.18 gives $b_{\nu,\alpha} = 2/\pi a^2$ for every mode.

However, in many cases, it is not possible to numerically integrate over the entire gel, as seen in Fig. 1.4D. All numerical integrals are instead over the gel mask, which encompasses as much of

the gel as fits into the field of view. The value of this mask is one within the gel and zero outside, so it serves to define the limits of numerical integration. Denoting the area covered by this mask as Ω , the normalization constants for $C_{\nu,\alpha}$ and $S_{\nu,\alpha}$, respectively, are

$$1/b_{\nu,\alpha}^C = \int_{\Omega} \frac{J_{\nu}^2(\alpha r') \cos^2(\nu\theta')}{(J'_{\nu}(\alpha a))^2} r' dr' d\theta' \quad (1.19)$$

$$1/b_{\nu,\alpha}^S = \int_{\Omega} \frac{J_{\nu}^2(\alpha r') \sin^2(\nu\theta')}{(J'_{\nu}(\alpha a))^2} r' dr' d\theta' \quad (1.20)$$

Likewise, the numerical integrals in Eqns. 1.15 and 1.16 are more precisely written

$$C_{n,\alpha} = b_{\nu,\alpha}^C \int_{\Omega} \cos(\nu\theta') J_{\nu}(\alpha r') f(r', \theta') r' dr' d\theta' \quad (1.21)$$

$$S_{n,\alpha} = b_{\nu,\alpha}^S \int_{\Omega} \sin(\nu\theta') J_{\nu}(\alpha r') f(r', \theta') r' dr' d\theta' \quad (1.22)$$

The degree to which the resulting coefficients deviate from the ideal case of a perfect circle can be roughly quantified by the ratio $b_{\nu,\alpha}/(2/\pi a^2) = \pi a^2 b_{\nu,\alpha}/2$. For small hydrogels, which entirely fit into the field of view, these values range from 1.01 to 1.03, signifying deviations of up to 3%. Large hydrogels whose top and bottom were cut from the field of view had deviations of up to 10%. However, using Eqns. 1.19 and 1.19 instead of the uniform mode weighting $b_{\nu,\alpha} = 2/\pi a^2$ should compensate for the irregular area of integration.

For every hydrogel, Bessel order ν , and Bessel zero α , mode coefficients were calculated numerically. The polar coordinates (r, θ) were converted to Cartesian (x, y) and a sum taken over all the pixels of the initial post-bleach image $f(x, y)$, using the hydrogel mask to set the limits. The center of the coordinate system was set at the center of the gel and the value of the gel radius a calculated as described in Sec. 1.3.1. Before the sum was calculated, the average intensity of the equilibrated portions of the hydrogel was subtracted from the entire image, effectively setting the equilibrium concentration at $c = 0$ as required by the series solution.

The sum was scaled using the area represented by each pixel (using the scale 1.58 $\mu\text{m}/\text{pixel}$) and normalized using the weighting constants $b_{\nu,\alpha}$. The final values of $C_{\nu,\alpha}$ and $S_{\nu,\alpha}$ were stored in two arrays for use in constructing the equation to which the experimental data was fit.

1.5.3.3 Reconstruction of initial image

To verify the mode coefficients, I reconstructed the initial post-bleach image from which they were calculated. The image was reconstructed by numerically integrating Eqn. 1.17 using a similar method to that discussed in the previous section. The area outside the gel mask was not reconstructed. Figure 1.12 shows the resulting reconstructions as the number of terms in the series increases. The relative magnitudes of those terms are plotted in Fig. 1.13. The reconstruction is adequate for our purposes by the time $N_\nu = 20$ and $N_\alpha = 20$, i.e. twenty Bessel orders and twenty zeros are used. Note that the Bessel orders are given by $\nu = -10, -9, \dots, 0, \dots, 8, 9$. A coefficient array of this size allowed a dataset of 43 hydrogels to be entirely processed and fit in approximately 24 hours on a desktop computer; larger coefficient arrays would require the use of a computing cluster.

1.5.3.4 Fitting recovery curves

Once the mode coefficients had been calculated, a fit string was constructed using the full series equation and fed into the Matlab curve fitter. I used a Bessel function identity to deal with the derivative in the denominator.

In order to fit the results to the experimental data, built a fit string for both the bleach spot only and the entire gel mask. I added the offset concentration value back to both of them and then divided them to mimic the way I account for photobleaching in the experimental data processing. There's no photobleaching in the analysis but I needed to make the strings match. Finally, I put in a multiplicative and additive parameter by hand, because I can't get it to fit right otherwise. I'm pretty close even without those parameters, all things considered, but definitely not correct.

As an initial guess at the diffusion constant, I input the diffusion constant calculated using the simple no-exchange fit.

Figure: I should show an example plot for NTF2 and mCherry.

I also have a similar script that simulates data for a given diffusion constant instead of producing a fit string. The math is the same, but I can't fit the simulated data. On the other

Figure 1.12: Reconstruction of initial concentration distribution. Reservoir is masked and not included. (A) Heatmap of original image with average equilibrated gel intensity set to zero. (B) Reconstruction using two Bessel orders ($N_\nu = 2$) and two zeros ($N_\alpha = 2$) in each order. (C-E) Reconstructions with $N_\nu = N_\alpha = 4, 10$, and 20 .

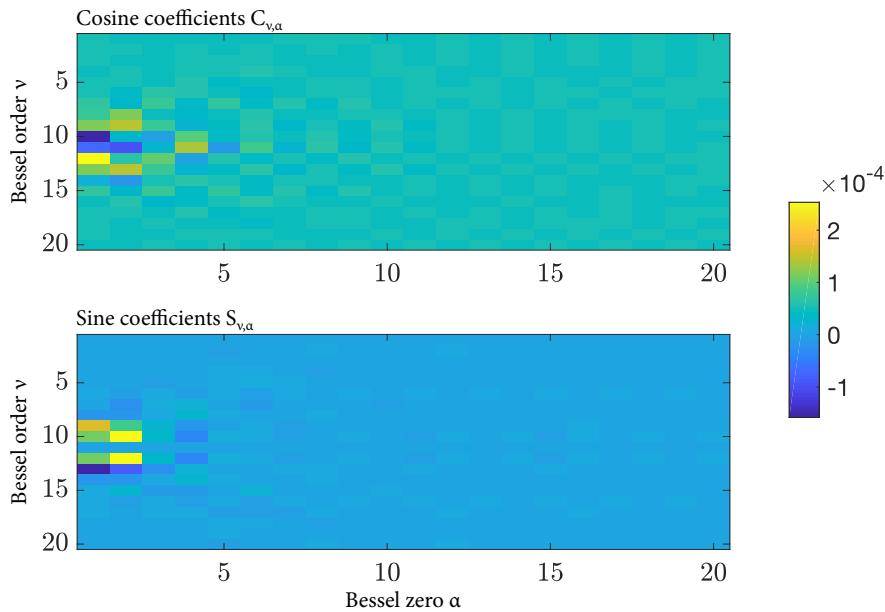
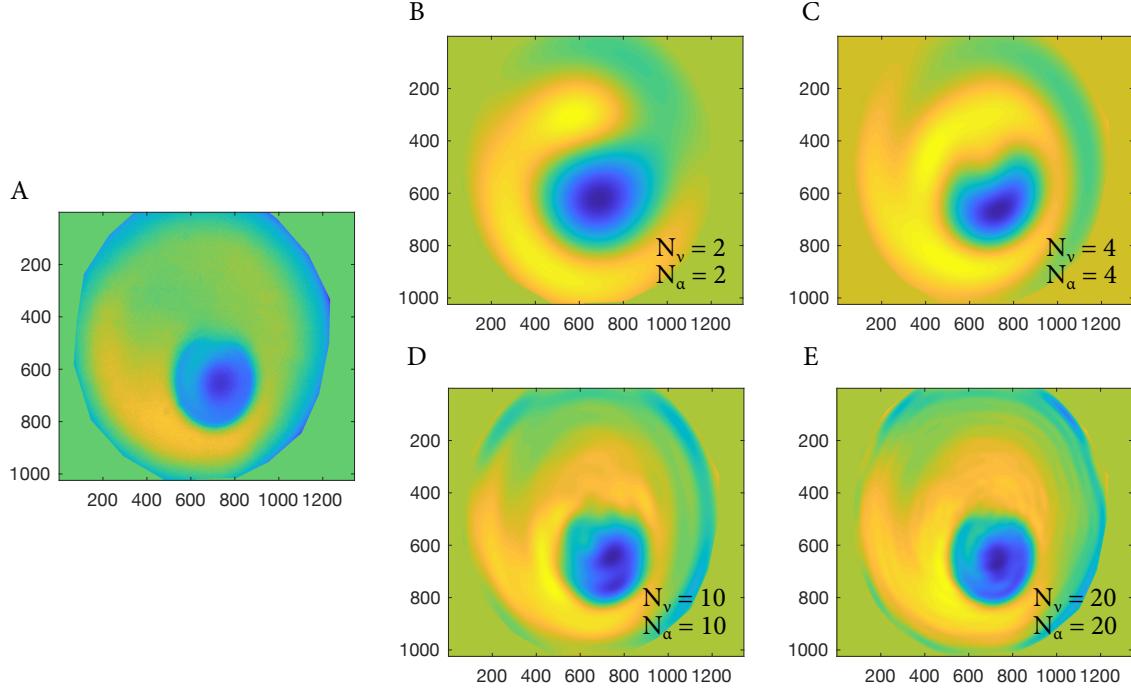
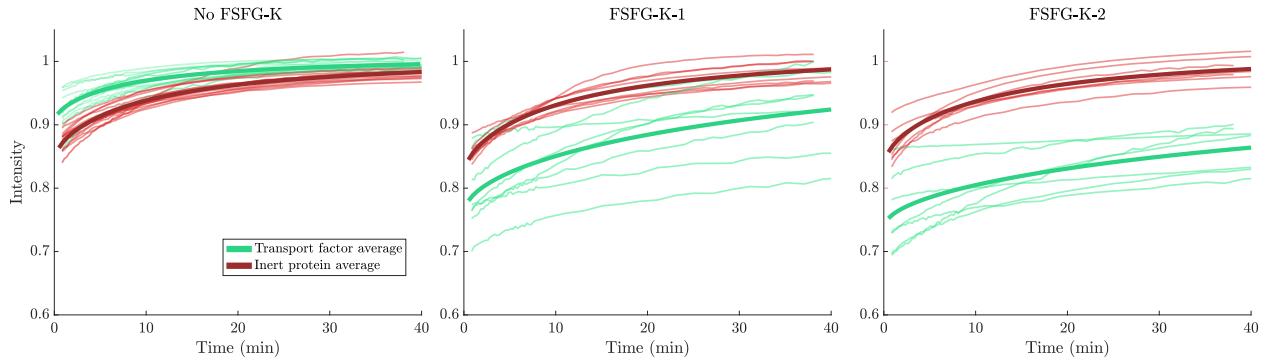


Figure 1.13: Heatmap of cosine and sine mode coefficients $C_{\nu,\alpha}$ and $S_{\nu,\alpha}$ for the reconstruction shown in Fig. 1.12E. Values are shown for each Bessel order ν and each zero α .

Figure 1.14: Heatmap of cosine and sine mode coefficients $C_{\nu,\alpha}$ and $S_{\nu,\alpha}$ for the reconstruction shown in Fig. 1.12E. Values are shown for each Bessel order ν and each zero α .



hand, I can plot it. I used this script for testing while I was getting the fitting working.

1.6 Results

My results are shown in the following figures. The partition coefficients and fraction of time bound did not rely on complicated analysis. As expected, the partition coefficients were very similar for NTF2-F and mCherry in the control, No Nup gels, but the NTF2-F coefficient increased as a result of binding in the FSFG gels. It's higher in the cct2 gels because they had more FG repeats. I need to carefully go through and see which had what concentration. Maybe split them into two datasets if possible.

The observed diffusion coefficients are shown as well. NTF2 and mCherry are (don't say it like this) not significantly different in the control gels but they are in the others. The calculated bound diffusion constant is nonzero for both, give p values. We can't tell the difference between concat 1 and concat 2. In an appendix, put the table with all of the data.

Somewhere I should talk about the bright rings around the bleach region and include a figure.

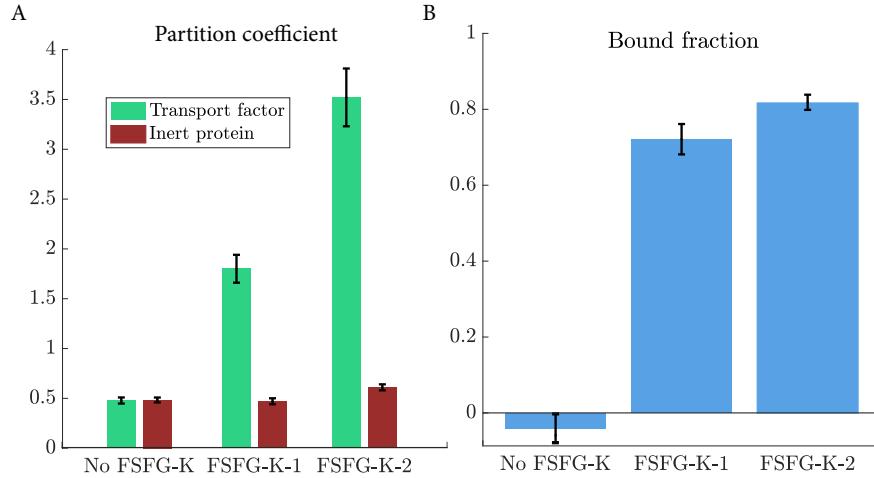


Figure 1.15: Heatmap of cosine and sine mode coefficients $C_{\nu,\alpha}$ and $S_{\nu,\alpha}$ for the reconstruction shown in Fig. 1.12E. Values are shown for each Bessel order ν and each zero α .

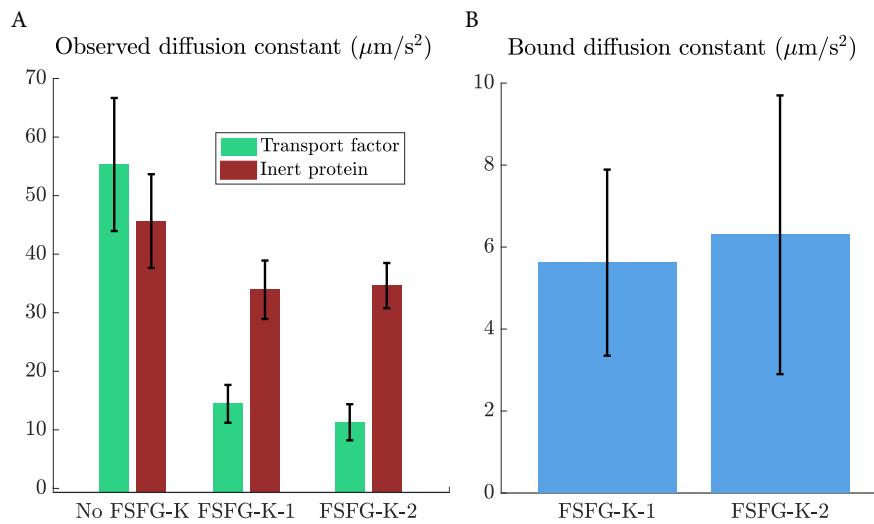


Figure 1.16: Heatmap of cosine and sine mode coefficients $C_{\nu,\alpha}$ and $S_{\nu,\alpha}$ for the reconstruction shown in Fig. 1.12E. Values are shown for each Bessel order ν and each zero α .

1.7 Discussion

We can measure bound diffusion in these gels, although we can't tell the difference between concat 1 and concat 2. Higher tethered Nup concentrations should make our analysis more sensitive. I did a really lot of math for the analysis and at this point we can't make it better without including binding.

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Appendix A

Predicted selectivity of nuclear pore mimics

Nup fragment	Nup concentration	Molecule	MW	Partition coeff.	Diffusion coeff. in gel	Diffusion coeff. free	K_D	D_B	D_B/D_F	S	S with partition coeff.	10x	Notes
Nsp1 (2-601)	3 mM	IBB-MBP-mEGFP-ImpB MBP-mCherry	510 kD 70 kD	100 0.16	0.17 $\mu\text{m}^2/\text{s}$	4.03 $\mu\text{m}^2/\text{s}$	4.8 μM	0.17 $\mu\text{m}^2/\text{s}$	0.04	42	50		[6]
Nup57 (1-223)-Nup49 (1-246)	3.7 mM	IBB-MBP-mECFP-ImpB MBP-mCherry	510 kD 70 kD	400 0.15	0.1 $\mu\text{m}^2/\text{s}$	2.7 $\mu\text{m}^2/\text{s}$	1.4 μM	0.1 $\mu\text{m}^2/\text{s}$	0.04	69	45		[6]
Nup57 (1-223)-Nsp1 (2-601)-Nup49 (1-246)	1.7 mM	IBB-MBP-mEGFP-ImpB MBP-mCherry	510 kD 70 kD	350 0.1	0.24 $\mu\text{m}^2/\text{s}$	4.03 $\mu\text{m}^2/\text{s}$	0.48 μM	0.24 $\mu\text{m}^2/\text{s}$	0.06	38	16		[6]
Nsp1 (2-175)	3.0 mM	IBB-MBP-mEGFP-ImpB MBP-mCherry	510 kD 70 kD	100 3	0.04 $\mu\text{m}^2/\text{s}$	12.1 $\mu\text{m}^2/\text{s}$	90 μM	0.04 $\mu\text{m}^2/\text{s}$	0.003	1.4	4.3		[7]
Nsp1 (2-601)	3.0 mM	IBB-MBP-mEGFP-ImpB MBP-mCherry	510 kD 70 kD	60 0.4	0.22 $\mu\text{m}^2/\text{s}$	6.94 $\mu\text{m}^2/\text{s}$	20 μM	0.22 $\mu\text{m}^2/\text{s}$	0.03	15	40		[7]
Nsp1 (1-601)	2.2 mM	IBB-Redstar-ImpB IBB-Redstar	530 kD 150 kD	1000 0.3	0.1 $\mu\text{m}^2/\text{s}$	0.2 $\mu\text{m}^2/\text{s}$	0.66 μM	0.1 $\mu\text{m}^2/\text{s}$	0.5	230	100		[8]
Nsp1 (1-601)	2.2 mM	GFP-ImpB IBB-Redstar	124 kD 150 kD	100 0.3	0.1-0.2 $\mu\text{m}^2/\text{s}$	0.2 $\mu\text{m}^2/\text{s}$	6.6 μM	0.1-0.2 $\mu\text{m}^2/\text{s}$	0.5-1	210-250	230-240		[8]
Nsp1 (1-601)	2.2 mM	GFP-ImpB acRedStar	124 kD 117 kD	100 0.05	0.1-0.2 $\mu\text{m}^2/\text{s}$	0.2-1 $\mu\text{m}^2/\text{s}$	1.1 μM	0.1-0.2 $\mu\text{m}^2/\text{s}$	0.1-1	94-260	53-130		[8]
P - Nsp1 (274-601) - P *	4.4 mM	IBB-MBP-mEGFP-ImpB IBB-MBP-mEGFP	510 kD 100 kD	7 0.9	2.78 $\mu\text{m}^2/\text{s}$	16.0 $\mu\text{m}^2/\text{s}$	560 μM	2.42 $\mu\text{m}^2/\text{s}$	0.15	5.3	25		[9]

Table A.1: Predicted selectivity of FG Nup hydrogels in previous work. We took partition and diffusion coefficients from tables in references or calculated them using concentration plots. We determined the dissociation constant K_D from the partition coefficient of the binding species (P_B) and non-binding species (P_N) and the Nup concentration N_t using $K_D \approx (P_I/P_B)N_t$. Note that the measured P_B is an underestimate of the true partition coefficient. We estimated the bound diffusion coefficient from the in-gel (effective) diffusion coefficient (D_{eff}) and the probability of the binding species being bound ($p_b \approx 1 - K_D/N_t$) using $D_B = p_b D_{\text{eff}}$ (Sec. 1.3.2). We used the reaction-diffusion equations discussed in Sec. ?? to estimate the selectivity. Because partition coefficient estimates were lower bounds, we also calculated selectivity assuming that the reported partition coefficients were 10% of their actual value. P - Nsp1 (274-601) - P * refers to a fusion between Nsp1 (274-601) and a pentameric coiled-coil P which facilitates the aggregation of the Nsp1 domain into hydrogels. See [9].

Appendix B

Protein expression and purification

B.1 Purification of his-tagged protein with metal affinity column

Most proteins used in this work contain a C-terminal 6x histidine tag. These proteins can be purified using a cobalt or nickel affinity column. Proteins purified with this protocol include all Nup variants (FSFG, FG124, etc.), NTF2, mCherry, and GFP. This protocol is designed for the purification of one 0.5-L cell pellet. If running more than one column at once, adjust the total buffer volumes accordingly.

This protocol is used for all Nup variants. For FSFG variants, use the urea concentrations noted in the buffer table. For FG124 purifications, use 7M guanidine hydrochloride in all buffers (including elution) instead of urea. Always make urea and guanidine hydrochloride solutions the same day they will be used. **Do not use urea, guanidine hydrochloride, or other denaturants when purifying ordered proteins.**

To prevent aggregation, BME must be used for any protein that contains two cysteines. **Do not use BME if not necessary; any BME solutions must be disposed of in the hazardous waste container in the fume hood.**

Add the protease inhibitor cocktail (PIC) stock in DMSO immediately before using the buffer. PIC has a lifetime of about 30 minutes in aqueous solution. Store all buffers on ice before use. Incubations should be done at 4°, but the column can be used at room temperature.

Figure B.1: Buffer guide for cobalt or nickel affinity column purifications.

Buffer	Use (and volume)	Composition
Lysis Buffer	for cell lysis (15 mL)	Buffer of choice 1x (15** mL) 1:1000 PIC (15uL) (8M Urea) (7.2 g) (3mM BME) (3 uL)
Wash I	for wash I (20 mL)	Buffer of choice 1x (20** mL) 1:1000 PIC (20uL) (3M Urea) (4 g) (3mM BME) (4uL)
Wash II *the imidazole is optional; can be useful for removing junk bound to column	for wash II (20 mL)	Buffer of choice 1x (20** mL) 1:1000 PIC (20 uL) (10mM Imidazole) (.013 g) (3mM BME) (4uL)
Elutions	20 mL total	Buffer of choice 1x (20** mL) 1:1000 PIC (20uL) 250mM Imidazole (.32 g) (3mM BME) (4uL)
Handy Molecular Weights	Imidazole: 68.08 g/mol Urea: 60.06 g/mol BME: 14.3 M GuHCl: 95.53 g/mol	**Bring up to volume with buffer. Will not need full 20- <u>25mL</u> if using urea

- (1) Remove the periplasmic matrix, if not already done (Sec. B.2).
- (2) Lyse the cells. Add 15 mL of lysis buffer to thawed 0.5-L pellet. Resuspend pellet by pipetting up and down or vortexing and then lyse by sonicating. Keep solution on ice. lookup: sonicator brand. Sonicate for at least two minutes total, in 30s-on, 60s-off pulses. Power delivered to sample should be at least 20 W. Centrifuge resulting lysate for about 15 mins on top speed of either floor centrifuge to pellet cellular debris.
- (3) Prepare the metal-affinity column. Gently resuspend the resin into a slurry by slowly turning the bottle. The beads will be crushed if shaken vigorously. Into a disposable plastic column, pipette enough slurry to contain 3.5-4 mL of beads once the storage buffer has drained out (typically 6-8 mL of slurry, if beads and buffer are stored in a one-to-one mixture).
Equilibrate the column by running 5-10x the bed volume (25-50 mL) of buffer through the column. Do not let the column run dry at any point in the purification.
- (4) Add supernatant to column and nutate for one hour at 4°C.
- (5) Drain the column. Add wash I buffer and nutate at 4°C for 10 minutes.
- (6) Drain the column. Add Wash II buffer, nutate at 4°C for 10 minutes, and drain.
- (7) Elute and collect the protein. Three elution methods can be used:
 - (a) *Fractional elution:* Prepare a row of eppendorfs. Add 5-10 mL elution buffer to open column and catch the draining liquid in fractions with 0.5-1.0 mL (8-16 drops) per eppendorf. Do not let the column dry. After all elutions are completed, use the Bradford test to pool the fractions with similar protein concentrations. **The Bradford test is unreliable for Nup variants and batch elution should be used.** Fractional elution gives the highest protein concentration and should be used where possible.

- (b) *Batch elution:* Add a bed volume of elution buffer to column and let drain to remove waste buffer from column. Watch resin color change carefully so as not to lose protein. Add 3-5 mL of elution buffer and collect flow-through.
- (c) *Nutated elution:* Add 5-10 mL of elution buffer to sealed column and nutate for 10-30 minutes. Collect flow-through.

Early elutions should be fractional or batch elutions.

9) Clean and store the column. Run 5 bed volumes of MES (lookup: precise components) through the column, then 5 bed volumes DI water. Run about 20 mL MES buffer through the column, then 20 mL DI water. Store 1:1 in 20% ethanol.

B.2 Periplasmic matrix removal (PPMR)

The periplasmic matrix (PPM) contains proteases and debris that binds to metal affinity columns. Removing the PPM before protein purification significantly increase the yield of his-tagged disordered proteins.

Note: Keep both solutions on ice. Resuspension of pellets should be done by gently pipetting up and down and swirling the tubes. Do not vortex to resuspend. Rough treatment of the cells may lyse them.

- (1) Spin down cell culture at 4000g (Sorvall centrifuge, GSA rotor) for 10 minutes at 4°C.
- (2) Resuspend pellets in at least 50 mL cold SHE buffer (20% Sucrose, 50mM HEPES, 1mM EDTA pH 7.9) per liter of culture. Keep tubes on ice.
- (3) Spin down for 10 minutes at 5000 rpm at 4°C.
- (4) Resuspend pellets in at least 50 mL cold 5mM MgSO₄ per liter of culture.
- (5) Incubate tubes on ice for 10 minutes.
- (6) Spin down for 10 minutes at 5000 rpm at 4°C.

- (7) Proceed to purification or flash-freeze tubes in liquid nitrogen and store in ultra-low freezer.

B.3 Lyophilization

Lyophilization refers to freeze-drying proteins. It's a good way to store a known mass of protein before resuspension in hydrogel precursor solution. All lyophilization in this work was done using a Labconco freeze dry system. Lyophilization concentrates any salts in the sample buffer. Therefore, whenever possible, a decomposing buffer should be used. A 25 mM ammonium bicarbonate buffer was used for all lyophilized Nup variants. This buffer decomposes into carbon dioxide, ammonia, and water when lyophilized or above 36°C.

- (1) Dialyze sample into 25 mM ammonium bicarbonate if possible.
- (2) Perform a BCA to quantify the protein concentration in the sample. Prepare eppendorf aliquots that contain the desired mass of protein (usually 100 or 200 μg).
- (3) Cover the aliquots with parafilm and use a needle to punch a hole in the covering.
- (4) Flash-freeze the aliquots.
- (5) Follow lyophilizer instructions. Keep samples frozen and load as quickly as possible to avoid thawing. Ensure that the vacuum is below 50×10^{-3} mBar.
- (6) Leave aliquots on lyophilizer at least 12 hours.
- (7) Remove aliquots from lyophilizer. Remove parafilm and close eppendorfs. Store with desiccant in ultra-low freezer.

Appendix C

Calculation of PEG hydrogel pore size

The following equation was used to estimate pore size in a 10 wt % PEG hydrogel with 20-kDa 8-armed PEG-norbornene and 1-kDa PEG-dithiol crosslinker [10].

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\left(\frac{\bar{v}}{v_1}\right) (\ln(1 - v_{2,s} + v_{2,s} + \chi v_{2,s}^2))}{v_{2,r} \left(\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3} - \frac{1}{2} \left(\frac{v_{2,s}}{v_{2,r}}\right) \right)} \quad (\text{C.1})$$

with:

The average molecular weight between crosslinks as M_c .

The average polymer molecular weight before crosslinking $M_n = 20$ kDa.

The specific volume of polymer $\bar{v} = 0.8$ cm³/g for 1.5K PEG.

The molar volume of the swelling agent $v_1 = 18$ g/mol for water.

The Flory PEG-water interaction parameter $\chi = 0.4$ [11].

The polymer volume fraction before swelling $v_{2,r} = 0.10$ for a 10 wt % hydrogel.

The polymer volume fraction after swelling $v_{2,s} = (\rho_p(\frac{Q_m}{\rho_s} + \frac{1}{\rho_p}))^{-1}$ [12] with:

The solvent density $\rho_s = 1$ g/cm³ for water.

The polymer density $\rho_p = 1.2$ g/cm³ for 20-kDa PEG (Santa Cruz Biotech).

The mass ratio of solvent to polymer $Q_m = 9$.

This calculation gives me $M_c \approx 210$ Da as the average molecular weight between crosslinks.

The mesh size ξ can be estimated using [10]

$$\xi = v_{2,s}^{-1/3} \ell \left(\frac{2M_c}{M_r} \right)^{1/2} c_n^{1/2} \quad (\text{C.2})$$

with:

The carbon-carbon bond length $\ell = 1.54 \text{ \AA}$.

The molecular weight of the repeating polymer unit $M_r = 67 \text{ Da}$ for PEG.

The “characteristic ratio” for PEG $c_n \approx 4$.

The result is an estimated mesh size on the order of 1 nm.

Appendix D

Bis-labeling Nup fragments for incorporation into acrylamide hydrogels

Before FSFG-cys or any other cys-labeled Nup variant can be tethered to an acrylamide hydrogel, it needs to be labeled with bisacrylamide or PEG-DA. This protocol describes the labeling procedure for either chemical group. The cysteines must be fully reduced for the labeling to occur. No BME, TCEP, or other reducing agent can be present in the reaction mixture. FSFG and other disordered peptides form disulfide bonds within minutes of being removed from reducing agents, so begin the reaction as quickly as possible after reducing. This protocol was developed with the help of Benjamin Fairbanks. Following labeling, the extent of labeling can be quantified using an Ellman's reagent assay.

D.1 Bis-labeling reaction protocol

- (1) Begin with a stock of at least 1 mg/mL FSFG with a terminal cysteine in PBS pH 7.8. Typical reactions use 1 mL of approximately 2 mg/mL FSFG.
- (2) Equilibrate an equal amount of immobilized TCEP resin slurry in a disposable 5-mL column which can be spun in a conical tube. Refer to Thermo-Pierce product reference sheet for resin volume and nutation time if necessary. Equilibrate with 20-30 bed volumes of PBS pH 7.8 using gravity, then spin at 161g for 10 s to remove remaining buffer.
- (3) Immediately add the FSFG stock and nutate at 4°C for 1 hour.

- (4) Prepare a conical tube with 40 μL of triethanolamine (TEA, in fume hood) and 50 μL 2% bisacrylamide solution. This will lead to a 10-fold molar excess of bisacrylamide over protein and 300 mM TEA in the final reaction mixture. Place column in conical tube and spin down 1000 rpm for 1 minute.
- (5) Immediately vortex thoroughly. Nutate at room temperature for 30 minutes.
- (6) Dialyze into 25 mM ammonium bicarbonate buffer to remove excess bisacrylamide and prepare for lyophilizing.
- (7) Perform a BCA to quantify protein concentration. Prepare 100 or 200 μg aliquots, freeze, and lyophilize. Store lyophilized protein with a desiccant in ultra-low freezer.

D.2 Ellman's reagent assay protocol

This protocol was taken from Thermo-Fisher's protocol and modified for a microwell plate. Due to the rapid disulfide bond formation of FSFG, the reactants must be mixed very rapidly once reducing agent is removed.

- (1) Equilibrate 80 μL TCEP resin slurry in each of two disposable 1-mL spin columns which can be spun in an eppendorf centrifuge. Refer to Thermo-Pierce product reference sheet for resin volume and nutation time if necessary. Equilibrate with 20-30 bed volumes of PBS pH 7.8 using gravity, then spin at 2300g for 10 s to remove remaining buffer.
- (2) Add 80 μL of bis-labeled FSFG in 25 mM ammonium bicarbonate to one column and a known concentration of unlabeled FSFG cys (as a control) in 25 mM ammonium bicarbonate to the other. Nutate for two hours.
- (3) While incubation proceeds, prepare the reaction buffer (0.1 M sodium phosphate buffer pH 8.0 with 1 mM EDTA) and the Ellman's reagent solution (ERS, 4 mg/mL Ellman's reagent in reaction buffer).

- (4) Prepare a 96-well plate with wells containing 1.8 μM ERS and 29.5 μM reaction buffer. Prepare wells for the FSFG-bis and FSFG cys samples but do not add the protein until everything has been prepared. Prepare a well containing ERS, reaction buffer, and 68 μL of 25 mM ammonium bicarbonate buffer. Finally, prepare a well containing ERS, reaction buffer, and 68 μL of unlabeled FSFG cys of a known concentration that has not been reduced.
- (5) Spin down both columns of TCEP solution 2300g for 30 s in an eppendorf centrifuge and collect the flow-through. Very rapidly, add 68 μL of flow-through to the appropriate wells and mix by pipetting up and down.
- (6) Incubate plate at room temperature for 15 minutes.
- (7) Measure the absorbance at 412 nm using a plate reader.
- (8) Calculate labeling efficiency. Use the buffer well's absorbance to blank the absorbance of the reduced FSFG cys sample and the labeled FSFG sample. Divide the blanked absorbance of the labeled sample by that of the reduced FSFG cys sample and subtract the resulting ratio from 1. The unreduced FSFG sample is not directly used but is a good check on the results.

Appendix E

Hydrogel precursor solutions

This appendix contains details of hydrogel precursor solutions. Lyophilized protein should be resuspended in the buffer component of the precursor solution and incubated at room temperature for at least 20 minutes before adding the other components. No-Nup control gels simply omit the protein. Precursor solutions must be degassed for 10 minutes and promptly polymerized. All solutions containing photoinitiator must be protected from light at all times. Mix under red light only. Prepare precursor solutions immediately before use.

E.1 PEG hydrogel precursor recipes

All PEG hydrogels were 10 wt % PEG with and 0.5 thiol-ene ratio. For more accurate pipetting, stock solutions were designed to be combined in equal volumes.

Table E.1: PEG hydrogel precursor stocks

Component	Concentration	Buffer
20-kDa PEG-norbornene	438 $\mu\text{g}/\mu\text{L}$	Water
1-kDa PEG-dithiol crosslinker	45 $\mu\text{g}/\mu\text{L}$	Water
8-kDa PEG-dithiol crosslinker	360 $\mu\text{g}/\mu\text{L}$	Water
Irgacure 2959	2 mM	Water
LAP	2 mM	Water
TCEP	4 mM	4x PTB

E.2 Acrylamide hydrogel precursor recipes

The monomer and crosslinker were bought premixed from BioRad (acrylamide/bisacrylamide 30% 29:1) but potentially could be prepared in different ratios and mixed separately. Any additional components, such as dextran or photoinhibitor, should be made into a stock with PTB and used instead of the buffer component in the recipe. Acrylamide hydrogels were mostly crosslinked with LAP photoinitiator, but sometimes with the APS/TEMED chemical crosslinking system. Results of several APS/TEMED concentrations in a 6% acrylamide precursor solution are shown in Table E.5.

Table E.2: Sample precursor soution recipe (10 wt % PEG, 10 mg/mL nominal Nup concentration)

Stock	Amount
20-kDa PEG-norbornene	2.5 μ L
1-kDa PEG-dithiol crosslinker	2.5 μ L
Irgacure 2959	2.5 μ L
TCEP in 4x PTB	2.5 μ L
Lyophilized FSFG cys	100 μ g

Table E.3: Acrylamide hydrogel precursor stocks

Component	Concentration	Buffer
Acrylamide monomer	30%	Premixed
Bisacrylamide crosslinker	1%	Premixed
LAP	20 mM	Water
APS	10% w/v	PTB
TEMED	1% w/v	PTB
PTB buffer	1x	PTB

Table E.4: Sample precursor soution recipe (6% final acrylamide concentration)

Stock	Amount
Premixed acrylamide/bis	2 μ L
LAP	1 μ L
PTB	7 μ L
Lyophilized FSFG cys	100 μ g

Table E.5: APS/TEMED chemical crosslinking tests

APS concentration	TEMED concentration	Degas time (min)	Results
1%	0.5%	0	Gelled in under 10 s.
0.1%	0.5%	0	Gelled in 5 minutes.
0.1%	0.1%	5	Gelled in 5 minutes (while degassing).
0.1%	0.1%	0	Gelled in 10 minutes.
0.1%	0.05%	0	Did not gel.
0.1%	0.05%	5	Gelled in 10 minutes.

Appendix F

Protein and peptide sequences

Table F.1: FG Nup fragments

Description	Notes	Sequence
FSFG his	('FSFG concat-1')	MGTSATSKPAFSFGAKSDENKAGATSKPA FSFGAKPEEKDDNSSKPAFSFGAKSN EDKQDGTAKPASFSGAKPAEKNNNET SKPAFSFGAKSDEKKDGDASKPAFSF GAKPDENKASATSKPASHHHHHH
cys FSFG his		MGCTSATSKPAFSFGAKSDENKAGATSKPA FSFGAKPEEKDDNSSKPAFSFGAKSNED KQDGTAKPASFSGAKPAEKNNNETSKPA FSFGAKSDEKKDGDASKPAFSFGAKPDEN KASATSKPASHHHHHH
cys FSFG cys his	Aggregates unless kept in reducing agent.	MGCTSATSKPAFSFGAKSDENKAGATSKPA FSFGAKPEEKDDNSSKPAFSFGAKSNED KQDGTAKPASFSGAKPAEKNNNETSKPA FSFGAKSDEKKDGDASKPAFSFGAKPDEN KACATSKPASHHHHHH
ybbR FSFG cys his	Most often used for hydrogel experiments; ybbR tag is intended for site-specific labeling but not used in the hydrogel experiments.	MGDSLEFIASKLATSATSKPAFSFGAKSDEN KAGATSKPAFSFGAKPEEKDDNSSKPA FSFGAKSNEDKQDGTAKPASFSGAK PAEKNNNETSKPAFSFGAKSDEKKDGDAS KPAFSFGAKPDENKACATSKPASHHHHHH
ybbR FSFG concat- 2 cys his		MGDSLEFIASKLATSATSKPAFSFGAKSDEN KAGATSKPAFSFGAKPEEKDDNSSKPA FSFGAKSNEDKQDGTAKPASF FGAKPAEKNNNETSKPAFSFGA KSDEKKDGDASKPAFSFGAKP DENKASATSKPASATSKPAFS FGAKSDENKAGATSKPAFSF GAKPEEKDDNSSKPAFSFGAKSNED KQDGTAKPASFSGAKPAEKNNNET SKPAFSFGAKSDEKKDGDASKPAFSF GAKPDENKACATSKPASHHHHHH

Table F.2: FG Nup fragments, continued.

Description	Notes	Sequence
ybbR FSFG concat- 3 cys his	Did not express well in my hands.	MGDSLEFIASKLATSATSKPAFSFGAKSDEN KAGATSKPAFSFGAKPEEKKK DDNSSKPAFSFGAKSNEDKQDGTAK PAFSFGAKPAEKNNNETSKPAFSF GAKSDEKKDGDASKPAFSFGAKPD ENKASATSKPASATSKPAFSFGAKS DENKAGATSKPAFSFGAKPEEKKDDN SSKPAFSFGAKSNEDKQDGTAKPAFSF GAKPAEKNNNETSKPAFSFGAKSDEKK DGGASKPAFSFGAKPDENKASATSKPA SATSKPAFSFGAKSDENKAGATSKPAFS FGAKPEEKKDDNSSKPAFSFGAKSNEDKQ DGTAKPAFSFGAKPAEKNNNETSKPAF SFGAKSDEKKDGDASKPAFSFGAKPDEN KACATSKPASHHHHH
ybbR SSSG cys his	Nonbinding control.	MGDSLEFIASKLATSATSKPASSSGAKSDEN KAGATSKPASSSGAKPEEKKDDNSSKPA SSSGAKSNEDKQDGTAKPASSSGAK PAEKNNNETSKPASSSGAKSDEKKDGDAS KPASSSGAKPDENKACATSKPASLEHHH HHH
FG124 his	Aggregates unless kept in denaturant	MAPNNTNNANSSITPAFGSNNTGNTAFGN SNPTSNVFGSNNSTTNTFGSNSAGT SLFGSSSAQQTKSNGTAGGNT FGSSSLFNNNSTNSNTTKPAFGLNFGGGN NTTPSSTGNANTSNNLFGATASHHHHHH
cys FG124 his	Aggregates unless kept in denaturant.	MGCTSAPNNTNNANSSITPAFGSNNTGN TAFGNSNPTSNVFGSNNSTTNT FGSNSAGTSLFGSSSAQQTKSNGTAGGNT FGSSSLFNNNSTNSNTTKPAFGLNFGGGN NTTPSSTGNANTSNNLFGATASHMHHH HHH

Table F.3: Other sequences

Description	Notes	Sequence
NTF2 his	Wild-type yeast NTF2.	MALDFNTLAQNFTQFYNNQFDTDRSQLGN LYRNESMLTFETSQLQGAKDIVEKLVS LPFQKVQHRITTLDAQPASPNGDVLVMIT GDLLIDEEQNPQRFSQVFHLIPDGN SYYVFNDIFRLNYSAAHHHHH
Pho4	Nuclear localization sequence.	KVDKLGGSGSANKVTKNKSNSSPYLNKRR GKPGPDSLE
Spo12	Nuclear localization sequence.	KVDKLGGSGKSTSNLKSSHTTSNLVKKT FKRDLLKQDPKRKLQLQQRFASPTDR LVSPCSLKLE

Appendix G

Dye-labeling protocols

The following protocols were used to label proteins with various fluorophores. Dyes should always be stored in the ultra-low freezer in anhydrous DMSO. Labeled proteins should be aliquoted within 24 hours of labeling and stored in the ultra-low freezer until just before use. These protocols were adapted from Thermo-Fisher amine and cysteine labeling protocols with help from Eric Verbeke and Annette Erbse.

If better efficiency is needed, the next improvement would be to carry out the protocols in a glove bag under nitrogen.

G.1 Labeling NTF2 with fluorescein-NHS

- (1) Resuspend lyophilized fluorescein-NHS at 100 mg/mL in anhydrous DMSO in the dark-room. Discard the remaining DMSO aliquot. Make 100- μ L aliquots of dye solution. Store with desiccant in ultra-low freezer, protected from light.
- (2) Mix NTF2 in PTB pH 7.0 and 100 mg/mL fluorescein-NHS in DMSO with around 15-fold molar excess dye. Several other buffers can be used as well (see Thermo protocol). A typical labeling reaction used 0.5 mL of 16 mg/mL NTF2 and 18.6 μ L dye stock mixed in an Eppendorf with an Eppendorf stir bar.
- (3) Incubate mixture, stirring, protected from light, at room temperature for one hour.

- (4) Equilibrate TALON cobalt resin with PTB in a column that can be spun in a centrifuge. TALON resin has a stated capacity of 5-15 mL protein per mL resin. Using significantly more than needed can lead to nonspecific binding of free dye. A typical reaction required 1.6 mL of the resin slurry. Equilibrate with at least 10 bed volumes PTB.
- (5) Cap column and add reaction mixture. Nutate at 4°C for one hour, protected from light.
- (6) Allow column to drain and wash with approximately 100 bed volumes of PTB, protected from light as best as possible. Occasionally cap column and resuspend resin to remove free dye from column cap and sides. Test flow-through using UV light to ensure that no dye is visible by the end of the wash. Resin should still be bright yellow.
- (7) Spin column at 160g for one minute to remove remaining wash buffer. Immediately cap and elute with 1 mL of 300 mM imidazole in PTB. Nutate 4°C for half an hour and collect elution. Resin should return to pink.
- (8) Dialyze elution against PTB to remove imidazole. No more than 24 hours after labeling, aliquot and freeze labeled NTF2.
- (9) Run a sample of labeled NTF2 on a native PAGE gel along with a sample of fluorescein-NHS. Use the Typhoon to compare the concentration of free dye to labeled protein in the protein sample.

G.2 Labeling NTF2 with Alexa Fluor 488 - SDP or FSFG with Alexa Fluor 647 - SDP

- (1) Resuspend lyophilized Alexa Fluor 488 at 10 mg/mL in anhydrous DMSO in the darkroom. Discard the remaining DMSO aliquot. Make 10- μ L aliquots of dye solution. Store with desiccant in ultra-low freezer, protected from light.
- (2) Mix NTF2 in 0.1 M sodium bicarbonate buffer and 10 mg/mL fluorescein-NHS in DMSO. A typical labeling reaction used 200 μ L of 16 mg/mL NTF2 and 20 μ L dye stock mixed in

an Eppendorf with an Eppendorf stir bar.

- (3) Incubate mixture, stirring, protected from light, at room temperature for one hour.
- (4) Equilibrate TALON cobalt resin with PTB in a column that can be spun in a centrifuge. TALON resin has a stated capacity of 5-15 mL protein per mL resin. Using significantly more than needed can lead to nonspecific binding of free dye. Equilibrate with at least 10 bed volumes PTB.
- (5) Cap column and add reaction mixture. Nuteat at 4°C for one hour, protected from light.
- (6) Allow column to drain and wash with approximately 100 bed volumes of PTB, protected from light as best as possible. Occasionally cap column and resuspend resin to remove free dye from column cap and sides. Test flow-through using UV light to ensure that no dye is visible by the end of the wash. Resin should still be bright yellow.
- (7) Spin column 2300g for 20 s to remove remaining wash buffer. Immediately cap and elute with 300 μ L of 500 mM imidazole in PTB. Nuteat 4°C for half an hour and collect elution. Resin should return to pink.
- (8) Dialyze elution against PTB to remove imidazole. No more than 24 hours after labeling, aliquot and freeze labeled NTF2.
- (9) Run a sample of labeled NTF2 on a native PAGE gel along with a sample of fluorescein-NHS. Use the Typhoon to compare the concentration of free dye to labeled protein in the protein sample.

G.3 Labeling NTF2-cys with Alexa Fluor 488 - maleimide

- (1) Resuspend lyophilized Alexa Fluor 488 at 10 mg/mL in anhydrous DMSO in the darkroom. Discard the remaining DMSO aliquot. Make 10- μ L aliquots of dye solution. Store with desiccant in ultra-low freezer, protected from light.

- (2) Mix protein in PTB pH 7.0 and 50 μ M TCEP (with no other reducing agent present) and 10 mg/mL dye in DMSO. Typical amounts were 50 μ L of 100 μ M NTF2-cys into 50 μ L dye stock.
- (3) Incubate mixture, stirring, protected from light, at room temperature for two hours.
- (4) Equilibrate TALON cobalt resin with PTB in a column that can be spun in a centrifuge. TALON resin has a stated capacity of 5-15 mL protein per mL resin. Using significantly more than needed can lead to nonspecific binding of free dye. Equilibrate with at least 10 bed volumes PTB.
- (5) Cap column and add reaction mixture. Nutate at 4°C for one hour, protected from light.
- (6) Allow column to drain and wash with approximately 100 bed volumes of PTB, protected from light as best as possible. Occasionally cap column and resuspend resin to remove free dye from column cap and sides. Test flow-through using UV light to ensure that no dye is visible by the end of the wash. Resin should still be bright yellow.
- (7) Spin column 2300g for 20 s to remove remaining wash buffer. Immediately cap and elute with 300 μ L of 500 mM imidazole in PTB. Nutate 4°C for half an hour and collect elution. Resin should return to pink.
- (8) Dialyze elution against PTB to remove imidazole. No more than 24 hours after labeling, aliquot and freeze labeled NTF2.
- (9) Run a sample of labeled NTF2 on a native PAGE gel along with a sample of fluorescein-NHS. Use the Typhoon to compare the concentration of free dye to labeled protein in the protein sample.