**Supporting Information for**

**Confocal analysis of CNC based hydrogels and suspensions**

Aref Abbasi Moud,a,b† Amir Sanati-Nezhad,b\* Seyed Hossein Hejazi,a\*

*a Department of Chemical and Petroleum Engineering, University of Calgary, 2500 University Dr NW, Calgary, Alberta T2N 1N4, Canada*

*b* *BioMEMS and Bioinspired Microﬂuidic Laboratory, Center for Bioengineering Research and Education, Department of Mechanical and Manufacturing Engineering, University of Calgary, Calgary, Alberta T2N 1N4, Canada*

\*Corresponding Authors: E-mail: shhejazi@ucalgary.ca (Seyed Hossein Hejazi), amir.sanatinezhad@ucalgary (Amir Sanati-Nezhad)

**S1. Theory of fluorescence recovery after photobleaching (FRAP)**

Fluorescence recovery after photobleaching (FRAP) as a common confocal imaging technique for assessing dynamics of entities in situ, holds an important role among other methods, such as fluorescence photoactivation, single particle tracking (SPT), fluorescence correlation spectroscopy (FCS), and image correlation spectroscopy (ICS) (Alcor et al. 2009; Bancaud et al. 2010; Haustein and Schwille 2007; Kolin and Wiseman 2007; Lippincott-Schwartz and Patterson 2003; Toprak and Selvin 2007). While each technique has its own weaknesses and points of strength, FRAP has many advantages over alternative methods. For instance, measuring the ratio of mobile to immobile particles within total population is accessible using FRAP, a variable not easily acquired with other techniques. Furthermore, without any special modifications, FRAP can be performed on most commercially available confocal setups.

All the parameters and their definitions are tabulated in **Table S1**. For simplicity, we assume that cellulose nanocrystal (CNC) gels or clusters are planar enough so that they can be viewed as 2-D objects.

**Table S1**. List of parameters and their definitions

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| ***Parameter*** | ***Definition*** | ***Parameter*** | ***Definition*** |
| $$D$$ | Diffusion coefficient | $$F\_{i}$$ | Pre-bleach fluorescence intensity |
| $$τ\_{1/2}$$ | Half-time of recovery | $$F\_{0}$$ | Initial post-bleach fluorescence intensity |
| $$r\_{n}$$ | Nominal radius = bleaching spot radius = sampling region radius | $$F\_{\infty }$$ | Post-bleach steady-state fluorescence intensity |
| $$r\_{e}$$ | Effective radius = spreading radius of post-bleach profile | $$F\_{1/2}$$ | Fluorescence intensity at the half of recovery |
| $$q$$ | Quantum yield | $$M\_{F}$$ | Mobile fraction |
| $$\in $$ | Attenuation factor for excitation laser | $$τ\_{D}$$ | Diffusion time |
| $$K$$ | Bleaching depth parameter | $$D\_{re}$$ | D from Soumpasis equation  |

It is assumed that the bleaching spot size is small, compared to the gel size, so that we can treat the gel as an infinite plane. Having considered these assumptions, laser intensity profiles for photo-bleaching lasers in the infinite plane as either a Gaussian laser (equation S1) or uniform laser (equation S2).

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| $$I\_{rn}\left(x,y\right)=\frac{2l\_{0}}{πr\_{n}^{2}}exp⁡(-\frac{2(x^{2}+y^{2})}{r\_{n}^{2}})$$ | (S1) |
| $$I\_{rn}\left(x,y\right)=\frac{2l\_{0}}{πr\_{n}^{2}}H⁡(r\_{n}^{2}-x^{2}-y^{2})$$ | (S2) |

where H acts as the Heaviside function. The concentration of FB28 tagged CNCs (C (x,y,t)) changes with Cartesian coordinates and time, therefore equation S3 can be used to connect changes in time and coordinates:

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| $$C\_{t}=D∆C$$ | (S3) |

where D (μm2/s) is diffusion coefficient of CNCs and $∆=\left(\frac{δ^{2}}{δx^{2}}\right)+\left(\frac{δ^{2}}{δy^{2}}\right)$, therefore the solution of the diffusion equation can be obtained form a convolution of the fundamental solution of the diffusion equation and under the initial condition.

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| $$C\left(x,y,t\right)=∬\_{}^{}C(x-x^{'},y-y^{'},0)ϕ\_{Dt}\left(x^{'},y^{'}\right)dx^{'}dy^{'}$$ | (S4) |

where the fundamental solution of the diffusion equation in the infinite plane is strictly defined as:

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| $$ϕ\_{Dt}\left(x,y\right)=\frac{1}{4πD\_{t}}exp⁡(-\frac{x^{2}+y^{2}}{4D\_{t}})$$ | (S5) |

Therefore, the fluorescence intensity from the bleached region of interest (ROI) can be calculated from equation S6:

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| $$F\left(t\right)=q∬\_{}^{}εI\_{rn} (x, y)C (x, y,t) dx dy$$ | (S6) |

where q is quantum yield of the fluorophores and C(x,y,t) describes the concentration of fluorescent particles within the confocal volume at time.

It has been empirically shown that a confocal post-bleach profile can be described as a Gaussian function (constant minus Gaussian).

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| $$C (x, y, 0) = C\_{i} ( 1- K exp (-\frac{-2(x^{2}+y^{2})}{r\_{e}} )$$ | (S7) |

where $C\_{i}$ is pre-bleach fluorescent particle concentration, and $r\_{e}$ is the half width at the approximately 14% of bleaching depth from the top. We define $r\_{e} $as the effective radius of a post-bleach profile, in contrast to the nominal radius ($r\_{n}$) from a user-defined bleaching spot radius. In line with the computation work of Axelrod et al. (Axelrod et al. 1976). for the diffusion equation with unknown diffusion coefficient D and an initial condition given by equation S7, a diffusion FRAP equation for the confocal FRAP can be formulated as equation S7.

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| $$F\left(t\right)=F\_{i}\left\{1-\frac{K}{1+γ^{2}+^{2t}/\_{τ\_{D}}}\right\}M\_{f}+(1-M\_{f})F\_{0}$$ | (S7) |

where $τ\_{D} = re^{2} / (4D)$ and $γ = r\_{n}/r\_{e}$. $M\_{f}$ is defined as:

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| $$M\_{f}=\frac{F\_{\infty }-F\_{0}}{F\_{i}-F\_{0}}$$ | (S8) |

where $F\_{i}$ is pre-bleach fluorescence intensity, $F\_{0} $is postbleach initial fluorescence intensity, and $F\_{\infty }$ is postbleach steady-state fluorescence intensity. K can be computed from equation S7 at t = 0 (i.e. F(0) = F0):

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| $$F\_{0}=F\_{i}\left\{1-\frac{K}{1+γ^{2}}\right\}$$ | (S9) |

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| $$K=\frac{\left(F\_{i}-F\_{0}\right)}{F\_{i}}(1+γ^{2})$$ | (S10) |

We let $F\_{1/2}=\frac{F\_{0}+F\_{\infty }}{2}$ (i.e. fluorescence intensity at the half time of recovery), then by definition, F($τ\_{1/2}$) = $F\_{1/2}$. $F\_{1/2} $is related to $M\_{f} $by equation S11.

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| $$F\_{1/2}=\frac{\left(F\_{i}-F\_{0}\right)}{2}M\_{f}+F\_{0}$$ | (S11) |

by setting F($τ\_{1/2}$) = $F\_{1/2}$:

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| $$F\_{1/2} = F\_{i} \left\{1-\frac{K}{1+γ^{2}+2τ\_{1/2}/τ\_{D}}\right\}M\_{f}+(1-M\_{f})F\_{0} $$ |  (S12) |

where the definition of K in equation S10 is applied. By multiplying 2Fi to both the denominator and numerator in the right hand side, we obtain:

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| $$\frac{2τ\_{1/2}}{τ\_{D}}=(1+γ^{2})$$ | (S13) |

Finally, by solving for D in $τ\_{D}=\frac{r\_{e}^{2}}{4D}$, after applying $γ=\frac{r\_{n}}{r\_{e}}$, we reach:

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| $$D=\frac{r\_{e}^{2}+r\_{n}^{2}}{8τ\_{1/2}}$$ | (S14) |

With the assumption $r\_{e}=r\_{n}$, we obtain $D=0.25\frac{r\_{n}^{2}}{τ\_{1/2}}$, which is essentially identical to soumpasis equation. The approximately 3% difference in the proportionality constants in equations S1 and S14 is due to the different assumptions on either Gaussian or uniform laser profiles.

**S2. Protocols for performing FRAP tests**

The general microscope configurations that are necessary for the FRAP data acquisition are presented here. Finding suitable photo-bleaching parameters for each dye helps to store and reused the dye on subsequent days, with few or no adjustments.

1. Set the image in confocal software with a suitable zoom to the zone of interest. Generally, when working with CNC gel, a 1024 × 1024-pixel image at 10X Apo lens electronic zoom is a good starting point, and the electronic zoom can be adjusted from there depending on the FRAP application.
2. Using the software ROI selection tool, trace a circular region for bleaching in the center of the image window. Once this ROI is created, it should be saved on the computer for seeking and adjusting the proper ROI size.

Selecting and adjusting the proper size for ROI for each system takes trial and error, and it needs to be determined on a case by case. If the circular ROI is too small, fast recovery leads to a quick recovery in FRAP, and this causes the measurement difficult or the FRAP is too noisy. On the other hand, the ROI should not be selected too large so that a significant fraction of total fluorophores is deactivated due to the bleaching protocol, as this will artificially downplay the mobile fraction assessment. Reaching this balance takes a lot of trial and error. Take into consideration that the geometry and size of ROI can be varied depending on the final goal of a study. For the FRAP measurement, circular geometry is highly recommended.

1. Use the software to perform the bleaching event, after scanning of multiple pre-bleach micrographs. These pre-bleach pictures can provide steady-state fluorescence intensity prior to photobleaching, which will be needed to normalize the FRAP intensity.

A general rule of thumb to establish a stable fluorescence baseline before FRAP is to gather 3 pre-bleach images if taking pictures at a rate of roughly 1 frame/second is used.

1. Set the number of bleach repetitions between the number of 5 and 20, with no delay between them. This number implies the number of times the laser goes over each pixel in the bleach region during the protocol.

The number of repetitions will depend on the laser power, the scanning rate, the photo-stability of the fluorescent dye, and the quickness of diffusion of the material under investigation.

1. Choose a bleach laser wavelength and fix it on the maximum power allowed based on the software.
2. The bleaching phase must be sufficiently short to minimize recovery during bleaching. As a rule of thumb, it is recommended that the total bleaching time be at least 15 times shorter than the period of recovery.(Phair et al. 2004)

**S3. Dye binding to CNCs**

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**Figure S1.** Filtered cellulose nanocrystal (CNC)-FB28 dye **(a)** Before and **(b)** after exposure to ultraviolet (UV) light.

Carbohydrate-aromatic (CA) interactions from van der Waals forces (CH–p interactions) and the hydrophobic effect also have been reported to explain the surface adsorption of aromatic molecules to carbohydrate-based polymers such as cellulose in aqueous environments. These CA interactions as opposed to electrostatic interactions, likely account for most of the observed binding in aqueous systems. The most notable example of observed CA interactions has been the adsorption of aromatic residues to cellulose in the cellulose-binding domains of proteins. Since electrostatic interactions, van der Waals forces, and hydrophobic effects have all been reported to affect aromatic molecule adsorption to cellulose or similar molecules. It is likely that all these could affect dye adsorption, requiring individual investigation of each dye desired for tagging.

The binding mechanism in our samples is mostly Carbohydrate-aromatic (CA) interactions from van der Waals forces (CH–p interactions). Nonetheless, the adhesion between the dye and the CNC is strong enough to resist the effect of rinsing and centrifugation.

**S4. Normalization techniques**

To facilitate FRAP analysis, recovery curves can be normalized in two different ways. Firstly, the values are normalized to make the pre-bleached intensity value equal to 1. This modification allows for making comparisons between samples with varying levels of brightness. Secondly, for simplification purposes, the curve can be fully normalized so that the initial intensity becomes 1 and the intensity at the time of bleaching equals to 0. This can represent the data as a proportion of recovery with 1 being full recovery and 0 being no recovery. If measurements are accessible for each bleach set-up, a background and a suitable reference region would be selected to characterize unintentional bleaching region, a typical approach called double normalization (equation S15):(Phair et al. 2004)

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| $$Norm\left(t\right)=\frac{Ref\_{pre-bleach}}{ref(t)}\frac{FRAP(t)}{FRAP\_{pre-bleach}}$$ |  (S15) |

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