A PROJECT REPORT

ON

"Fluorescence Correlation Spectroscopy Analysis of Diffusion across the Nuclear Membrane"

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BY

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Ishaant Agarwal

ABSTRACT

This project involved a comprehensive investigation into the diffusion of Dextran molecules across the nuclear membrane. It included analysis of single photon as well as time-averaged fluorescence data obtained through a confocal microscope examination of cells during the diffusion process.

In this work we have used different bio molecules for studying nuclear transport rates and permeability of nuclear membrane.We have employed times lapse confocal fluorescence imaging to study the transport of dye labeled dextran molecules of different sizes through the nuclear pore complexes. Several non-trivial findings were observed and probed.

Keywords: Dextran, fluorescence correlation spectroscopy, passive transport, active transport

Contents

1 Introduction **2**
- 1.1 Nucleus . . . 2
  - 1.1.1 Nuclear Structure . . . 2
  - 1.1.2 Diffusion in Nucleus . . . 2
- 1.2 Fluorescence Correlation Spectroscopy . . . 3
  - 1.2.1 Confocal Microscope . . . 3
  - 1.2.2 Diffusion and FCS . . . 3
- 1.3 Autocorrelation Analysis . . . 4
  - 1.3.1 Autocorrelation Function . . . 4
  - 1.3.2 Autocorrelation function for diffusing particles . . . 4

2 Implementation **6**
- 2.1 Autocorrelation function Code . . . 6
- 2.2 Model and Curve Fitting Code . . . 8

3 Results **12**
- 3.1 Diffusion Model . . . 12
  - 3.1.1 Non-Zero Slope at Steady State . . . 12
- 3.2 Fluorescence levels not equalising at steady state . . . 13
- 3.3 Kink for some sizes after initial rise . . . 14

4 Conclusion and Future Scope **15**
- 4.1 Conclusion . . . 15
- 4.2 Future Scope . . . 15

List of Figures

2.1 **Autocorrelation lots obtained for different nuclei** 7
2.2 **Curves fitted for 3 parameter and 4 parameter diffusion model** 11

3.1 **Diffusion curve fitted to intensity data Sample 1** 13
3.2 **Diffusion curve fitted to intensity data Sample 2** 13
3.3 **Kink is observed at around the 20 second mark** 14
3.4 **Kink is observed at around the 50 second mark** 14

Chapter 1

Introduction

1.1 Nucleus

1.1.1 Nuclear Structure

Studies on biomolecular transport through nuclear membranes are of interest from a fundamental physics perspective as well as from the point of view of biological applications. The nuclear pore complexes (NPCs) in the nuclear membrane are the only known passage way for macromolecules to enter into and exit from the nucleus.The nucleus is surrounded by a double layered membrane which separates the nucleus from the cytoplasm. The nuclear membrane is a phospholipid bilayer, with its outside surface continuous with the endoplasmic reticulum and its internal surface facing the nucleus.

1.1.2 Diffusion in Nucleus

The nuclear pore complex (NPC) is the principal gateway between the nucleus and the cytoplasm. The NPC can function as a passive diffusion channel, as an ion channel, or as a macromolecule shuttle channel, transporting materials bidirectional between the cytoplasm and the nucleus.Passive diffusional transport is a movement of biochemicals and other molecular or atomic substances across nuclear envelopes. The passive transport takes place through simple diffusion through the concentration gradient and the rate of diffusion will critically depend on the size on the pores. The size of inactively transported substances may range from that of freely penetrable mono and divalent ions up to 40 kDa .[1] Active transport is the movement of molecules across the nuclear envelope against their concentration gradient

First, we give a brief explanation of the concepts involved in fluorescence correlation spectroscopy, its application to our experiment and finally analysis of the results obtained.

1.2 Fluorescence Correlation Spectroscopy

1.2.1 Confocal Microscope

A confocal microscope makes use of a laser as the excitation source so that it can be tightly focused on the sample. Fluorescence from the focal point alone is passed to a photodetector kept at the conjugate focal plane of the objective lens and an aperture of appropriate dimension blocks all out of focus light from reaching the detector.[2] This confocal detection helps in improving the axial resolution of the microscope and results in sharper images. Two dimensional images of the sample slices are acquired point by point either by moving the laser beam over the sample (laser scanning) or by moving the sample (stage scanning) in the X-Y plane. The stage scanning typically requires larger time to acquire an image and is more expensive compared to the laser scanning.

Confocal optical fluorescence microscopy records the fluorescence from a specific axial volume of interest and thus can be used to selectively study a particular constituent or section of an appropriately labeled cell nucleus. In time lapse confocal fluorescence imaging one monitors a cross sectional area of interest as a function of time. The technique can be used to study the temporal evolution of a microscopic system. In this project we have employed times lapse confocal fluorescence imaging to study the transport of dye labeled dextran molecules of different sizes through the nuclear pore complexes.

1.2.2 Diffusion and FCS

FCS may be used to measure dynamical properties of particles. One of the most important properties is the diffusion coefficient D, as it contains information about the size and weight of the moving particle.

The diffusion coefficient D (units $\mu m/s^2$) describes how far a particle may reach due to its diffusive motion. Diffusion can also be described as a random walk in the solution.[3]x The larger the diffusion coefficient is, the faster the particle moves. The trajectory may be characterized by its so called mean squared displacement (MSD) $\langle r^2 \rangle$, which describes the area that the particle covers in a certain time. It is linear in time and the proportionality is given by the diffusion coefficient:

$$\langle r^2 \rangle = 6.D.\tau$$

In FCS the measure signal is the fluorescence intensity I(t) emitted by the observed particles. Each particle (if excited to the same level) adds the same amount of fluorescence to the measured signal, so the overall intensity is proportional to the number of particles N we observe. For the further analysis, we will therefore look at the particle number $N(t)$. In FCS we split up the overall particle number $N(t)$ over time, into an average value $\langle N \rangle$ and (small) fluctuations $\delta N(t)$ around $\langle N \rangle$

$$N(t) = \langle N \rangle + \delta N(t)$$

In FCS, only the fluctuations $\delta N(t)$ are analyzed, the average $\langle N \rangle$ is mostly left out during the data processing. These intensity fluctuations are caused by particles leaving and entering

the observation volume Vobs. If we know how long a particle stays inside the observation volume, we can also determine its diffusion coefficient, which corresponds to its speed. So if we observe particles with a large D, they will often enter and leave the observation volume in a fixed observation time, and thus show quick fluctuations $\delta N(t)$. On the other hand these fluctuations will be slower for small diffusion coefficients. The correlation analysis performed in FCS extracts the characteristic timescale of these fluctuations which may subsequently be converted into a diffusion coefficient.

1.3 Autocorrelation Analysis

1.3.1 Autocorrelation Function

As we cannot directly count the number of particles, we measure the fluorescence intensity $I(t)$ emitted by the particles currently inside the focus and analyse this. While a particle is inside the focus, it is excited by the laser of the microscope and therefore constantly cycles between its ground and excited state. In each cycle a fluorescence photon is emitted in a random spacial direction. A fraction of these photons is then collected by the objective lens and subsequently detected. We will call this output $I(t)$. Therefore the intensity I$I(t)$ fluctuates in the same way, as the particle number $N(t)$ and every property we derived for $N(t)$ is also valid for $I(t)$.

$$I(t) = \langle I \rangle + \delta I(t)$$

From this measured signal, we now have to extract how fast it fluctuates, in order to gain information on how fast the particles move in the sample. To do this, we use a mathematical tool, called **autocorrelation analysis** (therefore also the name fluorescence *correlation* spectroscopy, FCS).The autocorrelation function is mathematically defined as:

$$g(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle_t}{\langle I(t) \rangle_t^2}$$

where $\langle ... \rangle$ denotes a time average over the time variable t:

$$\langle I(t) \rangle_t = \frac{1}{T} \int_1^T I(t) \mathrm{d}t$$

It measures the self-similarity of the fluctuations dI(t), if compared to itself, a time t (lag time) later.

1.3.2 Autocorrelation function for diffusing particles

As already mentioned each FCS measurement yields an autocorrelation curve $g^{(\text{measured})}(\tau)$ which we have to further evaluate. To obtain the desired values – diffusion coefficient D and particle number N – we have to fit theoretical models $g(\tau)$ to the measured autocorrelation function $g^{(\text{measured})}(\tau)$. These models may be derived by plugging in the diffusion law into the autocorrelation function and doing all the integrals. For a solution of a single species with a diffusion coefficient D and N particles in a confocal focus (on average), we get:

$$g(\tau) = \frac{1}{N} \cdot \left(1 + \frac{4D\tau}{w_{\mathrm{xy}}^2}\right)^{-1} \cdot \left(1 + \frac{4D\tau}{z_0^2}\right)^{-1/2}$$

where w_{xy} is the width in the xy-plane and z_0 the length (z-direction) of the measurement volume (confocal focus). The focus is estimated to have the shape of an ellipsoid with two equal axes w_{xy} and a length z_0. Usually it is rewritten in terms of the average retention time or diffusion time $\tau_D = w_{xy}^2/(4D)$ of a particle in the focus:

$$\begin{aligned} g(\tau) &= \frac{1}{N} \cdot \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \cdot \left(1 + \frac{\tau}{\gamma^2 \tau_D}\right)^{-1/2} \\ D &= \frac{w_{xy}^2}{4\tau_D} \\ \gamma &= \frac{z_0}{w_{xy}} \end{aligned}$$

where γ is called structure factor or axial ratio of the focus.

Chapter 2

Implementation

2.1 Autocorrelation function Code

autocorr.m

```
%% GUI for selecting data
[fname, pname]=uigetfile('*.csv');
%Create fully-formed filename as a string
filename = fullfile(pname, fname);
id = find(fname == '.', 1, 'last');

% Loads the file as 'a'
A=load(filename);

%%

[R,C]=size(A);
t=A(:,1);%-A(1,1);

scale_fac=1; %scaling factor for time

t=t*scale_fac;

for i=2:size(A,2)

    if mod(i,4)==2
        figure
    end

    k{i}=A(:,i);
    [autocorr1,lags]=xcorr(k{i},'coeff');
    autocorr1=autocorr1(ceil(length(autocorr1)/2):end);
    lags=lags(ceil(length(lags)/2):end);
    plot(log10(t),autocorr1,'.');hold on
    xlabel('Delay (in log t seconds)')
    ylabel('Autocorrelation')
    title(['Nucleus number ' num2str((i-1)/4)])
end

%% Analysis of data quality
figure
 v=k1(randperm(length(k1)));
[autocorr1,lags]=xcorr(v,'coeff');
autocorr1=autocorr1(ceil(length(autocorr1)/2):end);
lags=lags(ceil(length(lags)/2):end);
plot(log10(t),autocorr1,'.');
xlabel('Delay (in log t seconds)')
ylabel('Autocorrelation')
title('Randomized data')
```

code/autocorr.m

This function acts as an all in one package for plotting autocorrelation curve for each nucleus. It plots the ratio of N/B intensities for three different background pixels.

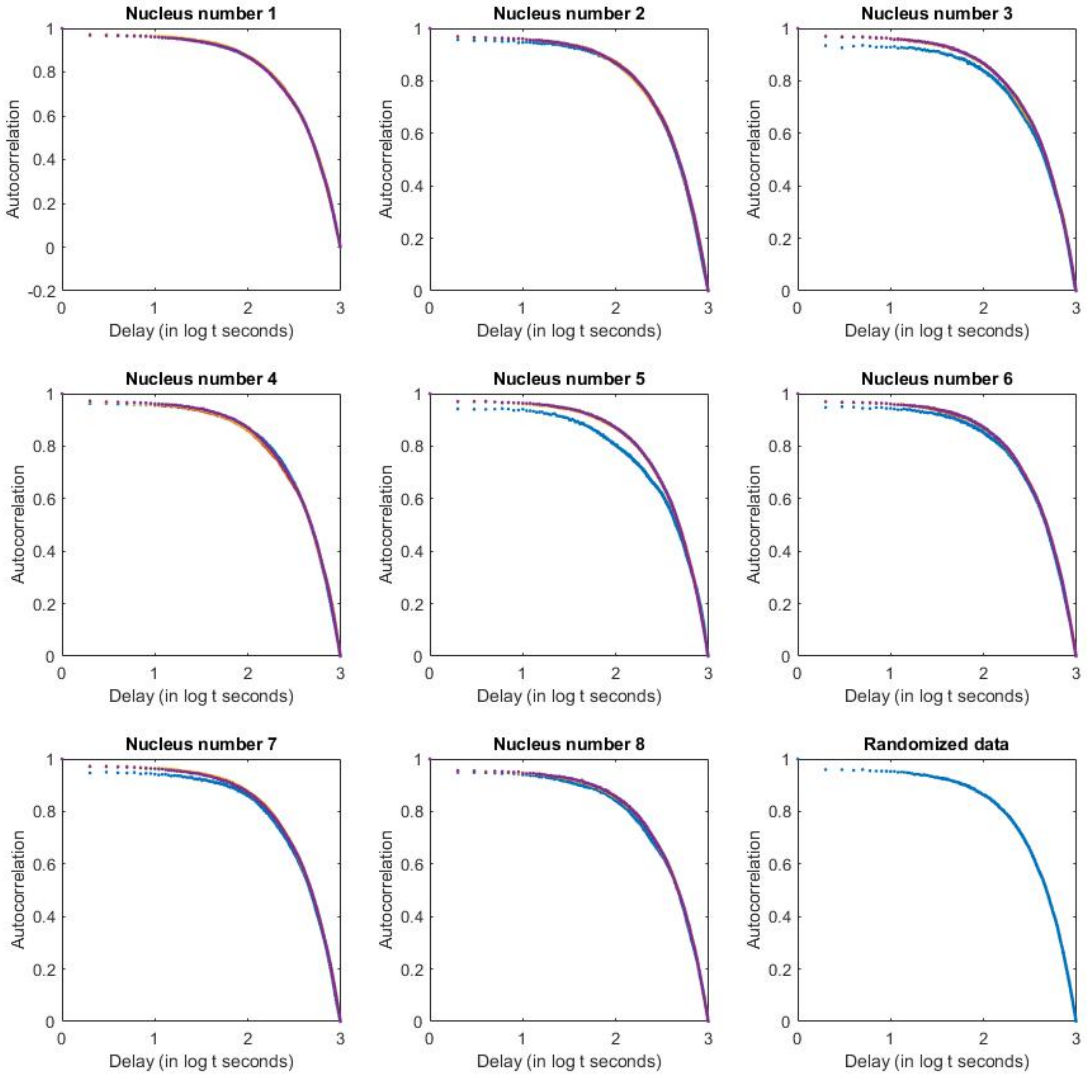

Figure 2.1: Autocorrelation lots obtained for different nuclei

2.2 Model and Curve Fitting Code

We modeled the diffusion process with a double exponential given by the following equation:

$$\frac{N}{B}(t) = F_{max_1}(1 - e^{-k_1 t}) + F_{max_2}(1 - e^{-k_2 t})$$

Corresponding code and graphs are displayed below:

curvefit_code.m

```
%% Init

clear A
clear coeffs
clear coeffs2
close all

% To make comparing different sets of data fits easier

    if exist('fits','var') ~= 1
        j=1;
    end

%% Stuff to make laoding files easier

%Create fully-formed filename as a string
[fname, pname]=uigetfile('*.xlsx');
filename = fullfile(pname, fname);
id = find(fname == '.', 1, 'last');

% Loads the file as 'A'
A=xlsread(filename,'fin');

%Initializing cells for storing calculated curve paramters
fits={}; % Stores curve fits
gofs={}; % Stores goodness of fits


for i=2:size(A,2)

    [fitresult, gof]=createFit_ish(A(:,1),A(:,i),i-1);

    fitresult

    fits{i-1}=fitresult;
    coeffs(i-1,:)=coeffvalues(fitresult);

    [fitresult2, gof2]=createFit2(A(:,1),A(:,i),i-1,[coeffs(i-1,1),coeffs(i-1,2),1-coeffs(i
        -1,1),coeffs(i-1,3)]);

    fits2{i-1}=fitresult2;
    coeffs2(i-1,:)=coeffvalues(fitresult2);

    fitresult2

end
figure
subplot(3,1,1)
plot(coeffs(:,1))
title('Fm')
subplot(3,1,2)
plot(coeffs(:,2))
title('D1')
```

```
subplot(3,1,3)
plot(coeffs(:,3))
title('D2')

%% Stores coefficient values across sessions
coeff_array{j}=coeffs;
coeff_array2{j}=coeffs2;
j=length(coeff_array)+1;
```

code/curvefit_code.m

createFit.m

```
function [fitresult, gof] = createFit2(x, y,iter, x_init)

%CREATEFIT(X,Y)
%  Create a fit.
%
%  Data for fit:
%      X Input : x
%      Y Output: y
%  Output:
%      fitresult : a fit object representing the fit.
%      gof : structure with goodness-of fit info.
%
%  See also FIT, CFIT, SFIT.



%% Fit: 'untitled fit 2'.
[xData, yData] = prepareCurveData( x, y );

% Set up fittype and options.
ft = fittype( 'a*(1-exp(-b*x))+c*(1-exp(-d*x))', 'independent', 'x', 'dependent', 'y' );
opts = fitoptions( 'Method', 'NonlinearLeastSquares' );
opts.Display = 'Off';
opts.StartPoint =x_init;

% Fit model to data.
[fitresult, gof] = fit( xData, yData, ft, opts );

% Plot fit with data.
figure( 'Name', ['Diffusion Fit ' num2str(iter)] );
h = plot( fitresult, xData, yData );
legend( h, 'y vs. x', 'untitled fit 2', 'Location', 'NorthEast' );
% Label axes
xlabel x
ylabel y
grid on
```

code/createFit2.m

createFit_ish.m

```
function [fitresult, gof] = createFit(x, y,iter)

%CREATEFIT(X,Y)
%  Create a fit.
%
%  Data for fit:
%      X Input : x
%      Y Output: y
%  Output:
%      fitresult : a fit object representing the fit.
%      gof : structure with goodness-of fit info.
%
%  See also FIT, CFIT, SFIT.



%% Fit: 'untitled fit 2'.
[xData, yData] = prepareCurveData( x, y );

% Set up fittype and options.
ft = fittype( 'a*(1-exp(-b*x))+(1-a)*(1-exp(-c*x))', 'independent', 'x', 'dependent', 'y' );
opts = fitoptions( 'Method', 'NonlinearLeastSquares' );
opts.Display = 'Off';
opts.StartPoint = [ 0.8 0.05 0.000193];

% Fit model to data.
[fitresult, gof] = fit( xData, yData, ft, opts );

% Plot fit with data.
figure( 'Name', ['Diffusion Fit ' num2str(iter)] );
h = plot( fitresult, xData, yData );
legend( h, 'y vs. x', 'untitled fit 2', 'Location', 'NorthEast' );
% Label axes
xlabel x
ylabel y
grid on
```

code/createFit_ish.m

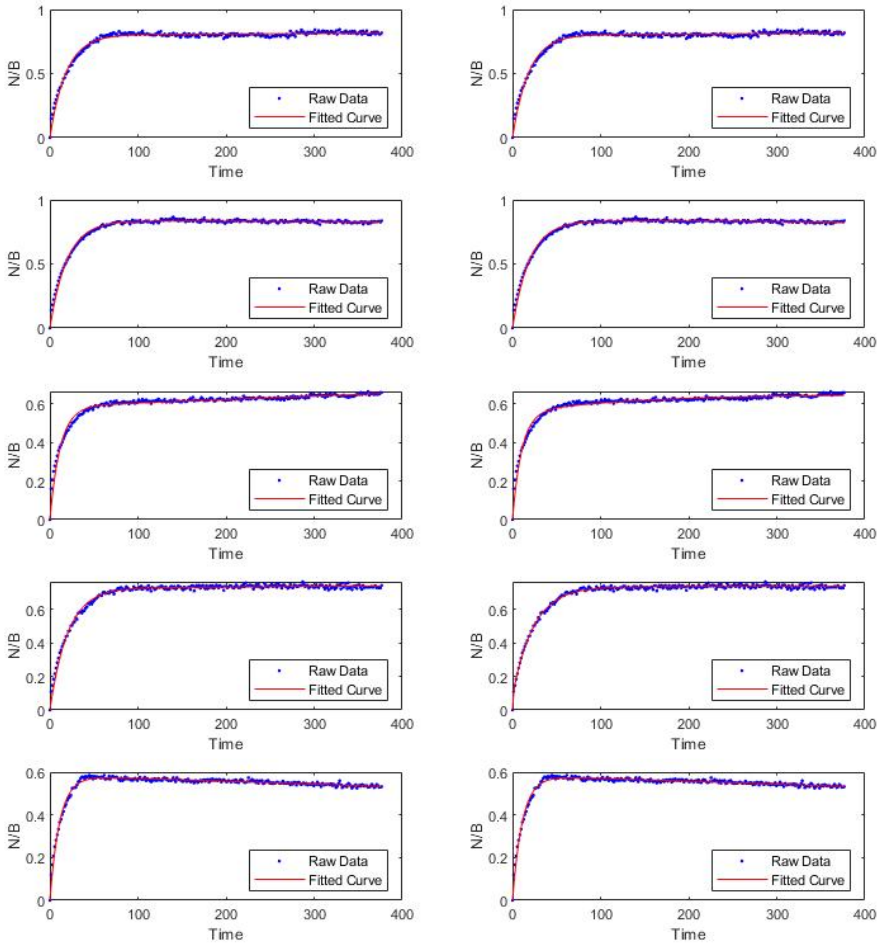

Figure 2.2: Curves fitted for 3 parameter and 4 parameter diffusion model

Chapter 3

Results

After carefully screening the data we are coming up with a model for the diffusion process. We also observed and are currently analysing some anomalies.

3.1 Diffusion Model

3.1.1 Non-Zero Slope at Steady State

It was observed that for most experiments the N/B ratio did not saturate but instead had a small slope even after a duration several times the expected time constant.

This led to the hypothesis that their might exist two simultaneous diffusion processes- one which accounts for the larger overall transfer and has a small time constant. The other would account for the non zero slope at steady state with a relatively much larger time constant.

Some calculated values, extracted from the data fits are given in the table below:

Fmax1	k1	T1(=1/k1)	Fmax2	k2	T2(=1/k2)
0.80108344	0.054629714	18.30505643	0.687314347	5.44E-05	1.84E+04
0.547934883	0.096928798	10.31685139	0.098671642	0.007993047	1.25E+02
0.65371146	0.04199967	2.38E+01	0.08376438	1.312681125	0.761799634
0.581859644	0.070141226	14.25695064	0.115947002	0.006034477	1.66E+02
0.525002008	0.064351707	15.53960323	0.413454292	0.000225422	4.44E+03

where

$$\frac{N}{B}(t) = F_{max_1}(1 - e^{-k_1 t}) + F_{max_2}(1 - e^{-k_2 t})$$

We largely observed that the values for k_1 and k_2 remained similar (in terms of order of magnitude) even across different datasets. k_1 also showed the expected trend with respect to size of the molecules, scaling down with size, lending credence to the model. We want to analyse the second slower process and figure out if it represents an active process(for which the values should be dependent on/independent of size) or a passive process.

3.2 Fluorescence levels not equalising at steady state

This could be either because the diffusion rates into and out of the nuclear membrane are different. This would assume that the membrane is selective and lets Dextran move out more easily than in. We will need to investigate if there exists an active process that accounts for this difference in rates or if its merely a matter of diffusion processes being different.

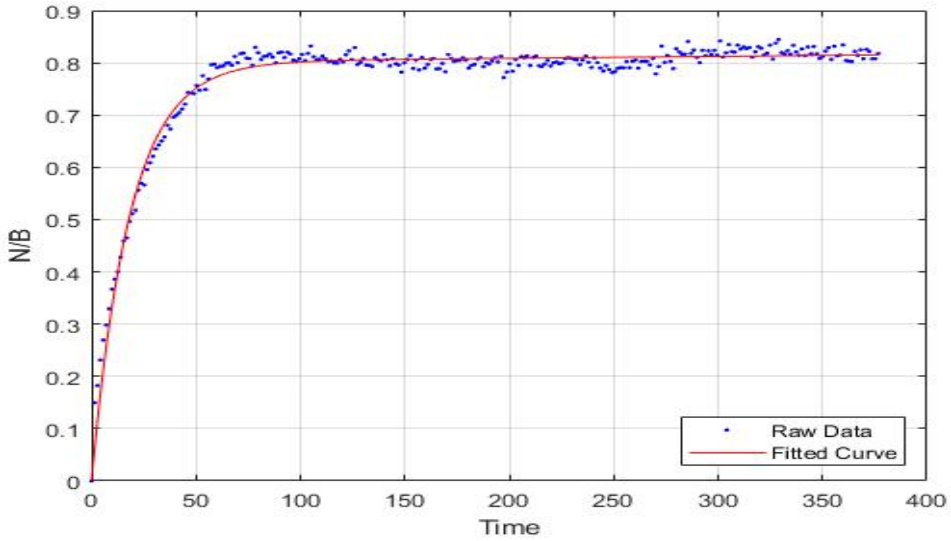

Figure 3.1: Diffusion curve fitted to intensity data Sample 1

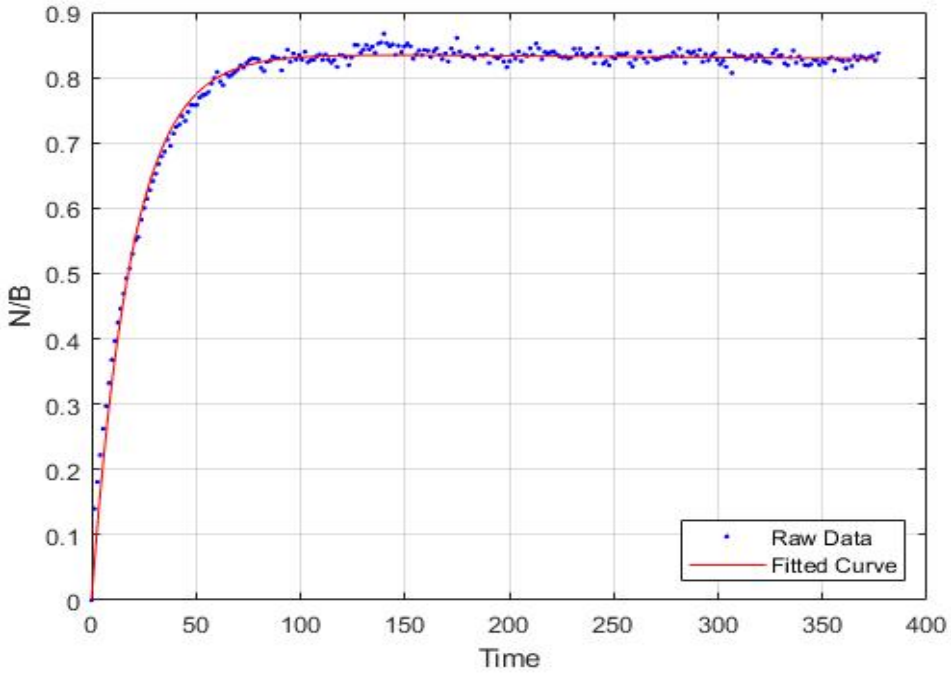

Figure 3.2: Diffusion curve fitted to intensity data Sample 2

3.3 Kink for some sizes after initial rise

As seen in the two plots above, often times kinks were observed right after the initial rise. This could be due to artifact reasons (like focus drift) or it might signify some more complex underlying phenomenon. We need to rule out experimental errors before investigating this further.

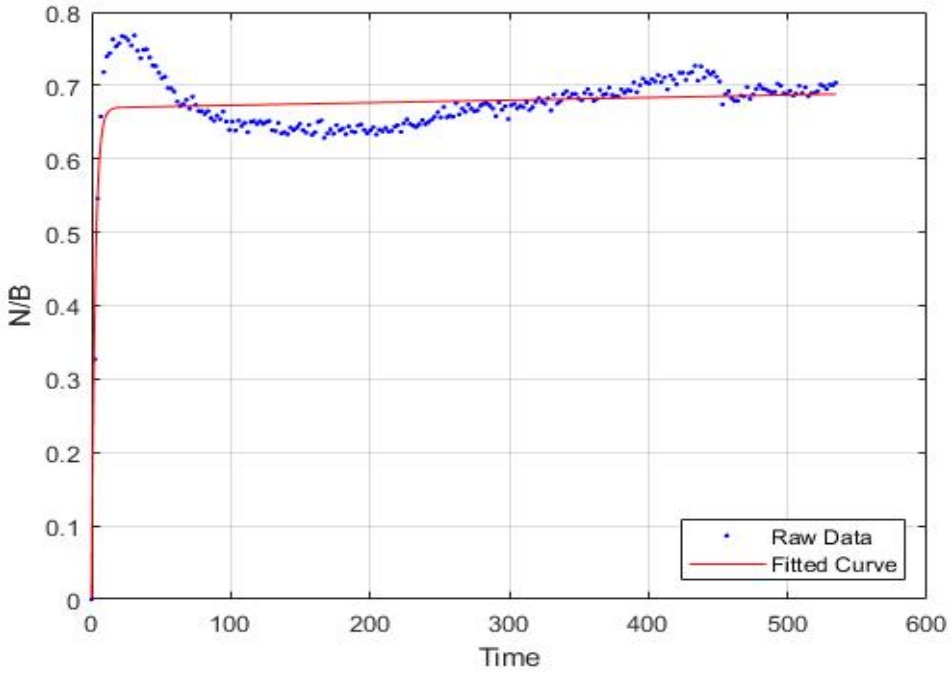

Figure 3.3: Kink is observed at around the 20 second mark

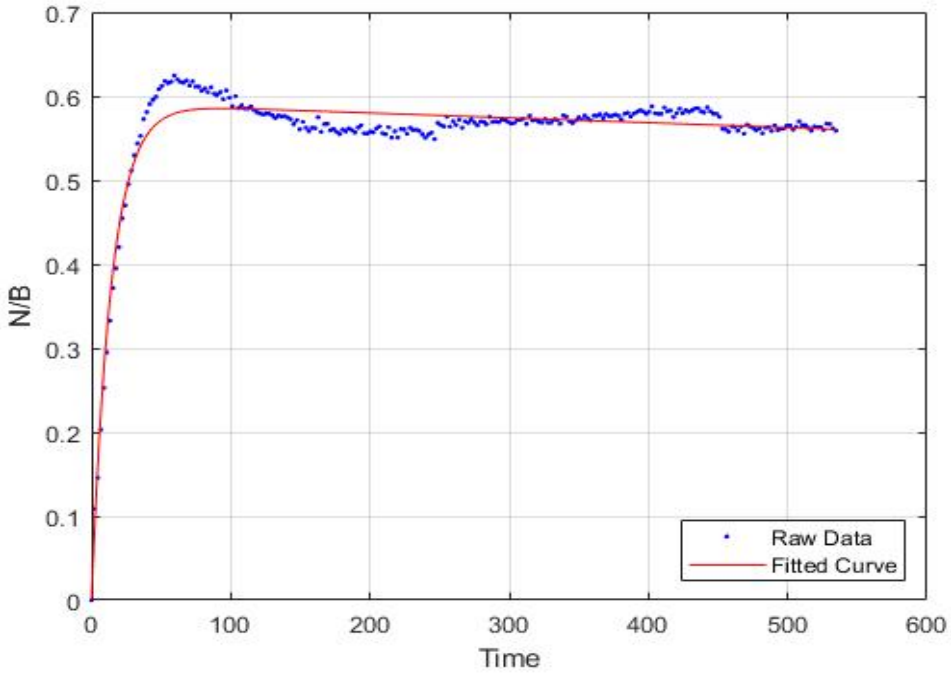

Figure 3.4: Kink is observed at around the 50 second mark

Chapter 4

Conclusion and Future Scope

4.1 Conclusion

In conclusion we have tried to extract diffusion constants for Dextran across the nuclear membrane using a variety of approaches and then fit a model to the same. WE have observed several interesting results such as the active process, kink and deviation from a unity intensity ratio at steady state, all of which warrant further investigation and accurate explanations.

4.2 Future Scope

We need to devise experiments and test out the possible hypotheses we have put forward and look if we observe similar phenomena in different nuclei and/or with different molecules.

References

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