1. a) Assume free diffusion in two dimensions:

$$\langle r^2 \rangle = 4Dt$$

$$t = \frac{\langle r^2 \rangle}{4D} = \frac{(2\mu m)^2}{4 \cdot 5 \cdot 10^{-3} \frac{\mu m^2}{s}} = 200s = 3.33min$$

b) $\langle \theta^2 \rangle = 2D_r t$

$$\theta = (2D_r t)^{\frac{1}{2}} = (2 \cdot 10^4 rad^2 s^{-1} \cdot 200s)^{\frac{1}{2}} = 2000\, rad = 114591.6° = 318.3\, rotations$$

2. The definition of γ is: $\gamma = \frac{\int dr\, [\omega(r)/\omega(0)]^2}{\int dr\, \omega(r)/\omega(0)}$

a) For a cubic probe volume of constant intensity:

$\omega(r) = \begin{cases} \omega(0) & \text{for } x, y, z \le a \\ 0 & \text{for } x, y, z > a \end{cases}$ where a is the side of the cube

Integrating in cartesian coordinates: dr = $dx \cdot dy \cdot dz$

$$\gamma = \frac{\int_0^a dx \int_0^a dy \int_0^a dz}{\int_0^a dx \int_0^a dy \int_0^a dz} = 1$$

b) For a spherical probe volume of constant intensity:

$\omega(r) = \begin{cases} \omega(0) & \text{for } |r| \le R \\ 0 & \text{for } |r| > R \end{cases}$ where R is the radius of the sphere

Integrating in spherical coordinates: dr = $\frac{dr\, d\theta\, d\varphi}{r^2\, sin\theta}$

$$\gamma = \frac{\int_0^R dr \int_0^\pi d\theta \int_0^{2\pi} d\varphi \frac{1}{r^2\, sin\theta}}{\int_0^R dr \int_0^\pi d\theta \int_0^{2\pi} d\varphi \frac{1}{r^2\, sin\theta}} = 1$$

c) For a 3-D Gaussian: ω(r) = $I_0\, e^{-2\frac{(x^2+y^2)}{\omega_r 2} - \frac{2z^2}{\omega_z 2}}$

$$\int dr\, [\omega(r)/\omega(0)]^2 = \int_{-\infty}^{\infty} dx \int_{-\infty}^{\infty} dy \int_{-\infty}^{\infty} dz\, e^{-4\frac{(x^2+y^2)}{\omega_r 2} - \frac{4z^2}{\omega_z 2}} =$$

$$= \int_{-\infty}^{\infty} dx\, e^{-4\frac{x^2}{\omega_r 2}} \int_{-\infty}^{\infty} dy\, e^{-4\frac{y^2}{\omega_r 2}} \int_{-\infty}^{\infty} dz\, e^{-\frac{4z^2}{\omega_z 2}} = \frac{\omega_r\sqrt{\pi}}{2} \frac{\omega_r\sqrt{\pi}}{2} \frac{\omega_z\sqrt{\pi}}{2} =$$

$$= \frac{\omega_z \omega_r{}^2 \pi^{3/2}}{8}$$

$$\int dr\, \omega(r)/\omega(0) = \int_{-\infty}^{\infty} dx\, e^{-2\frac{x^2}{\omega_r 2}} \int_{-\infty}^{\infty} dy\, e^{-2\frac{y^2}{\omega_r 2}} \int_{-\infty}^{\infty} dz\, e^{-2\frac{z^2}{\omega_z 2}} = \frac{\omega_r\sqrt{\pi}}{\sqrt{\pi}} \frac{\omega_r\sqrt{\pi}}{\sqrt{\pi}} \frac{\omega_z\sqrt{\pi}}{\sqrt{\pi}} =$$

$$= \frac{\omega_z \omega_r{}^2 \pi^{3/2}}{2^{3/2}}$$

3. a) $\tau_D = \frac{\omega_r{}^2}{4\, D}$ => $D = \frac{\omega_r{}^2}{4 \cdot \tau_D} = \frac{(200 \cdot 10^{-9} m)^2}{4 \cdot 185 \cdot 10^{-3} s} = 5.4 \cdot 10^{-14}\, m^2/s$

r = $\frac{k_b T}{6\,\pi\,\eta\,D} = \frac{1.38 \cdot 10^{-23}\frac{J}{K} \cdot 298.15\,K}{6\,\pi\,0.8904 \cdot 10^{-3} kg/(m \cdot s) \cdot 5.4 \cdot 10^{-14} m^2/s}$ = $4.54 \cdot 10^{-6}$ m = 4.54 µm

b) G(0) = γ/<N> => <N> = γ/G(0) = $\frac{1}{2^{3/2}}$: 2.7 = 0.131 molecules

$<c> = \frac{<N>}{<V>\,N_A}$

$V_{eff} = \left(\frac{\pi}{2}\right)^{\frac{3}{2}} \cdot \omega_r{}^2 \omega_z = 6.69 \cdot 10^{-20}\ m^3$

$<c> = \frac{0.131}{6.69 \cdot 10^{-20} \cdot 6.022 \cdot 10^{23}}$ mol/m^3 = 3.25 nM

4. Expanding $\Im_A - \frac{\Im_B}{K}$ using the definition for $\Im$:

$$\Im_A - \frac{\Im_B}{K} = \frac{\varepsilon_A \langle N_A \rangle}{\varepsilon_A \langle N_A \rangle + \varepsilon_B \langle N_B \rangle} - \frac{1}{K}\left(\frac{\varepsilon_B \langle N_B \rangle}{\varepsilon_A \langle N_A \rangle + \varepsilon_B \langle N_B \rangle}\right)$$
$$= \frac{1}{\varepsilon_A \langle N_A \rangle + \varepsilon_B \langle N_B \rangle}\left(\varepsilon_A \langle N_A \rangle - \frac{\varepsilon_B \langle N_B \rangle}{K}\right)$$

In equilibrium, $K = \frac{k_+}{k_-} = \frac{\langle N_B \rangle}{\langle N_A \rangle}$, hence

$$\varepsilon_A \langle N_A \rangle - \frac{\varepsilon_B \langle N_B \rangle}{K} = \varepsilon_A \langle N_A \rangle - \frac{\varepsilon_B \langle N_B \rangle}{\frac{\langle N_B \rangle}{\langle N_A \rangle}} = \varepsilon_A \langle N_A \rangle - \varepsilon_B \langle N_A \rangle$$

When states A and B have the same molecular brightness, $\varepsilon_A = \varepsilon_B$ and $\varepsilon_A \langle N_A \rangle - \varepsilon_B \langle N_A \rangle = 0$

Thus $\Im_A - \frac{\Im_B}{K} = 0$ and the relaxation term $K\left(\Im_A - \frac{\Im_B}{K}\right)^2 e^{-\lambda\tau}$ disappears.

5. a) From RICS autocorrelation functions, we can extract the concentration of the proteins from the correlation amplitude G(0), as well as their diffusion coefficients (in the microsecond to second timescale) from the rate of decay of the correlation function. The faster diffusion coefficient of GFP is evident from the quick decay of its autocorrelation function in the y-axis, resulting in a 'flat' ACF.

   b) ICS and RICS are performed on a frame-by-frame basis whereas TICS and STICS are performed in between frames.

   - ICS performs spatial correlation analysis of fluorescence fluctuations within an image
   - RICS exploits the temporal information present within single images obtained by a laser-scanning microscope to spatially correlate adjacent pixels that are a few microseconds apart along a line and a few milliseconds apart in successive lines
   - TICS performs time autocorrelation at one pixel across a stack of successive images
   - STICS is an extension of TICS and relies on a complete calculation of both temporal and spatial correlation lags for intensity fluctuations in different frames of an image series.

6. a) $\frac{dI}{I} = \frac{-P \cdot \pi \cdot r^2 \cdot c \cdot N_A}{1000} \cdot dl = \frac{-\sigma \cdot c \cdot N_A}{1000} \cdot dl$

   Integration over a finite path-length l gives: $\ln\left(\frac{I}{I_0}\right) = \frac{-\sigma \cdot c \cdot N_A}{1000} \cdot l$

Using Lambert-Beer's-Law we get: $A = -\lg\left(\frac{I}{I_0}\right) = \frac{-ln\left(\frac{I}{I_0}\right)}{2.303} = \varepsilon\, c\, l$

Hence: $\frac{\sigma \cdot c \cdot N_A}{1000 \cdot 2.303} \cdot l = \varepsilon\, c\, l$

⇨ $\varepsilon = \frac{\sigma \cdot N_A}{2303}$

b) Extinction coefficient: $\varepsilon = \frac{6.537 \cdot 10^{-14} cm^2 \cdot 6.022 \cdot 10^{23} \frac{1}{mol}}{2303} = 17093 \frac{1}{M\ cm}$

Absorbance of a 9 µM solution: $A = \varepsilon\, c\, l = 17093 \frac{1}{M\ cm} \cdot 9 \cdot 10^{-6}\ M \cdot 1\ cm = 0.154$

7. a) First, we need to determine the concentrations of Cy3 and Cy5, so we can subtract their contribution from the absorption at 280 nm.

$$c_{Cy3} = \frac{Abs_{552}}{\varepsilon_{552}\ell} = \frac{0.105}{150,000\ \mathrm{M}^{-1}\mathrm{cm}^{-1} \cdot 1\ \mathrm{cm}} = 7 \times 10^{-7}\ \mathrm{M} = 700\ \mathrm{nM}$$

$$c_{Cy5} = \frac{Abs_{647}}{\varepsilon_{647}\ell} = \frac{0.055}{250,000\ \mathrm{M}^{-1}\mathrm{cm}^{-1} \cdot 1\ \mathrm{cm}} = 2.2 \times 10^{-7}\ \mathrm{M} = 220\ \mathrm{nM}$$

The concentration of RNAP is determined from the absorption at 280 nm after subtracting absorption from Cy3 and Cy5.

$$c_{RNAP} = \frac{Abs_{280} - \varepsilon_{Cy3,280} c_{Cy3} \ell - \varepsilon_{Cy5,280} c_{Cy5} \ell}{\varepsilon_{280}\ell}$$

$$= \frac{0.275 - 12{,}000\mathrm{M}^{-1}\mathrm{cm}^{-1} \cdot 7 \times 10^{-7}\ \mathrm{M}^{-1} \cdot 1\ \mathrm{cm} - 12{,}500\mathrm{M}^{-1}\mathrm{cm}^{-1} \cdot 2.2 \times 10^{-7}\ \mathrm{M}^{-1} \cdot 1\ \mathrm{cm}}{250{,}000\ \mathrm{M}^{-1}\mathrm{cm}^{-1} \cdot 1\ \mathrm{cm}}$$

$$= 1.06 \times 10^{-6}\ \mathrm{M}^{-1} = 1.06\ \mu\mathrm{M}$$

b)/c) The labeling efficiency of Cy3 and Cy5:

$$eff = \frac{c_{Cy3}}{c_{RNAP}} = \frac{7 \times 10^{-7}\ \mathrm{M}^{-1}}{1.06 \times 10^{-6}\ \mathrm{M}^{-1}} = 0.66 \text{ or } 66\%$$

$$eff = \frac{c_{Cy5}}{c_{RNAP}} = \frac{2.2 \times 10^{-7}\ \mathrm{M}^{-1}}{1.06 \times 10^{-6}\ \mathrm{M}^{-1}} = 0.21 \text{ or } 21\%$$