Answers to Question Set 2

Optics on the small scale

These answers are not always complete, but they do indicate what are the important points. It is necessary to read and understand the papers. The lecture handouts are also helpful to understand what is important in the course. Note that the questions may require you to go beyond the papers specified here in order to find your answers.

For generalized optics see for example:

https://www.microscopyu.com/

http://micro.magnet.fsu.edu/

Questions for Review paper (Moerner)

Most of the questions can be answered by reading the paper[1], but it might be necessary to check the web as well.

1. How large is a typical fluorescent single molecule? E.g. fluorescein or GFP?

One example is GFP (238 amino acids, 28kDa) which is the shape of a barrel and roughly 3nm x 4nm. Flourescein, a common dye molecule, is around 1nm in size.

Note that the fluorescence cross section is not identical to the size of the molecule. It is rather related to the probability of a photon absorption event.

http://www.rcsb.org/pdb/explore/jmol.do?structureId=1GFL&bionumber=1

2. What are the benefits of single-molecule measurements over bulk measurements?

Information about *heterogeneous systems* – By measuring one molecule at a time any information that would otherwise be lost due to ensemble averaging can be accessed. The exact distribution of properties can be measured and subpopulations can be detected and visualized in histograms. On the other hand, in an ensemble measurement, only the average and possibly the standard deviation can be measured.

Local probe – The excitation and emission spectra of a single molecule is dependent on its local environment. A molecule is essentially a point source of light and can be used for precise measurements of position.

Time trajectories – if a molecule goes through several consecutive states, the order of these states can be determined only if one single molecule is observed over the time it takes for the states to evolve. In ensemble measurements this type of information may be accessible if the molecules can be synchronized, but synchronization is in general very difficult if not impossible. Furthermore, rare intermediate states are more easily detected on a single-cell basis.

3. How are single-molecule measurements implemented? What are the most common single-molecule techniques used to make it possible to see single molecules? What do I need to consider in order to succeed in detecting single molecules?

Some examples of techniques that are used to enable SMD: total internal reflection (TIR), confocal microscopy, two-photon confocal microscopy, localization enhancement, nearfield microscopy (NSOM). Localization microscopy relies on diffusion of the light emitters. If diffusion is hindered, the light is not spread out over a large area during exposure. In that way the S/N of the emitter is large enough that the emitter can be detected.

The main point to consider is background fluorescence. The fluorescence from water is very week, but in the focal volume of a microscope there are a lot of water molecules and only one single fluorescent molecule. It is therefore important to keep the excitation volume as small as possible. Background fluorescence is stronger at shorter wavelengths. Therefore, one should try to choose a dye that can be excited with reasonably long wavelengths such as in the green and upwards. The development of dyes that operate in the red or NIR addresses this problem. Secondly, the samples must be dilute enough to allow only one single molecule in the excitation volume.

It is important to adjust the excitation level such that the S/N is at a maximum. Increasing the excitation power above the optimum level (where the dye saturates) will not give rise to any more signal. It will just increase the background level.

4. How much light can I get from one single molecule? At what rate? What is the maximum total number of photons that I can get from one single fluorescent molecule?

The amount of light that can be extracted from one single molecule is limited by the lifetimes of the excited states, the probability of photodamage and the occurrence of dark states.

A typical maximum emission rate is $<10^8$ photons/second, $\tau_F \sim 10$ns

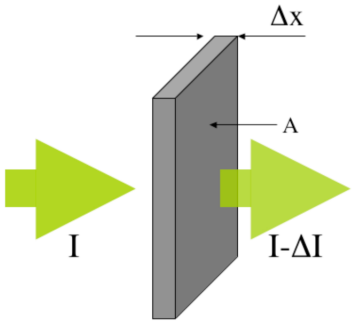
The probability of photodamage (*i.e.* bleaching) is typically $\sim 10^{-6}$. On average we can therefore expect every molecule to emit 10^6 photons and with a typical detection efficieny of 1% - 10% one can expect to detect 10^4 - 10^5 photons from each molecule.

5. (**) What is extinction coefficient and absorption cross-section? How can the absorption cross section be calculated from the extinction coefficient?

The molar extinction coefficient ε is used in bulk measurements. Consider a cuvette and a beam of light with photon flux I_0. After passage through the cuvette, the light will have a photon flux of $I(x) = I_0\ 10^{-\varepsilon c x}$, where c is the concentration of the solution and x is the distance the light traveled through the solution.

The absorption cross σ section is used in single-molecule measurements and it gives the effective size of the molecule. If I_0 is the incoming photon flux (number of photons per second) and focused to an area A, we have for the absorption rate $I_{abs} = \frac{I_0}{A}\sigma$.

We have $\sigma \sim \frac{\varepsilon\ \ln 10}{N_A}$. Derive the expression! The difficulty is in keeping track of the units of the involved volumes and concentrations.



BULK (Chemistry) - Lambert-Beer's law and take the derivative with respect to x:

$I = I_0\ 10^{-\varepsilon c x} \Rightarrow$

$$\frac{dI}{dx} = I_0\ \frac{d}{dx}10^{-\varepsilon c x} = I_0\ \frac{d}{dx}e^{\ln 10^{-\varepsilon c x}} = I_0\ \frac{d}{dx}e^{-\varepsilon c x \ln 10} = -\varepsilon c\ \ln 10\ I_0 10^{-\varepsilon c x} = -\varepsilon c\ \ln 10\ I$$

This gives us for small Δx:

$$\Delta I = -I\ \varepsilon c\ \ln 10\ \Delta x$$

Add dimensions to the expression:

$$\Delta I = -I\left(\frac{\varepsilon}{M^{-1}cm^{-1}}\right)M^{-1}cm^{-1}\left(\frac{c}{M}\right)M\ \ln 10\left(\frac{\Delta x}{cm}\right)cm = \Delta I = -I\left(\frac{\varepsilon}{M^{-1}cm^{-1}}\right)\left(\frac{c}{M}\right)\ln 10\left(\frac{\Delta x}{cm}\right)$$

SINGLE MOLECULE (Physics) - Consider the absorption cross-section and create a similar expression.

$$\Delta I = -\frac{I}{A}\sigma\Delta N$$

Here ΔN is the number of molecules in the volume:

$$\Delta N = c\,\Delta x\,A$$

where N_A = 6 10^{23} mol^{-1} is Avogadro's number and the concentration is expressed in moles per L = 10^{-3} m^3.

Now we have:

$$\Delta I = -\frac{I}{A}\sigma\, c\,\Delta x\, A = -I\,\sigma\, c\,\Delta x$$

Add dimensions to the expression:

$$\Delta I = -I\,\sigma\, c\,\Delta x = -I\left(\frac{\sigma}{m^2}\right)m^2\left(\frac{c}{m^{-3}}\right)m^{-3}\left(\frac{\Delta x}{m}\right)m = -I\left(\frac{\sigma}{m^2}\right)\left(\frac{c}{m^{-3}}\right)\left(\frac{\Delta x}{m}\right)$$

Combine the bulk and single-molecule expressions above:

$$\left(\frac{\sigma}{m^2}\right)=\left(\frac{\varepsilon}{M^{-1}cm^{-1}}\right)\left(\frac{c}{M}\right)\left(\frac{c}{m^{-3}}\right)^{-1}\left(\frac{\Delta x}{cm}\right)\left(\frac{\Delta x}{m}\right)^{-1}\ln 10$$

$$\Rightarrow\left(\frac{\sigma}{m^2}\right)=\left(\frac{\varepsilon}{M^{-1}cm^{-1}}\right)\left(\frac{cL}{mol}\frac{1}{N_A mol^{-1}}\frac{1}{1000Lm^{-3}}\frac{m^{-3}}{c}\right)\left(\frac{\Delta x}{cm}\frac{m}{\Delta x}\frac{100cm}{m}\right)\ln 10$$

$$\Rightarrow\left(\frac{\sigma}{m^2}\right)=\left(\frac{\varepsilon}{M^{-1}cm^{-1}}\right)\left(\frac{1}{N_A}\frac{1}{1000}\right)(100)\ln 10$$

$$\Rightarrow\sigma=\varepsilon\frac{1}{N_A}\frac{\ln 10}{10}\frac{m^2}{M^{-1}cm^{-1}}$$

where ε is expressed in units of $M^{-1}cm^{-1}$, σ is expressed in m^2 and $N_A=6\ 10^{23}$.

If we prefer to express σ in cm^2 instead, we have:

$$\sigma=\varepsilon\frac{1}{N_A}\frac{\ln 10}{10}\frac{m^2}{M^{-1}cm^{-1}}\frac{10000cm^2}{m^2}=\varepsilon\frac{1}{N_A}1000\ln 10\frac{cm^2}{M^{-1}cm^{-1}}$$

6. What is blinking? What is the physical basis for blinking?

A single molecule will switch from an emitting state to a dark state, ON-OFF, on a one-second timescale. For GFP the underlying reason is a photoisomerization (change in shape) of the fluorophore. It goes from an uncharged to a charged state and back.

A blinking behavior can also be observed for molecules that shift emission wavelength if the detection filter transmits one but not both wavelengths.

7. What is bleaching? What is the physical basis for bleaching? What can you do to avoid bleaching?

Bleaching is photoinduced permanent damage to the fluorophore. It is typically due to an oxidation of the fluorophore that damages the molecule permanently. It is also associated with the fluorophore molecule going into a triplet state, thereby exciting triplet ground-state oxygen to highly reactive singlet excited state oxygen that in turn will damage the fluorophore.

To avoid photobleaching there are four main approaches: (1) Create a reducing environment to counteract the oxidizing power of any oxygen present in the solution. This can be done by adding *e.g.* mercaptoethanol or dithiothreitol (DTT). (2) Remove oxygen by enzymatic processes (catalase and gluocose oxidase). (3) Remove oxygen by degassing using vacuum and ultrasonication of the solution and/or flushing the solution with an inert gas such as N_2 or Ar. (4) Use compounds that decrease the probability of ISC, *i.e.* reduces the probability of the fluorophore to assume a triplet state. The first three are standard approaches in molecular biology.

Using q-dots is another way to decrease any bleaching problems. They emit at least one order of magnitude more photons than standard organic dye molecules.

8. In a typical experiment I would see spots in the field of view of my CCD camera. How do I know that I am looking at a single molecule?

Reasonable number of photons – Calculate the number photons expected to be emitted from a typical molecule at the given excitation power. Calculate the detection efficiency of your microscope (typically 1-10%).

ON/OFF – A single molecule is either ON or OFF, not in between. If a gradual decay of the emission is observed, then it is most probably a large number of molecules. If a two-step decay is observed, we have probably two molecules. Blinking is common for single molecules. Bleaching will happen after sufficient number of photons have been emitted. In both cases the switch from ON to OFF is immediate.

Antibunching – A single fluorophore cannot emit more than one photon at the same time. By measuring the autocorrelation of the photon emission, a dip to zero in the autocorrelation at zero time delay indicates a single-molecule emitter.

$$c(\tau) \sim \int I(t) I(t-\tau) dt$$

9. What is FRET? What length scales can I probe using FRET? What are the limitations?

Fluorescence resonance energy transfer (FRET) is a very useful technique for measuring processes that take place on a length scale on the order of 1nm-10nm.

The principle is simple: Two fluorophores with overlapping emission and excitation spectra are used. The emission band of the donor fluorophore overlaps with the excitation spectra of the acceptor fluorophore. The two fluorophores are attached on two parts respectively on the molecule of interest, between which the distance needs to be measured. The donor is now excited and the donor and the acceptor fluorescence is measured. If the donor and the acceptor are in close proximity, the energy will flow directly to the acceptor and the acceptor emission will be enhanced, whereas the donor emission will be quenched. If the distance is far, mainly the donor will emit light. The energy transfer efficiency E is related to the distance R between the fluorophores by $E = \frac{I_{donor}}{I_{acceptor} + I_{donor}} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$. The characteristic length scale (Förster length) $R_0 \sim 5$nm.

The technique is limited to distance measurements in the range 1nm-10nm. It is sensitive to the orientation of the fluorophores and the local environment.

Questions for photoacoustic microscopy

Most of the questions can be answered by reading the paper[2], but it might be necessary to check the web as well.

1. Describe the key concept of the technique of photoacoustic microscopy! What property of the sample defines the contrast in the resulting image?

- Standard confocal microscope with ultrasound detector.
- Pulsed laser in infrared heats up the material. The heating causes expansion. The periodic expansions cause sound waves that are detected.
- The result can be viewed as a map of the *absorption* coefficient of a 3D object without any labels. The contrast mechanism is based on *absorption*. To be more precis the Grüneisen parameter Γ of the tissue should be considered. It relates the initial pressure p_0 to the light absorption: $p_0 = \Gamma \mu_a F$, where μ_a is the absorption coefficient of tissue, and F is the local light fluence. To determine and map the *absorption* coefficient, it is therefore necessary accurately know the Grüneisen parameter[3].

Questions on super resolution microscopy

Most of the questions can be answered by reading the review paper[4], but it might be necessary to check references that the papers does to the literature as well as the web.

1. What is the diffraction limit? What is the Rayleigh criterion? Make a drawing!

The ability to distinguish between two sources of light (or two objects) spaced close to each other is limited by diffraction – for a circular hole by the size of the Airy disk.

In a microscope with an objective of various numerical apertures (NA) and magnifications, the optical resolution limit is solely determined by the numerical aperture:

$$r = \frac{0.66\,\lambda}{NA}$$

where λ is the wavelength of the light and $NA = n \sin \theta$, where n is the index of refraction of the medium and θ is the collection angle of the objective.

2. Can you localize the position of a point-source of light without violating the diffraction limit? Elaborate! What limits the level of uncertainty that you can reach? In practice how low uncertainty can you reach? What is FIONA?

Yes! Diffraction limits the *resolution*, but not the ability to determine position. In other words, two light sources positioned close to each other are increasingly difficult to resolve as their distance decreases, but the position of a single light source can in fact be determined to an arbitrary precision provided enough photons are collected. This is the basic idea behind FIONA[5] (fluorescence imaging with one nanometer accuracy).

In the paper, the position was determined to a precision of 1.5nm of the fluorophore that was observed at two different points in time. To simplify (neglecting finite pixel size and background light), the uncertainty is determined by the diffraction limited resolution (the size of the Airy disk, which is the positional uncertainty of one photon) divided by the square root of the number of photons, *N*.

$$r = \frac{0.66\lambda}{NA}\frac{1}{\sqrt{N}}$$

This approach is used in various types of super-resolution techniques:

- Color multiplexing - Use two (or more) different fluorophores, not separated in time, but in wavelength.

- Blinking – Use the propensity of blinking to image a subset of the fluorophores at a given time. Repeat and combine resulting images to a final high-resolution image.

- STORM – Activate a random subset of the fluorophores and acquire an image. Repeat and combine the resulting data to a high-resolution image.

3. Describe the basic idea of STORM (aka FPALM). How is it performed in practice? Are there any special requirements on the dyes used?

STORM (Stochastic optical reconstruction microscopy) is based on the idea that a random subset of fluorophores is activated. The density of the fluorophores is such that the fluorophores can be resolved optically. In this way, using FIONA[5], the position of each fluorophore can be determined to a high accuracy. By repeating the activation several images is the result each with the positions of its fluorophores accurately determined. By combining the images, a high-resolution image is the result.

In practice for the dye system used in the early paper[6]:

-strong RED light to deactivate all fluorophores
then
-weak GREEN light to activate a small subset
-weak RED to image the activated fluorophores until switched off
- go back to weak GREEN and repeat the process until most of the fluorophores have been imaged at least once and/or most of the fluorophores are bleached so that no further measurements are possible.

Obviously, the dye system must be switchable. In the first paper a system of Cy5 and Cy3 in close proximity is used. The Cy3 must be as close as possible to the Cy5 to ensure high recovery rate.

4. What resolution is it possible to reach using STORM? What limits the attainable resolution?

The resolution demonstrated in the paper is 20nm, but this could be improved.

The number of photons emitted from each fluorophore limits the uncertainty attainable using the FIONA[5] approach, which in turn fundamentally limits the resolution attainable using STORM. In their experiment they found that they could detect ~3000 photons from each switching cycle. This corresponds to a FIONA uncertainty of 4nm, which in turn translates into a 20nm STORM resolution.

5. How long time does it take to acquire data of a typical object?

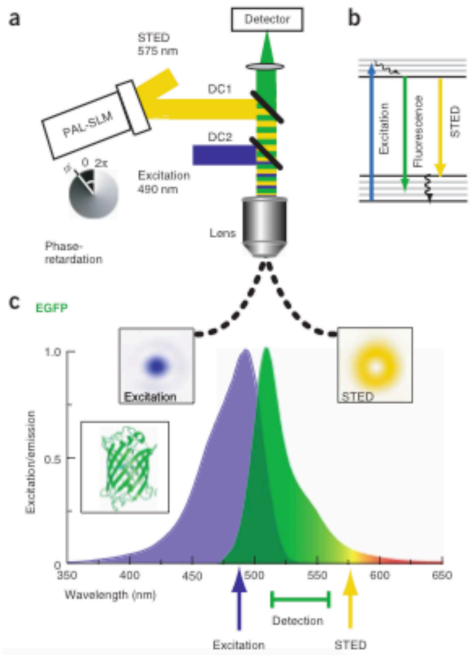
A few minutes, but it depends on how many fluorophores need to be imaged and how many cycles are needed. This can be improved by faster switching dyes and by stronger excitation. Note that saturation sets an ultimate limit.

6. How can 3D information be obtained in STORM?

The microscope is made astigmatic such that the focal spot is elliptic with a direction that depends on whether the focal plane is above or below the dye molecule.

7. How does STED work? What is the key idea? Does it need a pulsed laser?

Figure 1 from the paper [7] explains the principle fairly well. The key idea is that two lasers are combined. One is exciting fluorophores in a diffraction limited Gaussian spot. The other (donut-shaped) is "removing" the emission locally by deexcitation in a ring around the Gaussian spot. Both spots are diffraction limited, but through ***depletion of the stimulated emission*** a final subdiffraction spot size is obtained.



The de-excitation takes place through stimulated emission, which is the process where light of one wavelength stimulates the emission of light from excited molecules at the same wavelength. The excited molecules illuminated by the STED-beam (yellow in the figure) is thus emitting yellow light, which in turn is blocked by the choice of a filter that only transmits the green light.

In principle, STED does not require pulsed lasers, but in practice pulsed lasers have shown to give better results. In that case the excitation pulse is synchronized with the STED pulse such that the STED pulse illuminates the sample shortly after the excitation.

8. What resolution is attainable in STED? What limits the resolution?

Around 20nm is reported. The resolution is given by

$$r \cong \frac{0.66\lambda}{NA} \frac{1}{\sqrt{1+\frac{I_{STED}}{I_{SAT}}}}$$

In other words, the resolution is basically the diffraction limited resolution but decreased by the STED factor. By increasing the power of the STED-beam, I_{STED}, the STED factor is increased which in turn improves the resolution further. There is no fundamental limit as to how good resolution one can achieve. I_{SAT} corresponds to the intensity of the STED beam that decreases the fluorescence a factor of e~2.718.

However, if the power of the STED is too strong the probability of two-photon excitation becomes significant. Another effect of excessive STED power is heating and sample damage.

9. How well is the axial (along optical axis) resolution improved by STED? How can the situation be improved?

To improve the axial resolution, an additional STED beam can be used with lobes above and below the focus spot of the exciting laser beam[8]. Also, the 4Pi technology could be used, where the sample is placed in between two objectives thereby collecting the light from a solid angle that is double that of standard microscopy. Improvement of axial resolution to ~100nm combined with lateral resolution of 45nm.

10. How are STED, GSD, RESOLFT related? What is the main fundamental difference between the techniques? What is the main technological difference between the techniques?

These techniques have similar setups. However, the physical mechanisms are different. A donut-shaped focus of light is used to shift the fluorophores to a dark state around the main focus. The amount of light needed for the donut depends on the lifetimes involved. For short lifetimes, the dark state is short lived and must be "revived" often. In other words, a large intensity is required. For long lifetimes the dark state is long lived and so the number of photons in the donut do not need to be large per unit time.
The lowest power needed is for RESOLFT (dark state due to isoforms (dye changes shape); lifetime ~ms; mainly for fluorescent proteins). The highest STED (dark state due to stimulated emission; fluorescence lifetime ~ns). GSD in between (dark state due to intersystem crossing to triplet state; lifetime ~μs).
RESOLFT is also a term that is used to cover all techniques that are related to STED. It stands for REversible Saturable/Switchable Opticaly Linear Fluorescence Transitions. See links for first hand explanation:
http://zeiss-campus.magnet.fsu.edu/tutorials/superresolution/resolft/indexflash.html
See also link to Nobel lecture by Stefan Hell:
http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/hell-lecture.html

11. How long time does it take to acquire a typical image? In which ways has the frame rate been improved since the first STED experiments? Consider also other techniques that are related to STED.

Typical dwell time is ~1ms per pixel. For a 500x500 pixel image that corresponds to roughly 5 minutes acquisition time.

Video-rate acquisition with 35ms for each frame (28 frames per second) [9]. The pixel dwell time was between 3.8μs and 6μs (see suppl mtrl). Even faster in [10], 500fps. Extremely fast with resonant scanning, more than 1000 fps[11].

Multi-spot array of RESOLFT (low required intensities)[12] gives reasonable speed (1 fps) for very large areas and number of pixels. 100 000 STED donuts in parallel!

References

1. Moerner, W.E. and D.P. Fromm, *Methods for single-molecule fluorescence spectroscopy and microscopy*. Review of Scientific Instruments, 2003. **74**(8): p. 3597-3619.
2. Wang, L.V., *Multiscale photoacoustic microscopy and computed tomography*. Nat Photon, 2009. **3**(9): p. 503-509.
3. Yao, D.-K., et al., *Photoacoustic measurement of the Grüneisen parameter of tissue*. Journal of Biomedical Optics, 2014. **19**(1): p. 017007.
4. Huang, B., M. Bates, and X. Zhuang, *Super-Resolution Fluorescence Microscopy*. Annual Review of Biochemistry, 2009. **78**(1): p. 993-1016.
5. Yildiz, A., et al., *Myosin V walks hand-over-hand: Single fluorophore imaging with 1.5-nm localization*. Science, 2003. **300**(5628): p. 2061-2065.
6. Rust, M.J., M. Bates, and X.W. Zhuang, *Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM)*. Nature Methods, 2006. **3**(10): p. 793-795.
7. Willig, K.I., et al., *Nanoscale resolution in GFP-based microscopy*. Nature Methods, 2006. **3**(9): p. 721-723.
8. Wildanger, D., et al., *A compact STED microscope providing 3D nanoscale resolution*. Journal of Microscopy, 2009. **236**(1): p. 35-43.
9. Westphal, V., et al., *Video-rate far-field optical nanoscopy dissects synaptic vesicle movement*. Science, 2008. **320**(5873): p. 246-249.
10. Moneron, G., et al., *Fast STED microscopy with continuous wave fiber lasers*. Optics Express, 2010. **18**(2): p. 1302-1309.
11. Schneider, J., et al., *Ultrafast, temporally stochastic STED nanoscopy of millisecond dynamics*. Nature Methods, 2015. **12**: p. 827.
12. Chmyrov, A., et al., *Nanoscopy with more than 100,000 'doughnuts'*. Nat Meth, 2013. **10**(8): p. 737-740.