CCD & Fluorescence Imaging Essentials

Remarks

- *2013-10-31 - Version 1.7 by Sebastian Rhode*
- *No guarantee that the formulas are correct !!!*
- *Feel free to correct errors!*

Thermal and Photon noise

Thermally generated charges N_{dark} on the CCD array contribute to the overall signal and are referred to as dark charge. The generation of the dark charge follows a poisson distribution and the resulting dark noise per pixel σ_{dark} is proportional to the square root of the number of dark charge electrons in that pixel

$$\sigma_{dark} = \sqrt{N_{dark}} \ .$$

Another important source of noise is the inherent noise of every incoming photon σ_{signal} , usually called shot noise. The electrons generated by P photons on a pixel of the CCD chip with a quantum efficiency Q_E will have a noise given by poisson statistics

$$\sigma_{signal} = \sqrt{Q_E P} \ .$$

Dynamic Range

The dynamic range in mathematical terms defined as

$$dynamic\ range = \frac{full\ well\ capacity\ [e^-]}{\sigma_{read}\ [e^-]} \ .$$

The full well capacity represents the number of electrons that can be stored in each pixel and the read noise σ_{read} is the noise produced by the ADC procedure. Typically the dynamic range is given in “bit”, which refers to the number of intensity values that are distinguished in the ADC process. The actual dynamic range of a camera system is determined by the minimum ADC value – given in bit - and the dynamic range. For example 100ke^- full well capacity, 5e^- rms (root mean square) read noise and a 15 bit ADC achieves an actual dynamic range of $\log_2\left(\frac{1\times10^5}{5}\right) \approx 14.3\ bit$.

Detectability – SNR

The sensitivity of detection devices depends on the signal-to-noise ratio (SNR) that can be achieved within a given time. In fluorescence microscopy, the SNR is defined as the ratio between the background noise and the signal intensity. The background noise is calculated as the standard deviation of the background signal on a pixel to pixel basis. For signals spread over more than one pixel, the background noise is scaled with the square root of the area of the signal at half maximum because of the shot noise nature of the present noise sources. Sources of background noise are

photon noise and noise of the detection system. Photon arrival is a statistical process that is described by the Poisson distribution. The noise generated by this process is shot noise and can be calculated using

$$SNR = \frac{PQ_E\, t}{\sqrt{F_n^2 (P+B)\, Q_E\, t + F_n^2 N_{dark}\, t + \left(\sigma_{read} / gain\right)^2}}$$

$$SNR = \frac{F\, t}{\sqrt{F_n^2 \left(F + N_{dark}\right) t + \left(\sigma_{read} / gain\right)^2}}$$

- ***P*** = photon flux incident on the CCD (photons/pixel/second)
- ***B*** = background photon flux; when signal is the only light → B=0
- Q_E = quantum efficiency
- ***t*** = integration time (seconds)
- N_{dark} = dark current (electrons/pixel/second)
- F_n = noise factor (=1.41 for EM-CCDs, =1 for CCD or sCMOS cameras)
- σ_{read} = read out noise (electrons rms/pixel)
- ***gain*** = EM gain factor (= 1 for standard CCDs or sCMOS)

Here *F* is the detected fluorescence intensity in counts per second, *t* is the integration time in seconds, N_{dark} the dark current in electrons per second and σ_{read} the read noise in electrons rms. Saturation effects and photobleaching limit the number of photons per time frame and the total number of photons per dye until bleaching.

Binning

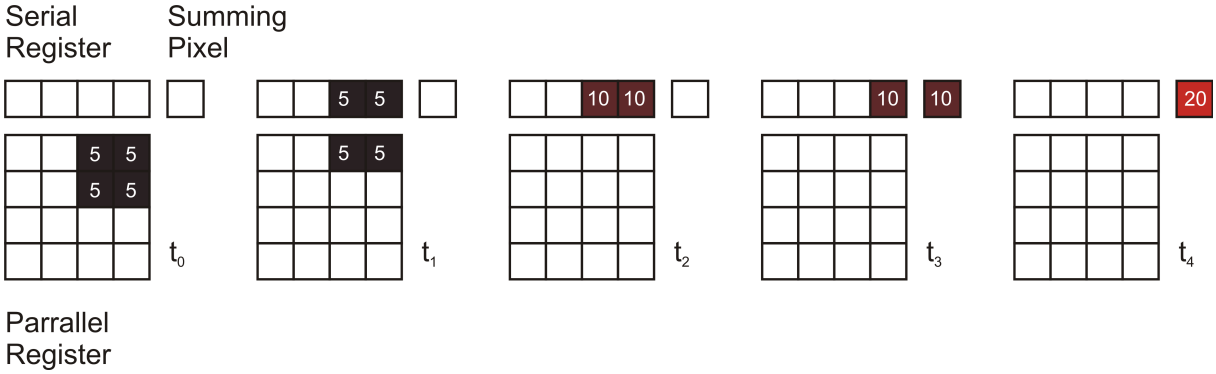
Binning combines neighbouring pixel of a CCD array to one superpixel. The resolution is reduced, while the SNR increases proportional to the binning factor. A binning factor of n_{bin} = 4 means you add up the intensity from 4 adjacent pixel. The signal *F* increases, while the read noise σ_{read} from the ADC process remains constant, when reading out the superpixel.

$$SNR = \frac{n_{bin}\, PQ_E\, t}{\sqrt{(P+B)\, n_{bin}\, Q_E\, t + n_{bin} N_{dark}\, t + \sigma_{read}^2}}$$

$$SNR = \frac{n_{bin}\, F\, t}{\sqrt{\left(F + N_{dark}\right) n_{bin}\, t + \sigma_{read}^2}}$$

Therefore adding up n_{bin} pixel, will increase the SNR by a factor of ≈ sqrt (n_{bin}) compared to a single pixel readout, where you sum the number of n_{bin} pixel

afterwards. For instance, a binning of 2x2 will increase the SNR ratio by a factor of sqrt(n_{bin}) = 2.



Scheme of signal summation for 2 × 2 binning. The information content of 4 adjacent pixels on the CCD chip is integrated into one output pixel. The resulting increase in signal improves the SNR when the read out noise is limiting.

For camera systems with dominating read noise, binning allows increasing the SNR and increases the speed of the camera system because of the lower number of superpixels that have to be digitised. One drawback is a reduced spatial resolution. Moreover, for samples with strong background signals (out of focus fluorescence, dark noise, etc.), the signal addition can cause overloads in the summing pixel because of its limited full well capacity. In contrast to the read noise, dark noise is not reduced while binning, therefore cooling of the CCD remains essential to achieve a good SNR.

SNR and Binning for CMOS cameras

It is important to point out that the improvement in SNR cannot be achieved when one is using a CMOS sensor. Here one cannot improve the SNR similar to a CCD camera since the signal cannot be summed up before readout but only afterwards.
Due to this fact the read noise of and sCMOS will also increase by a factor of sqrt(n_{bin}), e.g. by factor of two for 2x2 binning using an sCMOS camera.

Pixel Size Requirements for matching optical resolution

In order to optimize the information content of the resulting image, the resolution of the detector must closely match that of the microscope. The limits of spatial resolution are also dictated by the diffraction of light through the optical system, a term that is generally referred to as diffraction limited resolution. Investigators have derived several equations that have been used to express the relationship between numerical aperture, wavelength, and optical resolution:

$$r = \frac{0.61 \times \lambda}{NA} \quad (1) \qquad r = \frac{1.22 \times \lambda}{(NA_{OBJ} + NA_{COND})} \quad (2)$$

Here r is resolution (the smallest resolvable distance between two specimen points), NA equals the objective numerical aperture, λ equals wavelength, NA_{OBJ} equals the objective numerical aperture, and NA_{COND} is the condenser numerical aperture. These equations are based upon a number of factors, including a variety of theoretical calculations made by optical physicists to account for the behaviour of objectives and condensers, and should not be considered an absolute value of any one general physical law. The following equations are a vital importance for the understanding of the resolution limit in optical microscopy.

For a point light source, which is located within a finite distance from the aperture, the image will be located at a distance s_i from the lens. The radius of the corresponding Airy-Disk will be $p = 1.22 \frac{s_i \lambda}{D}$, where D is the aperture diameter. Within this context the formula is called Point-Spread-Function (PSF). For high performance objective the object is located very close to the focus. Therefore the following applies

$$\frac{p'}{p} \approx \frac{f}{s_i} \quad \Rightarrow \quad p' = 1.22 \frac{f \lambda}{D}.$$

With the definition of the aperture of an objective, $NA = \frac{R}{f}$ one can write

$$p' = 1.22 \frac{R \lambda}{D\ NA} \quad \Leftrightarrow \quad p' = 1.22 \frac{1}{2} \frac{\lambda}{NA}\ p' = 0.61 \frac{\lambda}{NA}.$$

Here *p`* can be interpreted as the smallest possible distance, which is required for the separate imaging of two point light source (Rayleigh-Criterion). In this case the centre of a light source will be imaged at the location of the first minimum of the second light source.
Another point the user must pay attention to is the Nyquist-Theorem. In order to correctly image periodic structures the radius of the Airy-Disk has to be imaged onto 2 pixels of your CCD chip.

$$\frac{0.61 \lambda}{NA} \times M_{Objective} = 2 \times pixelsize \quad (Nyquist - Theorem)$$

In some instances, such as confocal and multiphoton fluorescence microscopy, the resolution may actually exceed the limits placed by any one of these three equations. Other factors, such as low specimen contrast and improper illumination, may serve to lower resolution and, more often than not, the real-world maximum value of **r** (about 0.20µm using a mid-spectrum wavelength of 550nm) and a NA of 1.35 to 1.40 are not realized in practice.

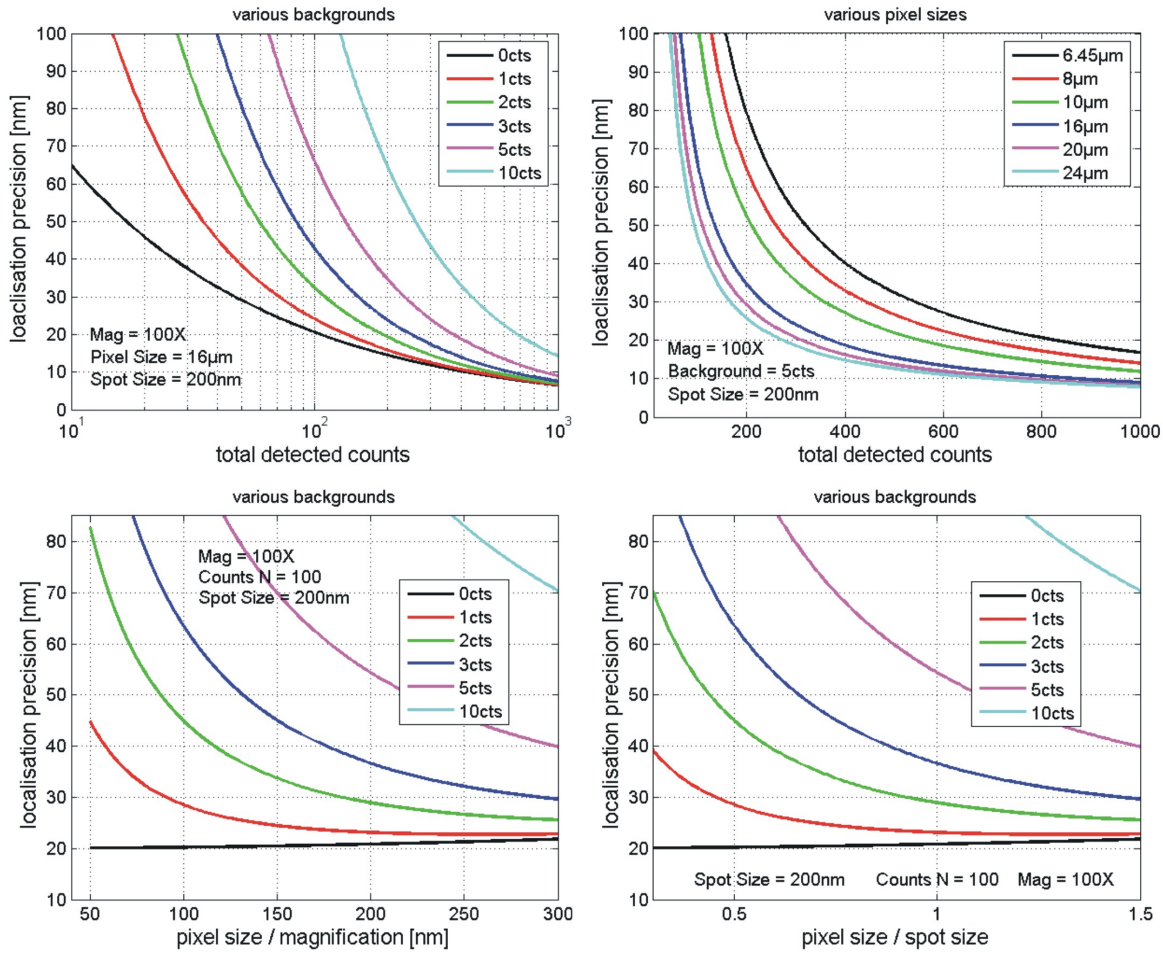
Note is that magnification does not appear as a factor in equations 1 or 2, because only numerical aperture and the wavelength of the illumination determine specimen resolution.

Objective (Numerical Aperture)	Resolution Limit (Micrometers)	Projected Size (Micrometers)	Required Pixel Size (Micrometers)
1x (0.04)	6.9	6.9	3.5
2x (0.06)	4.6	9.2	4.6
2x (0.10)	2.8	5.6	2.8
4x (0.10)	2.8	11.2	5.6
4x (0.12)	2.3	9.2	4.6
4x (0.20)	1.4	5.6	2.8
10x (0.25)	1.1	11.0	5.5
10x (0.30)	0.92	9.2	4.6
10x (0.45)	0.61	6.1	3.0
20x (0.40)	0.69	13.8	6.9
20x (0.50)	0.55	11.0	5.5
20x (0.75)	0.37	7.4	3.7
40x (0.65)	0.42	16.8	8.4
40x (0.75)	0.37	14.8	7.4
40x (0.95)	0.29	11.6	5.8
40x (1.00)	0.28	11.2	5.6
40x (1.30)	0.21	8.4	4.2
60x (0.80)	0.34	20.4	10.2
60x (0.85)	0.32	19.2	9.6
60x (0.95)	0.29	17.4	8.7
60x (1.40)	0.20	12.0	6.0
100x (0.90)	0.31	31.0	15.5
100x (1.25)	0.22	22.0	11.0
100x (1.30)	0.21	21.0	10.5
100x (1.40)	0.20	20.0	10.0

As mentioned above (and can be observed in the equations) the wavelength of light is an important factor in the resolution of a microscope. Shorter wavelengths yield higher resolution (lower values for r) and vice versa. The greatest resolving power in optical microscopy is realized with near-ultraviolet light, the shortest effective imaging wavelength. Near-ultraviolet light is followed by blue, then green, and finally red light in the ability to resolve specimen detail. For the calculation of the resolution values a wavelength of 450nm was used.

SNR and positional accuracy

One principal advantage of optical single molecule microscopy is the possibility to determine the position of single molecules beyond the diffraction limit as shown in the figures below. Depending on the SNR ratio it is possible to achieve positional accuracies down to a few nanometers, which is far below the diffraction limit in conventional optical microscopy $d = 0.61 \times \lambda / NA \approx$ 200–300nm. Structural information on this length scale is not accessible with normal optical microscopy. Moreover the use of two different fluorescent dyes which are spectrally separated gives the opportunity to carry out colocalization or binding studies at this length scale.



Theoretical results for the localization precision plotted as a function of the total number of detected counts N for background noise b = 0, 1, 2, 3, 5, 10 counts. The results also depend on the pixel size the spot size and the ratio pixel size / spot size. Note that the quantum efficiencies of different CCD systems were not taken into account for this calculation.

From these calculations it becomes obvious which parameters have to be optimized for (single) fluorescent-particle localization and tracking. The attention should be paid to maximizing the detected fluorescence and minimizing the measurement noise. Typical estimates for the overall photon-detection efficiency in modern microscopes are around 1-5%! The use of high NA objectives is essential both to decrease spot size and to maximize the number of detected photons. Always keep in mind that the optical resolution of your single molecule microscope remains unchanged but the positional accuracy can go beyond, if your system is well optimized.

CCD-Comparison

In order to make fair comparison between different CCD model and camera types one has to consider different sources of noise, the actual photon flux and the pixel size of the detection device. All these information are required to correctly calculate the SNR using the following formula:

$$SNR = \frac{Q_E * P}{\sqrt{N_F^2 * \left(Q_E * P + \delta_{dark}^2 + \delta_{cic}^2\right) + \frac{\delta_{readout}^2}{M^2}}}$$

Q_E	= quantum efficiency of the detection device
P	= number of photons falling on a pixel
N_F	= noise factor from amplifying the signal
δ_{dark}	= noise from thermally generated electrons
δ_{cic}	= clock induced charge or spurious noise
$\delta_{readout}$	= readout noise
M	= gain factor of EM-amplifier

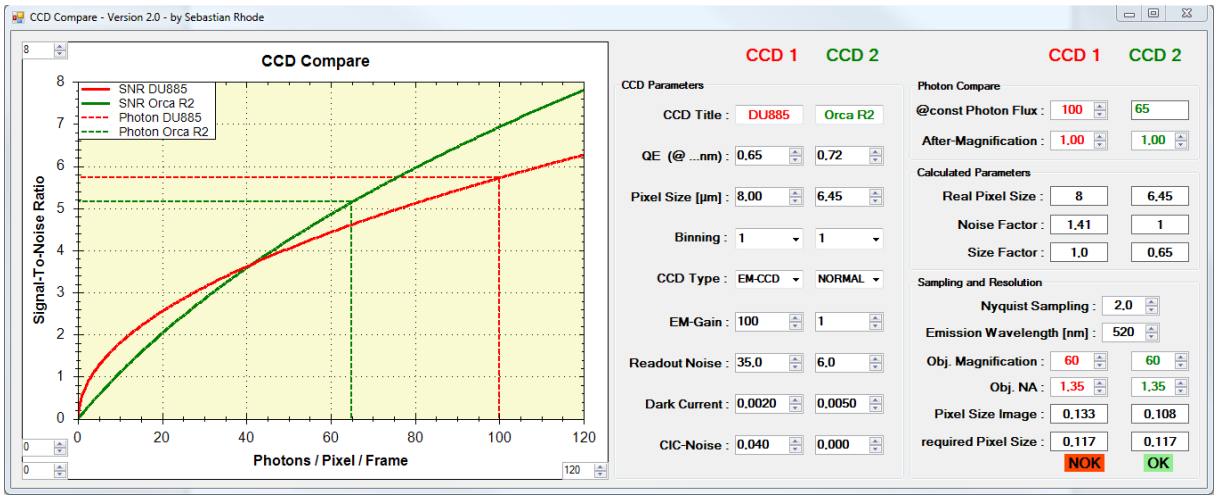
Typical parameters are shown in the following table:

	Ideal	CCD	EM-CCD	I-CCD
N_F	1	1	1.41	1.6
Q_E	100%	93%	93%	50%
δ_{dark}	0	0.001	0.001	0.001
δ_{cic}	0	0.05	0.05	0
$\delta_{readout}$	0	10	60	20
M	1	1	1000	1000

An import thing to consider in this calculation is the photon flux and the pixel size. Imagine a flux of 100 photons per pixel per frame for a pixel size of 16x16µm for CCD1. To compare the SNR you have to adapt the flux to the pixel size of CCD2. If that other model has 8x8µm pixels, the resolution photon flux for CCD2 will be:

$$\frac{100}{16\mu m * 16\mu m} * 8\mu m * 8\mu m = \frac{100}{256\mu m^2} * 64\mu m^2 = 25\,photons / pixel / frame$$

So the actual photon flux P for CCD2 will be only 25% compared to CCD1, which will strongly influence the result of the SNR calculation.



Measuring CCD readout noise

Measuring CCD readout noise is quite simple. Just use the following procedure:

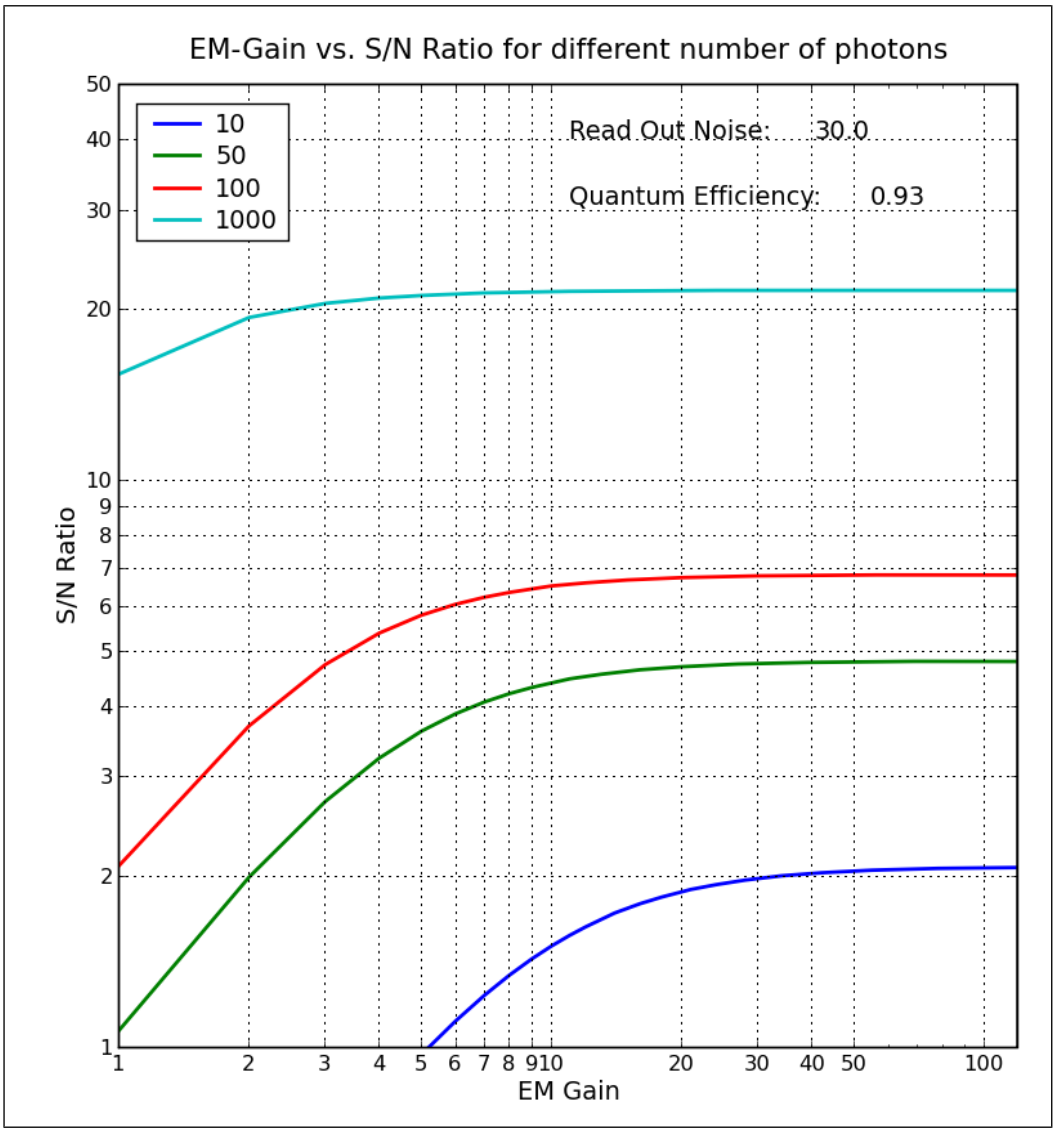
- record 2 dark frames with “zero” exposure time → DF1 and DF2
- calculate the standard deviation of the difference std(DF1-DF2)
- divide the result by sqrt(2) due to Gaussian error propagation to get the noise (RMS) → usually the mean of DIFF should be close to zero, so the assumption $RMS^2 = STD^2$ is justified
- To get the final readout noise in electrons, multiply with AD-Conversion factor and 1/EM-Gain

$$READOUT\ NOISE = \frac{stdDev(DF1 - DF2)}{\sqrt{2}} * ADconv * \frac{1}{EMGain}$$

- in case the AD-Conversion factor is unknown, one can use the following equation to estimate its value → ***G = 0.85 * Full Well Capacity / Bit Depth***

EM-Gain and Signal-to-Noise Ratio

Depending on the CCD and the experiment parameters, it might be not required to use the full EM-Gain. The reachable S/N ration will not increase any further. Using higher gains might just stress and damage the CCD chip.



EM-gain vs. S/N ratio for different number of input photons – for this example EM gains higher than ~50 do not yield to any further improvement in the S/N ratio.

Requirements for optimal detection of single molecules

For positional accuracy but also for continuous recording of the single molecule, the total number of detected photons is of key relevance. It depends on the specifications of the imaging setup, the excitation intensity, the dye properties, and the experimental environment, such as buffers and used materials. It is essential to optimize the setup and the overall detection efficiency. Therefore the used imaging setup has to fulfill some important demands.

- a strong monochromatic light source (monochromator or laser), with enough output intensity to produce a significant amount of photons within the illumination time and an optimized illumination optics

To illustrate this demand, it is helpful to estimate the number of photons finally reaching the CCD chip, when illuminating dye molecules like Cyanin 5. Assuming an intensity of 1kW/cm^2 at a wavelength of 647nm the photon flux will be 3.25x10^{21} photons/cm^{2}s. Cyanin 5 has 250000M^{-1}cm^{-1} bulk molar absorptivity, which converts to a molecular excitation cross section of 9.6x10^{-16}cm^2. Multiplying the photon flux with the cross section yields 3.12x10^6 absorption events per second. At a quantum efficiency of 27% (for Cyanin 5), 8.44x10^5 photons per second will be emitted. Assuming an overall detection efficiency of the used setup of 5%, the CCD chip will detect around 42 photons per millisecond.

- low-noise CCD camera with a high quantum yield and a fast acquisition rate

This is probably the most important part of the detection system. Modern back-illuminated, cooled CCD cameras or EM-CCDs with low readout noise should be selected. In order to achieve the highest possible localization precision the pixel size of the CCD chip should be about the size of the standard deviation of the point spread function.
If possible the fluorophores should be excited at the maximum of its absorption spectrum, because an excitation more or less far away from the absorption peak (in order to stay away from the spectral detection range) will lead to a smaller S/N ratio when using the same illumination time, as a short calculation shows: An often used laser line for Cyanin5 excitation is the 633nm line. At this wavelength Cyanin 5 has about 190000M^{-1}cm^{-1} bulk molar absorptivity. The above calculations yield in 32 photons per millisecond, a reduction in the number of photons by ~25% compared to the excitation at 647nm.

- appropriate filter combinations in order to improve or optimize the overall detection efficiency of the setup

To collect as much emitted photons as possible one has to carefully select the filters in the optical path. Excitation filters normally used in fluorescence microscopy are not required, if the illumination is carried out via monochromatic light source, such as a laser. Very important is the choice of the dichroic mirror and the emission filter. Especially the emission filter should reduce the leak-through intensity by a factor of at least 10^5 (OD5-OD6 at excitation wavelength)!

- optimization of all used materials in order to minimize any undesired fluorescence signals

Single molecule detection, especially in living cells, requires an optimization of materials used for cell culture, because buffers, glass slides and cell maintenance media can increase the background noise making it impossible to observe single molecules. Only if the setup is optimized for all these demands, the advantages of this non-invasive ultra-sensitive microscopy technique can be fully exploited.

A very important issue is the discrimination of the specific signal from cellular autofluorescence, especially in ‘‘dual-color’’ experiments. Living cells contain many

substances, which are capable of producing background or undesired signals. There are several techniques to reduce or discriminate autofluorescence.

- use of very bright dyes in combination with an excitation wavelength close to the absorption peak will produce a very strong signal, which can easily be distinguished from other signals by its intensity

- many auto-fluorescent cellular compounds can be effectively excited with UV or blue/green light; the emitted fluorescence light is typically spread over a broad spectral range

- the use of "red" excitation light can decrease the intensity of the autofluorescence signals

- fluorescent dyes usually emit in a well defined spectral range while auto-fluorescent substances produce a broad emission spectrum

The excitation of a red or green dye in it absorption peak will lead to fluorescence light detectable in a characteristic rather narrow spectral region. It is therefore possible to split the emission into two channels, one selective for the red dye, the second channel selective for the green dye. In contrast to these "pure" dyes the excitation of autofluorescence with green light will lead to broad emission signal detectable in both channels. This can be used to remove undesired signals from the recorded images.

Standard Deviation, Arithmetic Mean and Standard Error

The standard deviation σ is defined as the square root of the variance. This means it is the root mean square (RMS) deviation from the average $\bar{x}$. The standard deviation is always a positive number and is always measured in the same units as the original data. For example, if the data are distance measurements in meters, the standard deviation will also be measured in meters.

$$\bar{x} = \frac{1}{N}\sum_{i=1}^{N} x_i = \frac{x_1 + x_2 + \ldots + x_N}{N}$$

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(x_i - \bar{x}\right)^2}$$

$$error = \frac{\sigma}{N}$$

The standard error of a sample from a population is the standard deviation of the sampling distribution and may be estimated from the standard deviation *σ* and the size *N* of the population

RMS – Root Mean Square

In mathematics, root mean square (abbreviated rms), also known as the quadratic mean, is a statistical measure of the magnitude of a varying quantity. It can be calculated for a series of discrete values or for a continuously varying function. The name comes from the fact that it is the square root of the mean of the squares of the values. It is a power mean with the power $t = 2$.

The RMS for a collection of N values $\left\{ x_1^2 + x_2^2 + \ldots + x_N^2 \right\}$ is

$$x_{RMS} = \sqrt{\frac{1}{N}\sum_{i=1}^{N} x_i^2} = \sqrt{\frac{x_1^2 + x_2^2 + \ldots + x_N^2}{N}} \; .$$

If $\bar{x}$ is the arithmetic mean and σ_x is the standard deviation of a population, then

$$x_{RMS}^2 = \bar{x}^2 + \sigma_x^2$$