

Image Resolution in Optical Nanoscopy

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ABSTRACT

Super-resolution microscopy often employs asynchronous localizations of many single fluorescent emitters achieving resolution below the diffraction limit. This family of techniques typically uses statistical switching of emitters between dark and bright fluorescent states. Here we investigate how imaging repeated activations cycles of the same emitter influences the achieved image resolution. Furthermore, we ask the questions how long such a typical bright emitting state should be and is there an optimal number of switching events if the measurement time is fixed. We find that longer measurement times and hereby imaging more activation cycles is always beneficial for the attained image resolution. In the case of a fixed measurement time it turns out that there is a trade-off between the number of cycles and the product of localization density and uncertainty.

Keywords: Super-resolution imaging, image resolution, emitter localization, multiple activations

1. INTRODUCTION

One of the most important laws of conventional optics is that resolution is limited to a value on the order of λ/NA , with λ the wavelength of light and NA the numerical aperture of the imaging lens. Rayleigh and Sparrow described this law by empirical resolution criteria, which were later replaced by Abbe and Nyquist's approach, defining resolution as the inverse of the spatial bandwidth of the imaging system. This diffraction limit, however, has been overcome by numerous optical nanoscopy techniques in the last decade, notably STED,¹ RESOLFT,² the family of localization microscopy techniques such as PALM, STORM, GSDIM, and dSTORM³⁻⁷ and statistical methods as BLINK and SOFI.^{8,9}

These developments raise the fundamental question: what is the resolution? The two-point resolution criteria of conventional microscopy by Rayleigh and Sparrow have been given an exact meaning in the context of localization microscopy by Ram *et al.*¹⁰ Recently the general question has been investigated more rigorously mostly in the context of localization microscopy.¹¹⁻¹³ These approaches can combine both labeling density and localization uncertainty into one number, however, only our approach¹³ delivers a measure that can be computed directly from experimental data alone, i.e. without any a-priori information concerning the spatial structure of the underlying sample. More importantly, it is the only measure that can conceptually and practically deal with repeated activations of the same emitter. In any actual experiment in localization microscopy^{4,7} individual emitters will be recorded multiple times, even in PALM like experiments.^{14,15} Practitioners in the field typically record until they expect from known switching times that each emitter is seen at least a couple of times. They do this in order to ensure that they imaged as many emitters as reasonably possible. It does not seem feasible in practice to avoid recording multiple activations cycle of all individual emitters. Especially in view of the possibility of accurate fitting of emitter locations on fast sCMOS cameras¹⁶ and the application to time lapse recordings, it is important to understand the trade-off between temporal and spatial resolution in a super-resolution data acquisition and how it is related to the number of activation cycles.

Here, we raise the questions how the image resolution is affected by localization each emitter more than once due to multiple activations per emitter. To this end we employ the framework developed before by us.¹³ The image resolution is computed from the data as follows. We first divide the set of single emitter localizations that constitute a super-resolution image into two statistically *independent* sub-sets, yielding two sub-images $f_1(\vec{r})$ and $f_2(\vec{r})$, where \vec{r} denotes the spatial coordinates. Subsequent statistical correlation of their Fourier transforms

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$\hat{f}_1(\vec{q})$ and $\hat{f}_2(\vec{q})$ over circles of constant spatial frequency magnitude $q = |\vec{q}|$ gives the Fourier Ring Correlation (FRC):

$$FRC(q) = \frac{\sum_{\vec{q} \in \text{circle}} \hat{f}_1(\vec{q}) \hat{f}_2(\vec{q})^*}{\sqrt{\sum_{\vec{q} \in \text{circle}} \hat{f}_1(\vec{q})^2} \sqrt{\sum_{\vec{q} \in \text{circle}} \hat{f}_2(\vec{q})^2}}. \quad (1)$$

The image resolution R is defined as the inverse of the spatial frequency q_R for which the FRC curve drops below a certain threshold, the correct threshold is found to be $1/7$.^{13,17} I.e. the inverse of the effective bandwidth of the image content itself is used to define resolution.

For our further investigations below we need the expectation value of the FRC curve which can be computed:¹³

$$\langle FRC(q) \rangle = \frac{\sum_{\vec{q} \in \text{circle}} \left(Q + N \left| \hat{\psi}(\vec{q}) \right|^2 \right) \exp(-4\pi^2 \sigma^2 q^2)}{\sum_{\vec{q} \in \text{circle}} \left[2 + \left(Q + N \left| \hat{\psi}(\vec{q}) \right|^2 \right) \exp(-4\pi^2 \sigma^2 q^2) \right]}, \quad (2)$$

where N is the total number of localization events, σ is the average localization uncertainty, and $\hat{\psi}(\vec{q})$ denotes the spectrum of the spatial distribution of the fluorescent emitters. The parameter Q is a measure for spurious correlations due to multiple localizations of the same emitter. Each emitter contributing to the image is localized once for $Q = 0$ and in general $Q/(1 - \exp(-Q))$ times on average, provided the emitter activation follows Poisson statistics.

2. RESOLUTION AS A FUNCTION OF MULTIPLE LOCALIZATIONS

Now we will analyze the influence of multiple localization per emitter governed by the parameter Q on the expectation value of the FRC at the resolution frequency q_R or equally the resolution $R = q_R^{-1}$, defined by the equality $\langle FRC(q_R) \rangle = 1/7$. To this end we will start from the expectation value given by eq. 2 but assuming that all localizations of an emitter are either captured in image f_1 or f_2 will lead to the following expression, see Ref. 13 eq. S.28

$$\langle FRC(q_R) \rangle = \frac{NS(q_R) \exp(-4\pi^2 \sigma^2 q_R^2)}{2 + (2Q + NS(q_R)) \exp(-4\pi^2 \sigma^2 q_R^2)} = \frac{1}{7}, \quad (3)$$

where the integration over the rings of constant spatial frequencies is combined with the spectrum into:

$$S(q) = \frac{1}{K^2} \int d^2 q' \frac{\delta(|\vec{q}'| - q)}{2\pi q} \left| \hat{\psi}(\vec{q}') \right|^2, \quad (4)$$

where K is the total number of emitters in the images that are localized at least once. The relation between K and N is given by $N = K \langle M \rangle$, with M the average number an emitter is localized. The relation between M and Q is given by (see Ref. 13 Supplementary Information sec. 1.2)

$$Q = \frac{\langle M(M-1) \rangle}{\langle M \rangle} = \frac{\langle M^2 \rangle}{\langle M \rangle} - 1 \quad \text{with} \quad M = \sum_{i=1}^{M_{act}} L_i, \quad (5)$$

where each emitter is activated M_{act} times and localized L_i times in activation event i . In a typical experiment L_i is equal to a few camera frames but most researchers use ‘frame connection’ to group these events into one single effective localization event. Let us assume for this paragraph that each emitter is only localized once effectively, i.e. $L_i = 1$ and $M = M_{act}$. The implications of $L_i \neq 1$ will be discussed in the next paragraph. For a Poissonian distribution of M the variance is equal to the mean if we include the case $M = 0$ (no activations for this emitter). If we restrict the probability distribution to the group of events of at least one activation per emitter we find:

$$\langle M \rangle = \frac{Q}{1 - \exp(-Q)}, \quad (6)$$

and $N = KQ / (1 - \exp(-Q))$.

Continuing with equation 3 we can rewrite it into the following expression:

$$\frac{1 - \exp(-Q)}{Q} = (3KS(q_R) - 1 + e^{-Q}) \exp(-4\pi^2\sigma^2q_R^2). \quad (7)$$

For the limiting case $Q \rightarrow 0$ this reduces to:

$$1 = 3KS(q_R) \exp(-4\pi^2\sigma^2q_R^2), \quad (8)$$

leading to the results derived in Ref. 13, eq. S.36. For large Q we find:

$$\frac{1}{Q} = (3KS(q_R) - 1) \exp(-4\pi^2\sigma^2q_R^2). \quad (9)$$

We assume in the following that $S(q)$ is a monotonically decreasing function of q . It is a reasonable assumption for a typical, realistic biological structure that for higher spatial frequencies the spectral energy decays. Only for pathological structures such as periodic structures with only one period this assumption is violated. With this it is clear that the right hand side (RHS) of eq. 9 is a decaying function as $q_R = 1/R$ increases. If we now look at the left hand side (LHS) of eq. 9 and combine it with the previous observation, it becomes clear that the resolution R becomes better (smaller) as the number of localizations per emitter Q increases. Additionally, as the RHS of eq. 9 is always positive, there must be a lower bound for $Q \rightarrow \infty$. This is given by

$$S(q_R) = \frac{1}{3K} \Leftrightarrow R = \frac{1}{S^{-1}(\frac{1}{3K})}. \quad (10)$$

Here $S^{-1}(x)$ is the largest q for which $S(q) = x$. The lower bound on R also implies a maximum value for the exponential on the RHS of eq. 9. Therefore this LHS will go to zero for large Q and thus the aforementioned lower bound on R will be reached for infinite Q .

We will illustrate this case for an example where the correlation $S(q)$ can be computed analytically. Let us consider a sample consisting of m parallel lines with a sinusoidal emitter density profile with average density ρ . This sample, of course, violates the assumption of a monotonically decreasing correlation, but it serves well for our purpose of illustrating the lower bound of the resolution. We know for such a sample that the integrated correlation S at the resolution q_R is given by (compare Ref. 13 eq. S.37 and S.40)

$$KS(q_R) = \frac{m\rho}{4\pi q_R^2}. \quad (11)$$

Consequently, if we substitute this result into eq. 9, we obtain

$$\frac{1}{Q} = \left(\frac{3m\rho}{4\pi q_R^2} - 1 \right) \exp(-4\pi^2\sigma^2q_R^2). \quad (12)$$

The LHS of the above equation is always non-negative, which implies indeed that there will be a lower bound to the resolution $R = q_R^{-1}$. For the limiting case of $Q \rightarrow \infty$, the term in brackets of the RHS must be zero and we arrive at

$$R = \sqrt{\frac{4\pi}{3m\rho}}. \quad (13)$$

As would be expected, this is the same limit as we obtained previously for this sample when all emitters which have been localized exactly once with $\sigma \rightarrow 0$; compare Ref. 13 eq. S.52. Please note that this limit is also proportional to the Nyquist resolution of random sampling $R_{\text{Nyquist}} = 2/\sqrt{\rho}$. Therefore, it is always better to image longer and acquire more blinking cycles from the emitters in terms of the achieved image resolution.

2.1 Resolution trade-off for fixed measurement time

In the following we will investigate if there is a trade-off between acquiring more localization per emitter $\sim Q$ and the statistical certainty with which each emitter is localized σ . This case is particularly relevant for time-lapse recordings where only a limited time is available and one has to decide how to best use the available measurement time.¹⁶ For the localization uncertainty we know that it scales inversely with the square root of the number of detected photons n_{ph} .^{10,18} Longer activation times t_{act} cause more photons to be acquired per cycle which leads to a better localization precision σ . However, shorter activation times allow for more localizations per emitter $\sim Q$ on average, as the ratio between the on and off times must be kept constant to keep the density of simultaneously active emitters constant. This results in a lower relative variance in the number of localizations per emitter. If we assume that $Q \propto 1/t_{act}$ and $n_{ph} \propto t_{act}$, we find that the resolution is optimal (in terms of speed) when (compare Ref. 13 Supplementary Information section 3.2)

$$R = 2\pi\sigma. \quad (14)$$

If this condition is reached, and we substitute it into eq. 7, then

$$3KS(q_R) = (1 - e^{-Q}) \left(1 + \frac{e}{Q}\right). \quad (15)$$

Again we illustrate this relation for the case of m parallel lines, which leads to the relation

$$3\pi m \rho \sigma^2 = (1 - e^{-Q}) \left(1 + \frac{e}{Q}\right). \quad (16)$$

Another practical form of this equation is

$$3\pi m \rho_{loc} \sigma^2 = e + Q, \quad (17)$$

where $\rho_{loc} = \rho Q / (1 - \exp(-Q))$ is the density of localization events. The RHS of eq. 16 is a monotonically decreasing function of Q , implying that a higher value of Q means that the trade-off point between labeling density and localization uncertainty is shifted to lower values of the labeling density. For the limiting case $Q \rightarrow 0$ it follows that

$$3\pi m \rho \sigma^2 = e, \quad (18)$$

in agreement with Ref. 13 eq. S.50. For large Q we find

$$3\pi m \rho \sigma^2 = 1 + \frac{e}{Q}, \quad (19)$$

Combining both cases we see that the optimum resolution condition can only be met if $e/3\pi m > \rho \sigma^2 > 1/3\pi m$, so that the range of values for the trade-off point varies by no more than a factor of roughly 3 due to effects of multiple activations per emitter.

3. RESOLUTION EVOLUTION IN CASE OF MULTIPLE CONSECUTIVE LOCALIZATIONS

Up to now we only considered the case where each emitter was only localized once during each activation event. Of course, that is in practice not a realistic assumption given the switching on-times t_{on} and typical camera exposures t_{frame} . Raw localizations fitted from the individual camera frames can be condensed into localization events by grouping spatially nearby events into single localization events. We assume that all frames are sparse, therefore it is unlikely that localizations obtained from subsequent camera frames that are spatially close to each other, originate from different emitters. Spatially close is defined as having a distance less than three times the sum of the localization uncertainty of the two to-be merged localization events. The number of time frames to look ahead and merge these close by spots is typically between 1 and 5 frames. The advantage of this procedure is that very small off-times and missed localization events can be grouped. Then the resulting event has an improved localization certainty. The disadvantage is that small off-times are not seen and if the assumption that

the image content is sufficiently sparse is violated, localizations from different emitter can be merged which leads to an increased localization uncertainty instead of a decreased one and a loss of emitter density.

In the previous sections we have shown that the resolution improves by having multiple activations per emitter. This raises the question if increasing the total number of localizations by having multiple localizations per activation event ($L_i > 1$) due to a camera frame time smaller than the on-time of the activation event is advantageous as well. It turns out this is not the case because decreasing the frame time reduces the amount of signal photons captured per frame and so compromises the localization uncertainty per event. The net effect is that the resolution gets worse for $L_i > 1$. In the following we present a formal proof of this statement. We want to optimize eq. 3 as a function of number of localizations L_i per activation event i . Let us start by revisiting eq. 5. We need to compute the variance of the total number of localized emitters $Var(M) = \langle M^2 \rangle - \langle M \rangle^2$ in terms of M_{act} the number of times each emitter is activated and L_i . Now we compute $\langle M^2 \rangle$ with the assumption that M_{act} is fixed for all emitters and known, then we obtain

$$\langle M^2 \rangle = \left\langle \sum_{i=1}^{M_{act}} L_i \sum_{j=1}^{M_{act}} L_j \right\rangle = M_{act}(M_{act} - 1) \langle L_i \rangle^2 + M_{act} \langle L_i^2 \rangle. \quad (20)$$

The first term represent the off-diagonal elements that occur $M_{act}(M_{act} - 1)$ times and are independent and the second term represent the diagonal elements. From Ref. 19 we know that the duration in frames during which an emitter is active is geometrically distributed. Therefore, if molecules are successfully localized in each frame where they are active, the L_i is equivalent to the duration in frames during which an emitter is active and due the geometric distribution we have $\langle L_i^2 \rangle = 2 \langle L_i \rangle^2 - \langle L_i \rangle$. Plugging this into eq. 5 and assuming that M_{act} is a stochastic variable with $\langle M \rangle = \langle M_{act} \rangle \langle L_i \rangle$, we find

$$Q = \left(1 + \frac{\langle M_{act}^2 \rangle}{\langle M_{act} \rangle} \right) \langle L_i \rangle - 2. \quad (21)$$

We can now use the expression we obtained for Q to optimize the FRC resolution as a function of the number of activations per emitter L_i . To this end, it is useful to rewrite eq. 3 as follows:

$$\langle FRC(q_R) \rangle = \frac{S(q_R)}{S(q_R) + 2(Q + \exp(4\pi^2\sigma^2 q_R^2)) / N}. \quad (22)$$

The following expression must be minimal as a function of L_i for the resolution to be maximal

$$f(\langle L_i \rangle) = \frac{2}{N} \exp(4\pi^2\sigma^2 q_R^2) + 2 \frac{Q}{N}. \quad (23)$$

Using the relation $N = K \langle M_{act} \rangle \langle L_i \rangle$ we find that:

$$f(\langle L_i \rangle) = \frac{2}{K \langle M_{act} \rangle} \left(1 + \frac{\langle M_{act}^2 \rangle}{\langle M_{act} \rangle} \right) + \frac{2}{K \langle M_{act} \rangle \langle L_i \rangle} (\exp(4\pi^2\sigma^2 q_R^2) - 2). \quad (24)$$

Assume now that the average duration of activation events t_{act} is fixed. The time associated with a single localization is then $t_{loc} = t_{act}/L_i$. Previously we have shown²⁰ that the localization uncertainty can analytically be approximated by

$$\sigma^2 \approx \frac{\sigma_a^2}{n_{ph}} \left(1 + 4\tau + \sqrt{\frac{2\tau}{1 + 4\tau}} \right), \quad (25)$$

where n_{ph} is the number of signal photons, and $\tau = 2\pi b\sigma_a^2/(n_{ph}a^2)$ is the ratio between the background intensity b/a^2 and the peak signal intensity $N/2\pi\sigma_a^2$ with a the back projected pixel size and $\sigma_a^2 = \sigma^2 + a^2/12$. Since $n_{ph} \propto t_{loc}$, we find that $\sigma^2 \propto t_{loc}^{-1} \propto L_i$. If we define this proportionality factor as $\sigma^2 = \sigma_c^2 L_i$, then we find for the only term in eq. 24 that depends on L_i

$$\frac{1}{\langle L_i \rangle} (\exp(4\pi^2\sigma^2 q_R^2) - 2) = \frac{1}{\langle L_i \rangle} (\exp(4\pi^2\sigma_c^2 q_R^2 \langle L_i \rangle) - 2). \quad (26)$$

Using that the function $x^{-1}(\exp(x) - 2)$ is a monotonously increasing function of x , we finally find that the resolution is optimal if L_i is minimal, i.e. 1. In practice, this condition may be achieved by combining the localizations from one emitter activation event into a single localization.

4. DISCUSSION

We found that it is optimal to only localize each emitter once during each on-time, i.e. $L_i = 1$ in the above notation or each emitter should only be visible in one frame. From a theoretical standpoint it is not surprising as the localization of each emitter can be recorded already from one frame, and only the certainty of the position increases if more frames are acquired as the photon count increases. A longer frame time, however, can achieve exactly the same certainty, therefore in an ideal situation the blinking of the emitters should be in sync with the camera frame rate and only last one frame.

It is also desirable in an experiment as it makes it easier fulfilling the sparseness requirement of localization microscopy. On a more practical note, events that only are visible for one frame are discarded by most image pre-processing routines while analyzing the raw image frames,^{21,22} as these are considered too unreliable compared to false positives background localizations. Also keep in mind that it is very unlikely that the camera frame rate coincides with the blinking behavior of all emitters. Given these considerations, at least two consecutive frames in the on-state seem to be a good trade-off between theoretically optimal and practically feasible giving the experiment and image processing needed to obtain localizations.

On another practical note we consider the effect of frame-connection. This image processing technique is regularly applied to group spatially close-by events in consecutive frames and close gaps in the time traces due to missed events (false negatives). Now if $L_i \gg 1$ then many frames need to be connected and of course this connection on very noisy data is prone to errors. Incorrect frame-connection in these cases leads therefore to worse image resolution than without connection (data not shown).

The conclusion on the optimal number of switching events resulting in localizations (given by M and respectively Q in the above notation) is twofold.

Firstly, measuring longer and thereby seeing the same emitter more often (higher Q) results always in a better resolution. From the computational side of the resolution it is required that the spurious correlation parameter Q in the formula is either estimated from the data as shown by us before¹³ or assumed to be not present as different emitters are assigned to different sub-image halves for the computation as in eq. 2. There exists obviously a lower limit of the resolution even for an infinite number of repeated activations which is given by the density of localizations. In this case the resolution is equivalent to the case where the localization uncertainty is zero, i.e. $R(\sigma = 0) = R(Q \rightarrow \infty) \propto R_{\text{Nyquist}}$ and the resolution is then proportional to the Nyquist resolution of random sampling.

Secondly, the case changes if the measurement time is fixed and the interpretation of this case compared to the above is subtle. As shown before¹³ the optimal resolution in terms of time efficiency is achieved if $R = 2\pi\sigma$. In this optimum a trade-off exists described by eq. 16 between the labelling density ρ and the localization uncertainty σ , characterized by the quantity $\rho\sigma^2$ being above or below a critical value C . Along the lines of reasoning in Ref. 13 for optimizing the product $\rho\sigma^2$ against time, we see changing which quantity increases the image resolution relatively the best. For $\rho\sigma^2 > C$ the largest relative gain is obtained by improving the localization uncertainty, for $\rho\sigma^2 < C$ the largest relative gain is obtained by improving the labeling density. The critical value C describing the trade-off decreases with the number of times each emitter is localized ($\sim Q$) by a factor $e = 2.7$ when Q is increased from a value close to zero to a value much larger than one. The values for C derived in Ref. 13 for the case $Q = 0$ are thus smaller for many experimentally reported number of cycles.^{13,15,23} Our findings can help to plan the experimental conditions and/or increase labeling densities, e.g. by enabling multi-emitter fitting²⁴ to get close to the theoretical optimum.

In the above we neglected the relevant effect of photobleaching of emitters during the measurement. This introduces a major complication in the analysis; we can no longer assume a Poissonian distribution of the number of localization emitters M . It is clear that it will have an effect on the resolution and its optimum as longer imaging will again increase M but at the same time $\text{Var}(M)$ will probably increase stronger. The details depend on the photo physical switching model between the on, off and bleached state, assuming the simplest possible three state model.^{15,19} We will look into this complicated problem in a future study.

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