Dynamic speckle illumination microscopy with translated versus randomized speckle patterns

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Abstract: Dynamic speckle illumination (DSI) microscopy is a widefield fluorescence imaging technique that provides depth discrimination. The technique relies on the illumination of a sample with a sequence of speckle patterns. We consider an image processing algorithm based on a differential intensity variance between consecutive images, and demonstrate that DSI sectioning strength depends on the dynamics of the speckle pattern. Translated speckle patterns confer greater sectioning strength than randomized speckle patterns because they retain out-of-focus correlations that lead to better background rejection. We present a theory valid for arbitrary point-spread-functions, which we corroborate with experimental results.

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1. Introduction

Confocal fluorescence microscopy [1] is a well-established technique in the bioimaging community, mainly because it provides optical sectioning. Implementations with laser illumination usually involve scanning of a single laser focal spot, whereas implementations without laser illumination typically make use of multi-spot array scanning [2, 3]. More light-efficient alternatives have been proposed such as aperture correlation microscopy [4, 5], or programmable array microscopy [6] that involve illuminating the sample with patterns of light defined by illumination masks, and collecting the fluorescence through these same masks. Alternative strategies that allow direct collection of the fluorescence without a return passage through the illumination mask include the simple and well-established technique of incoherent structured illumination microscopy [7, 8].

We recently proposed a technique similar to structured illumination microscopy wherein the incoherent grid illumination pattern is replaced with a dynamic speckle illumination (DSI) pattern from a laser, and we demonstrated that this new technique confers optical sectioning in biological tissue [9]. In our initial implementation, the sample was illuminated with a sequence of statistically independent (random) speckle patterns. The root-mean-square (rms) of the corresponding sequence of fluorescent images comprised the optically sectioned image. A drawback of this technique is that its sectioning strength is weaker than that of a confocal microscope. In this paper, we show that the sectioning strength can be significantly improved by using the same image processing algorithm but with a better control of how the DSI pattern is rendered dynamic. While in our initial implementation, the speckle pattern was completely randomized between image acquisitions, here we apply small lateral translations to the speckle pattern, on the order of a speckle grain size, and demonstrate both theoretically and experimentally that the resulting sectioning strength is near-confocal.

2. Principle of dynamic speckle illumination microscopy

Figure 1 depicts speckle illumination inside a sample and the detection point-spread-function (PSF$_{\text{det}}$) for an arbitrary pixel in the imaging CCD camera. The photocounts recorded by this pixel are plotted as a function of time, or, more precisely, image frame $k$. These photocounts can be thought of as arising from two contributions: signal fluorescence arising from the in-focus plane (denoted by $S$) and background fluorescence arising from out-of-focus planes (denoted by $B$). Our goal with DSI microscopy is to estimate the fluorophore concentration at the in-focus plane based only on these photocounts. Our strategy for achieving this is to render the speckle illumination dynamic and to exploit an a-priori knowledge on how the temporal statistics of $S$ and $B$ differ.

In particular, there is a fundamental difference between how the photocounts from zones $S$ and $B$ are acquired. Because the in-focus PSF$_{\text{det}}$ is narrow and the out-of-focus PSF$_{\text{det}}$ is wider, we may roughly think of PSF$_{\text{det}}$ as locally sampling the in-focus fluorescence while spatially averaging the out-of-focus fluorescence. Correspondingly, when the speckle pattern is rendered dynamic, different realizations of the speckle pattern are locally sampled in zone $S$ whereas they are spatially averaged in zone $B$.

The temporal average of the detected photocounts is $\bar{I}_d = \bar{S} + \bar{B}$. Assuming that the different realizations of the speckle pattern are statistically independent for $S$ (though not necessarily for $B$) then $\bar{S}$ provides a direct estimate of the fluorophore concentration at the in-focus plane. Our goal, therefore, is to distinguish $\bar{S}$ from $\bar{B}$. The temporal average of the photocounts does not, by itself, provide enough information to do this. However, in the case of DSI the additional knowledge garnered from the temporal variance of the photocounts does provide enough information. In particular, it is known that detected photocounts from locally sampled independent speckle patterns obey Bose-Einstein statistics [10]. Accordingly, the temporal variance of $S$ is given by...
Fig. 1. Schematic view of the laser speckle (blue ovals) inside the sample and the detection PSF (green lines) for an arbitrary CCD pixel. We define two regions delimited by the dashed vertical lines: the in-focus region corresponding to the signal $S$ we want to measure, and the out-of-focus region corresponding to the background $B$ we want to reject. $I_d$ is the recorded pixel intensity at each camera frame $k$.

$\text{Var}(S) = S^2 + \bar{S}$, which can be interpreted as arising from both “classical”local intensity fluctuations of speckle (which obey negative-exponential statistics – first term) and “quantum ”shot noise associated with photodetection (which obeys Poissonian statistics – second term). In contrast, the local intensity fluctuations in $B$ are spatially averaged and hence contribute little to its temporal variance, giving $\text{Var}(B) \approx \bar{B}$. The total variance is then $\text{Var}(I_d) \approx S^2 + S + B$, and an estimate of $\bar{S}$ becomes straightforward: $\bar{S} \approx \sqrt{\text{Var}(I_d) - I_d}$. This estimate is our intended goal.

A caution should be made regarding this simple idea since the separation between zones $S$ and $B$ in the sample is nowhere near as clear-cut as suggested in Fig. 1. In particular, we have assumed that $\text{PSF}_{\text{det}}$ in zone $B$ is so wide as to completely spatially average out the “classical”intensity fluctuations of speckle in $B$ and extinguish their contribution to $\text{Var}(B)$. This is an approximation. In fact, a simple variance algorithm cannot completely extinguish residual intensity fluctuations, and better algorithms for their reduction constitute the main motivation for this paper.

3. DSI sectioning with translated versus randomized speckle patterns

The algorithm we consider in this paper is based not on an intensity variance, as suggested above, but rather on a structure function $D$ defined as half the differential intensity variance:

$$D = \frac{1}{2N} \sum_{k=1}^{N} (I_{d,k+1} - I_{d,k})^2$$  \hspace{1cm} (1)

where $I_{d,k}$ is the intensity recorded by the CCD camera on pixel $d$ in frame $k$ (we consider only “classical”intensities for the moment), and $N + 1$ is the total number of acquired images. This algorithm was introduced in ref. [9] and has the advantage of being insensitive to long-term power variations in the illumination beam. We note that if consecutive $I_{d,k}$’s are statistically independent, then $D$ is formally equivalent to the intensity variance $\text{Var}(I_d)$. Such statistical independence is achieved, for example, if the speckle pattern is completely randomized between camera frames, as was done in ref. [9]. However, if correlations are induced between consecutive $I_{d,k}$’s, then $D$ can be much smaller than $\text{Var}(I_d)$. In particular, if correlations are induced only in the out-of-focus background and not in the in-focus signal, they can lead to significantly enhanced background rejection. A simple technique for introducing such background correlations is with DSI based on speckle translation rather than speckle randomization. In this section
we present an intuitive argument for why background rejection is improved, and defer a more rigorous argument to section 4.

To quantify DSI sectioning strength, we consider as we did in ref. [9], a sample that is a thin uniform fluorescent plane and calculate the expected structure function \(D\) as a function of the sample’s axial position \(z_c\) (see Fig. 2). To gain an intuitive understanding, we adopt the following simplifications:

1) We approximate the lateral profile of \(\text{PSF}_{\text{det}}(\vec{\rho}, z)\) to be a circular top-hat with a diameter that expands linearly with distance \(z\) away from the focal plane. \(\text{PSF}_{\text{det}}(\vec{\rho}, z)\) is normalized such that \(\text{PSF}_{\text{det}}(0, 0) = 1\) and \(\int \text{PSF}_{\text{det}}(\vec{\rho}, z) d^2\vec{\rho} = A_d\), independently of \(z\). That is, \(A_d\) corresponds to the area of \(\text{PSF}_{\text{det}}\) at the focal plane.

2) We further approximate the speckle pattern to consist of a mosaic of speckle “grains” of equal lateral areas \(A_s\) (the speckle correlation area) and of uniform intensities within these areas. The intensity of each speckle grain is statistically independent of its neighbors’, as illustrated in Fig. 2(c). The speckle grain intensities are denoted \(I_{s,n}\) and obey the negative-exponential statistics of a fully developed speckle pattern [10]. Hence, the spatial variance of these grain intensities sampled over many grain areas is given by the average speckle intensity squared \(I_s^2\) (again, we neglect shot-noise for the moment). We note that for widefield speckle illumination, as is the case in DSI microscopy, the speckle correlation area (or grain area) does not significantly depend on \(z\) [9].

Fig. 2. Simple picture to evaluate the translation and random DSI signals produced by a uniform fluorescent plane located at defocus position \(z_c\). When the plane is in focus (a), \(\text{PSF}_{\text{det}}\) is narrow and the detected speckle pattern is completely renewed upon randomization (b) or translation (c). When the plane is out of focus (d), \(\text{PSF}_{\text{det}}\) is wide and the detected speckle pattern is completely renewed upon randomization (b) but only partially renewed upon translation (c). \(\Delta \rho_s\) is the translation step size.
To calculate the intensity detected by a CCD pixel, we define \( N_s \) to be the number of speckle grain areas “seen” by PSF_{det}. That is, when the sample plane is in focus then \( N_s = 1 \) (we assume \( A_d \leq A_s \)); when it is out of focus then \( N_s \) is the ratio of the PSF_{det} area to the speckle grain area, which increases as \( z_c^2 \). The intensity detected by a CCD pixel is then given by the spatial summation

\[
I_d = \frac{C A_d}{N_s} \sum_{n=1}^{N_s} I_{s,n} \tag{2}
\]

where \( C \) is the fluorophore concentration and \( I_{s,n} \) is the intensity of speckle grain \( n \). We can now readily calculate the structure function \( D \) for the cases when the speckle pattern is randomized \( (D_R) \) versus when it is translated \( (D_T) \). Our final DSI image in both cases is given by \( \sqrt{D} \).

We consider the random case first. As mentioned above, \( D_R \) can be directly interpreted as the variance of the detected intensities, and therefore reads:

\[
D_R = \frac{(C A_d T_o)^2}{N_s} \tag{3}
\]

When the sample is at the focal plane, the DSI image is simply given by the product of the averaged illumination intensity \( T_o \), the concentration \( C \) of fluorophores, and the area of PSF_{det} at the focal plane, as we would obtain with a confocal microscope. When the sample is out of focus, \( N_s \) is proportional to \( z_c^2 \) and the final DSI image \( (\sqrt{D_R}) \) decays as \( 1/|z_c| \). Spatial averaging caused by PSF_{det} therefore reduces the out-of-focus speckle fluctuations.

For the translation case, we first define \( \Delta I_{d,k} \) as the difference between two consecutive intensities \( (\Delta I_{d,k} = I_{d,k} - I_{d,k-1}) \). For a uniform fluorescent plane (as considered here), a translation of the speckle pattern relative to a fixed PSF_{det} is equivalent to a translation of PSF_{det} relative to a fixed speckle pattern, allowing us to write:

\[
\Delta I_d = \frac{C A_d}{N_s} \left[ \sum_{n=1}^{N_s} I_{s,n} - \sum_{n'=1}^{N_s} I_{s,n'} \right] \tag{4}
\]

where \( \Delta N_s \) is the (\( z_c \) dependent) number of speckle grain areas encompassed in either of the differential areas between PSF_{det} and its shifted replica. These differential areas are colored in green and red in Figs. 2(c) and 2(f), green corresponding to the gain in new speckle grain areas (left term in Eq. (4)) and red corresponding to the loss in old speckle grain areas (right term in Eq. (4)). The corresponding structure function \( D_T \) is given by:

\[
D_T = \frac{(C A_d T_o)^2 \Delta N_s}{N_s^2} \tag{5}
\]

If the lateral shift \( \Delta \rho \) of the speckle pattern is larger than the width of PSF_{det} (as is the case, for example, in Fig 2(f)), then \( \Delta N_s = N_s \) and \( D_T = D_R \). Two consecutive raw images \( I_{d,k} \) are then completely uncorrelated and the final DSI image is the same regardless of whether the speckle pattern is translated or randomized.

In contrast, if the lateral shift \( \Delta \rho \) is smaller than the width of PSF_{det}, then \( \Delta N_s < N_s \) and hence \( D_T < D_R \). In this case, two consecutive raw images are highly correlated because of the overlap in the areas covered by PSF_{det} and its shifted replica, meaning consecutive raw images comprise many of the same speckle grains. Assuming that \( \Delta \rho \) is small (in practice it is of order the speckle grain size) then \( \Delta N_s \) scales linearly with \( z_c \) for large \( |z_c| \) (i.e. out of focus) and the final DSI image \( \sqrt{D_T} \) decays as \( 1/|z_c|^{3/2} \). We therefore expect better DSI sectioning strength with translating speckle patterns than with randomized speckle patterns.

We recall that the signal from a fluorescent plane scales as \( 1/|z_c|^2 \) for a confocal microscope [1]. The DSI sectioning strength for randomized speckle patterns \( (1/|z_c|) \) was previously referred...
to as “quasi-confocal”[9]. For lack of a better term, we refer to the DSI sectioning strength for translated speckle patterns \(1/|z_c|^{3/2}\) as “near-confocal”.

4. DSI sectioning for arbitrary PSF

In this section, we present a rigorous calculation of the structure function \(D_T\) that is valid for arbitrary illumination and detection PSF’s (denoted PSF\(_\text{det}\) and PSF\(_\text{ill}\) respectively). This calculation assumes a translating speckle pattern; however we note that in the limit where the translation step size is infinite, then \(D_T\) reduces to \(D_R\). Hence, this calculation applies to both DSI techniques.

In general, the fluorescence intensity at a position \(\hat{\rho}_d\) on the CCD detector plane is given by

\[
I_d(\hat{\rho}_d) = \int \int \text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho}, -z)C(\hat{\rho}, z)I_c(\hat{\rho}, z)d^2\hat{\rho}dz
\]

(6)

where \(I_c(\hat{\rho}, z)\) is the speckle pattern in sample and \(C(\hat{\rho}, z)\) the fluorophore concentration. We normalize our PSF’s such that \(\text{PSF}_{\text{ill}}(0,0) = \text{PSF}_{\text{det}}(0,0) = 1. \int \text{PSF}_{\text{ill}}(\hat{\rho}, z)d^2\hat{\rho} = A_s\) and \(\int \text{PSF}_{\text{det}}(\hat{\rho}, z)d^2\hat{\rho} = A_d\).

Again we consider a uniform fluorescent plane located at axial position \(z_c\). The fluorophore concentration is then \(C(\hat{\rho}, z) = C\delta(z - z_c)\), and the intensity detected at the CCD camera can be simplified to

\[
I_d(\hat{\rho}_d) = C \int \text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho}, -z_c)I_c(\hat{\rho}, z_c)d^2\hat{\rho}.
\]

(7)

Denoting as \(\Delta I_d\) the difference between two consecutive images, we find,

\[
\Delta I_d(\hat{\rho}_d) = C \int \text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho}, -z_c)I_c(\hat{\rho}, z_c)d^2\hat{\rho} - C \int \text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho}, -z_c)I_c(\hat{\rho} - \Delta\hat{\rho}_s, z_c)d^2\hat{\rho}
\]

(8)

where \(\Delta\hat{\rho}_s\) is the speckle pattern translation vector. Equivalently,

\[
\Delta I_d(\hat{\rho}_d) = C \int [\text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho}, -z_c) - \text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho} + \Delta\hat{\rho}_s, -z_c)] I_c(\hat{\rho}, z_c)d^2\hat{\rho}.
\]

(9)

For ease of notation we recast the structure function \(D_T\) (Eq. (1)) as \(D_T(\hat{\rho}_d) = \frac{1}{2} \Delta I_d(\hat{\rho}_d)^2\), where the overline indicates an average over many successive speckle patterns. Expanding, we obtain

\[
D_T(\hat{\rho}_d) = \frac{C^2}{2} \int \left[ \text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho}, -z_c) - \text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho} + \Delta\hat{\rho}_s, -z_c) \right]
\]

\[
[\text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho'}, -z_c) - \text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho'} + \Delta\hat{\rho}_s, -z_c)]
\]

\[
I_c(\hat{\rho}, z_c)I_c(\hat{\rho'}, z_c)d^2\hat{\rho}d^2\hat{\rho'}
\]

At this point, we invoke \textit{a-priori} knowledge on the speckle statistics. Assuming that the speckle pattern is fully developed and that the speckle size (correlation length) is roughly constant over a large depth of field [9], we have [10]:

\[
I_c(\hat{\rho}, z_c)I_c(\hat{\rho'}, z_c) = I_c^2(1 + \text{PSF}_{\text{ill}}(\Delta\hat{\rho}, 0))
\]

(10)

where \(\Delta\hat{\rho} = |\hat{\rho} - \hat{\rho'}|\). Defining \(R_{\text{det}}\) as the lateral autocorrelation of PSF\(_{\text{det}}\):

\[
R_{\text{det}}(\Delta\hat{\rho}, z_c) = \int \text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho}, -z_c)\text{PSF}_{\text{det}}(\hat{\rho}_d - \hat{\rho} + \Delta\hat{\rho}, -z_c)d^2\hat{\rho}
\]

(11)
and noting that \( \int R_{\det}(\Delta \hat{p} + \Delta \hat{p}_s, z_c)d^2 \Delta \hat{p} = A_d^2 \), we obtain finally:

\[
D_T(\hat{p}_d) = T_s^2 C^2 \int \left[ R_{\det}(\Delta \hat{p}, z_c) - \frac{1}{2} R_{\det}(\Delta \hat{p} + \Delta \hat{p}_s, z_c) - \frac{1}{2} R_{\det}(\Delta \hat{p} - \Delta \hat{p}_s, z_c) \right] PSF_{\text{ill}}(\Delta p, 0)d^2 \Delta \hat{p}
\]

(12)

This expression is valid for arbitrary PSF_{\text{ill}} and PSF_{\det} and we can progress no further without specifying these. In the case where both PSF’s exhibit Gaussian-Lorentzian profiles, then \( D_T(\hat{p}_d) \) can be solved analytically (see below). However, in practice, PSF_{\det} is usually defined by a circular pupil. To consider this latter more realistic case, we assume that \( R_{\det}(\Delta \hat{p}, z_c) \) is smooth on the scale of a speckle grain size, defined by PSF_{\text{ill}}(\Delta p, 0) (we are mostly concerned here with out-of-focus background rejection, and for large enough \( |z_c| \) this assumption is reasonably valid). We then make the approximation \( \text{PSF}_{\text{ill}}(\Delta p, 0) \approx A_s \delta(\Delta p) \), and Eq. (12) readily simplifies to

\[
D_T(\hat{p}_d) = T_s^2 C^2 A_s \left[ R_{\det}(0, z_c) - \frac{1}{2} R_{\det}(\Delta \hat{p}_s, z_c) - \frac{1}{2} R_{\det}(-\Delta \hat{p}_s, z_c) \right]
\]

(13)

Equation (13) is equally valid for translated or randomized speckle patterns. In the case of randomized patterns (\( \Delta p_s \rightarrow \infty \)), then only the first term in Eq. (13) is non-zero, and we recover our previous result (Eq. (3)). Note: to recover this result, we invoke the general definition for the lateral area of PSF_{\det} at depth \( z_c \), given by

\[
\text{Area}[\text{PSF}_{\text{det}}] = \frac{\int \text{PSF}_{\text{det}}(\vec{\hat{p}}, z_c)d^2 \vec{\hat{p}}}{\int \text{PSF}_{\text{det}}^2(\vec{\hat{p}}, z_c)d^2 \vec{\hat{p}}} = \frac{A_d^2}{R_{\det}(0, z_c)}
\]

(14)

which is valid for arbitrary PSF_{\text{det}}.

Further insight into the behavior of \( D_T(\hat{p}_d) \) is gained by reformulating Eq. (13) in terms of the 2D Optical Transfer Function (OTF):

\[
\text{OTF}_{\text{det}}(k_\perp, z) = \frac{1}{A_d} \int \text{PSF}_{\text{det}}(\vec{\hat{p}}, z)e^{-i \vec{k}_\perp \cdot \vec{\hat{p}}}d^2 \vec{\hat{p}}
\]

(15)

where \( \vec{k}_\perp \) is a lateral spatial frequency. We note that

\[
R_{\det}(\Delta \vec{p}, z_c) = \frac{1}{(2\pi)^2 A_d^2} \int \left| \text{OTF}_{\text{det}}(\vec{k}_\perp, z_c) \right|^2 e^{-i \Delta \vec{p} \cdot \vec{k}_\perp}d^2 \vec{k}_\perp
\]

(16)

from which we readily obtain:

\[
D_T(\hat{p}_d) = \frac{T_s^2 C^2 A_s}{(2\pi)^2 A_d^2} \int \left| \text{OTF}_{\text{det}}(\vec{k}_\perp, z_c) \right|^2 \left[ 1 - \cos(\Delta \vec{p}_s \cdot \vec{k}_\perp) \right] d^2 \vec{k}_\perp
\]

(17)

Once again, this equation is equally valid for translated or randomized speckle patterns. In the case of randomized patterns (\( \Delta p_s \rightarrow \infty \)), then the term \( \cos(\Delta \vec{p}_s \cdot \vec{k}_\perp) \) can be replaced by its average value, namely 0. The random case expression for \( D_T \) thus simplifies to

\[
D_R(\hat{p}_d) = \frac{T_s^2 C^2 A_s}{(2\pi)^2 A_d^2} \int \left| \text{OTF}_{\text{det}}(\vec{k}_\perp, z_c) \right|^2 d^2 \vec{k}_\perp.
\]

(18)

The advantage of expressing \( D_T \) and \( D_R \) in terms of \( \text{OTF}_{\text{det}} \) is that a well-established analytical approximation for \( \text{OTF}_{\text{det}} \) is available for circular pupils, given by [11]

\[
\text{OTF}_{\text{det}}(k_\perp, z) = g \left( \frac{k_\perp}{\Delta k_\perp} \right) \frac{2J_1(\frac{1}{2}k_\perp(\Delta k_\perp - \frac{1}{2}k_\perp))}{\frac{1}{2}k_\perp(\Delta k_\perp - \frac{1}{2}k_\perp)}
\]

(19)
focus can be interpreted as the defocus where the width of PSF of small speckle translations then from Eq. (12), we find our simple model in section 3.

When $|z_c|$ is less than this transition defocus, the speckle translation is effectively seen as a randomization. However when $|z_c|$ is greater than this transition defocus, the speckle translation preserves correlations in consecutive speckle patterns. To keep this transition defocus as tightly confined to the focal plane as possible, $\Delta\rho_s$ must be chosen to be small (though no smaller than the speckle grain size!). In practice, $\Delta\rho_s$ is chosen to be of order the speckle grain size. The translation DSI out-of-focus background rejection ($\sqrt{D_T}$) therefore scales as 1/$|z_c|^3/2$, also as expected from our simple model in section 3. The reason for this improved background rejection is clear from Fig. 3(a), and stems from the extra factor $\left[1 - \cos(\Delta\rho_s k_\perp)\right]$ in the integrand of Eq. (17). This term effectively quenches the low-frequencies contributions to $\left|\text{OTF}_{\text{det}}(k_\perp, z_c)\right|^2$, which decay only weakly (if at all) with $|z_c|$.

Having developed a model that is valid for arbitrary PSF, we now examine how the DSI sectioning strength is influenced by the PSF shape. In the case of speckle randomization, DSI sectioning strength seems to depend little on the exact profile of PSF and PSF$^\text{ill}$ since both circular (see above) and Gaussian (see ref. [9]) pupil functions lead to quasi-confocal sectioning.

The situation is different, however, in the case of speckle translation. For example, if PSF$^\text{ill}$ and PSF$^\text{det}$ are both defined by a same Gaussian–Lorentzian profile: $\text{PSF}(\vec{\rho}, z) = \frac{1}{1+\gamma^2} e^{-\gamma^2/\psi_0^2(1+\gamma^2)}$ (where $\gamma = \frac{z_c}{\psi_0}$ and $\psi_0$ is the PSF waist), and we calculate $D_T$ directly from Eq. (12), we find

$$D_T(\vec{\rho}_d) = \frac{2A^2}{3+2\zeta^2} \left[1 - e^{-\frac{2A\Delta\rho_s^2}{\psi_0^2(1+3\zeta^2)}}\right]$$

(21)

where $A = \pi\psi_0^3/2$. (this expression reduces to that found in ref. [9] when $\Delta\rho_s \to \infty$). In the case of small speckle translations then $D_T(\vec{\rho}_d) \approx \left(\frac{2A\Delta\rho_s}{\psi_0(1+3\zeta^2)}\right)^2$ and we find that the DSI signal $\sqrt{D_T}$ exhibits truly confocal sectioning (as opposed to near-confocal sectioning for a circular pupil function). This suggests that we could improve out-of-focus background rejection by using Gaussian apodization in the detection pupil.

5. Experimental results

Our experimental setup, shown in Fig. 4, is based on a standard widefield microscope with an argon laser as a light source. A spatial light modulator (SLM - Holoeye LC-R-768) is used to apply a random binary phase mask to the laser beam, by randomly assigning to each pixel a
phase-shift of 0 or π. The SLM is imaged onto the back focal plane of the microscope objective, thereby producing widefield speckle illumination. The fluorescence emitted at the focal plane of the objective is then imaged onto a CCD camera (Retiga 2000R, Q-Imaging).

Various strategies can be used to translate the speckle pattern, the simplest of which is to apply a linear phase-gradient across the SLM (a phase slope in the objective back focal plane leads to an amplitude translation in the front focal plane). Unfortunately, the dynamic range of our SLM was limited to about π, and hence we had to resort to an alternative strategy: It is well known that a lateral translation of the random phase mask applied at the SLM plane will result in a translation of the speckle pattern everywhere except at the corresponding SLM Fourier plane [12, 13]. We therefore decided to displace this Fourier plane away from the objective focal plane by illuminating the SLM with a diverging beam instead of a collimated beam. The SLM Fourier plane is then located at the geometric image of the effective illumination point source (see Fig. 4), and with this configuration a simple translation of the SLM phase mask induces a translation of the speckle pattern in the region of the objective focal plane.

A more detailed schematic of this illumination configuration is shown in the inset of Fig. 4 where, for simplicity, we removed the 1 × 1 telescope and depicted the SLM directly at the objective back focal plane. The dotted lines illustrate the conjugation between the effective point source and the SLM Fourier plane. Moreover they provide a simple geometric picture relating a phase-mask translation Δρ_M at the SLM plane to the corresponding speckle pattern translations inside (or outside) the sample. At the SLM Fourier plane, this corresponding translation is equal to zero, and the speckle pattern is said to be “boiling”[12]. Away from the SLM Fourier plane, the speckle is almost purely translated. The amount of translation Δρ is linearly proportional to the distance d between the observation plane and the SLM Fourier plane: Δρ = Δρ_M d / f_o.

Fig. 3. DSI sectioning strength for a circular aperture OTF (Eq. (19)). Panel (a): |OTF(k, z_c)|^2 for z_c Δk / k = 0 (solid blue line), 10 (dashed red line) and 40 (dash-dotted green line). The dotted black line corresponds to the modulation factor [1 - cos(k_c Δρ_s)] for a speckle translation in the x direction of step size Δρ_s = π / Δk_⊥. Panel (b): Numerical evaluation of DT (Eq. (17)) for a uniform plane sample as a function of defocus z_c, for Δρ_s = π / Δk_⊥ (bottom blue solid line), Δρ_s = 12π / Δk_⊥ (middle black solid line) and Δρ_s = 80π / Δk_⊥ (top green solid line), on a logarithmic scale. For Δρ_s = π / Δk_⊥ and Δρ_s = 80π / Δk_⊥, the results are fitted by straight lines of slopes −2 and −3 respectively. For Δρ_s = 12π / Δk_⊥ there is a break between these slopes. Note: traces in panel (b) are normalized so that the random DSI signal is unity at z_c = 0.
Fig. 4. Experimental setup. A spatial light modulator (SLM) imparts a random phase mask on an argon laser beam. The SLM is imaged onto the back focal plane of the microscope objective, so as to create a widefield speckle illumination (the SLM is used in reflection but drawn here in transmission for simplicity). The fluorescent light emitted from the sample is imaged onto a CCD camera. The SLM is illuminated with a diverging beam so as to displace the SLM Fourier plane away from the objective focal plane. A translation of the SLM phase mask then results in a translation of the speckle pattern inside the sample (see text for details). The inset is a simplified schematic of the illumination geometry.

(where $f_o$ is the objective focal length)[12]. The SLM Fourier plane can thus be regarded as a pivot plane about which the 3D speckle pattern becomes sheared. In our experimental setup $f_o = 4.5\text{mm}$, $d_{FP} \approx 100\mu\text{m}$, and we choose $\Delta \rho_M$ such that the speckle translation $\Delta \rho_s$ at the objective focal plane is on the order of a speckle grain diameter ($<1\mu\text{m}$). We note that as long as $|\Delta \rho_s/d_{FP}| < \sin \alpha$ (the case here), then the speckle translation always remains smaller than the width of the out-of-focus PSF$_{det}$, as required to ensure out-of-focus correlations between consecutive speckle patterns.

We note that the theory presented in sections 3 and 4 is equally valid for translations comprising directed walks (fixed translation steps and directions) or random walks (fixed translation steps but random directions). The former was found to cause residual streaking in the final DSI image, and we generally opted for the latter in practice (we verified experimentally that both walks lead to the same depth sectioning strength).

For demonstration purposes, we directly compare randomization versus translation DSI imaging by applying a speckle update protocol that alternates between randomization and translation. In this way, $I_{d,k+1}$ and $I_{d,k}$ are statistically independent when $k$ is odd (by randomization) and statistically dependent when $k$ is even (by translation), and the corresponding $D_R$ and $D_T$ are evaluated from:

$$D_T = \frac{1}{2N} \sum_{k=1}^{N/2} (I_{d,2k+1} - I_{d,2k})^2 \quad \text{and} \quad D_R = \frac{1}{2N} \sum_{k=1}^{N/2} (I_{d,2k} - I_{d,2k-1})^2 \quad (22)$$
Fig. 5. Experimental measurement of the DSI sectioning strength $\sqrt{D}$ for a uniform fluorescent plane sample (thickness less than 1.5 $\mu$m), for a translation step size $\Delta \rho_s$ of approximatively one speckle grain size (blue trace) and 30 speckle grain sizes (green trace). The red trace corresponds to speckle randomization. The objective used in this experiment has a numerical aperture of 0.65 (Olympus 40 x dry). Traces are shown in linear scale (a) and logarithmic scale (b), and are normalized such that the random DSI signal is unity at $z_c = 0$. For a large $\Delta \rho_s$, the sectioning trace is identical to that obtained with random DSI. In panel (b), the experimental traces are fitted with straight lines of slope $-3/2$ for small $\Delta \rho_s$, and $-1$ for a large $\Delta \rho_s$ and random DSI.

Such an alternating speckle update protocol would not normally be used in practice, however it has the advantage here of allowing us to compare the sectioning strength of $D_R$ and $D_T$ with rigorously identical data sets.

We emphasize that we have only considered “classical” speckle fluctuations throughout sections 3 and 4, and have neglected the contributions of shot-noise to $D_R$ and $D_T$. In practice, to remove these shot-noise contributions we must subtract them from $D_R$ and $D_T$ before applying the square-root operation to retrieve our final DSI images, as prescribed in section 2 (and being careful to take into account CCD-camera gain and offset).

To experimentally quantify the DSI sectioning strength, we measure the signal from a thin fluorescent plane as a function of its axial position $z_c$. Figure 5 displays the measured signal $\sqrt{D_T}$ for two different translation step sizes $\Delta \rho_s$, and for randomization. The green trace corresponds to a step size $\Delta \rho_s$ that is larger than the maximum out-of-focus PSF$_{det}$ width. As expected, such a large step size is equivalent to randomization, and we observe the same quasi-confocal sectioning strength for translation and randomization (red trace), where $\sqrt{D_T}$ decays as $1/|z_c|$. In contrast, when $\Delta \rho_s$ is of order a speckle grain size (blue trace), we confirm that DSI sectioning is improved to near-confocal and that $\sqrt{D_T}$ decays as $1/|z_c|^{3/2}$, as expected from theory.

Finally, we present images of a fluorescent pollen grain (Fig. 6). From the same set of raw images, we compute three different images: the translation DSI image (a), the random DSI image (b) and the widefield image (c) which is obtained simply by averaging all the raw images. The translation $\Delta \rho_s$ is set to approximatively 1 speckle grain size. Again, we observe that translation DSI confers better sectioning than random DSI: the center of the pollen grain is darker, the details appear finer, and the overall image quality is significantly improved. Figure 6(d) illustrates a 3D image of the pollen grain reconstructed from a z-stack of 75 2D images.
Fig. 6. Images of a fluorescent pollen grain obtained using an objective of numerical aperture 1.3 (Olympus 40× oil). Panel (a): signal $\sqrt{D_T}$ obtained with translating speckle (one image from full z-stack - avi movie 2.27MB). Panel (b): signal $\sqrt{D_R}$ obtained with randomized speckle. Panel (c): widefield image (i.e. average of the raw images). Panel (d): 3D reconstruction from z-stack (using Image J - avi movie 1.75MB). 128 raw images (acquisition time 150 ms per image) were used for each sectioned DSI image. Images (a), (b) and (c) were calculated from the same set of raw images (see text for detail).

6. Conclusion

In conclusion, we have studied the sectioning capacity of DSI microscopy based on a (half) differential intensity variance algorithm, and have shown that this sectioning capacity can be improved by controlling the dynamics of the speckle. We have demonstrated both theoretically and experimentally that out-of-focus background rejection scales as $1/|z_c|^{3/2}$ when we slightly translate the speckle pattern and as $1/|z_c|$ when we randomize it, where $z_c$ is the defocus distance. The improved sectioning strength with translation DSI is near-confocal. In addition, we displayed images of a fluorescent pollen grain and demonstrated a significant improvement in image quality with translation DSI as compared to random DSI. This work is a step toward further improvements in DSI microscopy algorithms designed to increase sectioning strength while reducing the number of raw images required for DSI image processing.

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