Wide-field multispectral super-resolution imaging using spin-dependent fluorescence in nanodiamonds

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Abstract

Recent advances in fluorescence microscopy have enabled spatial resolution below the diffraction limit by localizing multiple temporally or spectrally distinguishable fluorophores. Here, we introduce a super-resolution technique that deterministically controls the brightness of uniquely addressable, photo-stable emitters. We modulate the fluorescence brightness of negatively charged nitrogen-vacancy (NV−) centers in nanodiamonds through magnetic resonance techniques. Using a CCD camera, this ‘deterministic emitter switch microscopy’ (DESM) technique enables super-resolution imaging with localization down to 12 nm across

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a $35 \times 35 \, \mu m^2$ area. DESM is particularly well suited for biological applications such as multi-spectral particle tracking since fluorescent nanodiamonds are not only cytocompatible but also non-bleaching and bright. We observe fluorescence count rates exceeding $1.5 \times 10^6$ photons per second from single NV$^-$ centers at saturation. When combined with emerging NV$^-$-based techniques for sensing magnetic and electric fields, DESM opens the door to rapid, super-resolution imaging for tracking and sensing applications in the life and physical sciences.

**Keywords:** Super-resolution, wide-field imaging, nitrogen-vacancy (NV) center, fluorescent nanodiamonds, multispectral imaging, particle tracking

The challenge in sub-diffraction limited microscopy is to localize multiple fluorescent emitters within a diffraction volume. To locate and discriminate several emitters, it is necessary to distinguish their fluorescence sufficiently to reconstruct individual spatial locations. The NV$^-$ center is appealing for fluorescence microscopy due to its exceptional photostability and brightness. These properties recently enabled stimulated emission depletion (STED) microscopy to resolve NV$^-$ centers down to 5.8 nm by scanning a high power ($\sim 1 \, \text{GW/cm}^2$) doughnut-shaped depletion spot across a sample. However, this serial scanning measurement results in a slow frame rate — approximately 25 seconds for a $0.3 \times 0.3 \, \mu m^2$ field of view — which precludes imaging of important dynamical process, especially in biological sciences. To address concerns related to high pump intensities, NV$^-$ spin manipulation techniques allow for a reduced laser excitation intensity, but at the cost of acquisition speed.

On the other hand, stochastic super-resolution techniques, such as photo-activated localization microscopy (PALM$^{4,5}$) and stochastic optical reconstruction microscopy (STORM$^{6,7}$), employ sequential activation of photo-switchable fluorophores for time-resolved localization. Such methods enable fast, parallel acquisition using 2D CCD arrays. For example, a recent demonstration of STORM reached a frame acquisition time of 30 seconds for 20 nm spatial resolution over a $13 \times 4 \, \mu m^2$ field of view using a bleaching laser power of approximately 15 kW/cm$^2$. However, stochastic super-resolution techniques suffer from the need for precise control of the maximum density of fluorophores, localization of stochastic switching events over diffuse background, and
trade-offs between photostability and imaging rate. By contrast, DESM employs deterministic modulation of emitters with spin-dependent fluorescence that are uniquely addressable, photostable, and bright with more than $1.5 \times 10^6$ photons observed per emitter per second. Through selective microwave excitation of the spin-triplet ground state, it is possible to control the fluorescence rates of tens to hundreds of uniquely addressable classes of NV$^-$ centers in nanodiamonds (See Supplemental). This multi-spectral probing in the microwave domain also presents new possibilities in multi-color particle tracking and imaging. Unlike a previous demonstration that used microwave addressability for sub-diffraction microscopy of two NV$^-$ centers in bulk diamond, we simultaneously image hundreds of centers in nanodiamonds with a resolution of 12 nm over a 35$\times$35 $\mu$m$^2$ field of view. This DESM technique enables high-speed, sub-diffraction limited imaging with low laser intensity across a wide field of view.

The fluorescence intensity of the NV$^-$ depends on occupation of the three sub-levels of its spin triplet. In the $|m_s = 0\rangle$ “bright” sub-level, the center is photostable and bright; in the $|m_s = \pm 1\rangle$ “dark” sub-levels, the center undergoes intersystem crossing into a metastable spin-singlet state that reduces the average fluorescence intensity. The energy of the $|m_s = \pm 1\rangle$ ground states exceeds the $|m_s = 0\rangle$ state by the crystal field splitting, $\Omega_{ZFS} \approx 2.87$ GHz, in the absence of external magnetic fields. The degeneracy of the $\pm 1$ states is lifted in the presence of a weak magnetic field via the Zeeman effect. In this regime, the energy difference between the two dark states is given by $2\Delta \omega \approx 2g\mu \cdot \vec{B}$, where $g$ is the electronic Landé g-factor, $\mu$ is the NV$^-$ magnetic moment, and $\vec{B}$ is the applied magnetic field (Fig. 1a). Continuous optical polarization of the NV$^-$ into $|m_s = 0\rangle$ using concurrent microwave excitation decreases the NV$^-$ fluorescence when the microwave field is resonant with the $|m_s = \pm 1\rangle$ transitions. This optically detectable magnetic resonance (ODMR) technique (Fig. 1b) enables us to measure the electron spin resonance frequencies of all centers within the optically excited region on the sample (Fig. 1c). If the centers within a diffraction-limited spot have non-overlapping resonances, we can individually address them by microwave excitation and deterministically drive them into the dark state.

Nanodiamonds are arbitrarily oriented on a surface, leading to a wide range of non-degenerate
Figure 1: a. NV$^-$ energy level diagram showing how preferential shelving of the $|m_s = \pm 1\rangle$ excited states ($^3E$) into the dark metastable state ($^1A$) gives rise to a typical electron spin resonance (ESR) spectrum. $^{11}$ b. Fluorescence of two NV$^-$ centers in the presence of a static magnetic field as a function of applied microwave frequency. The splitting of the two dips ($2\Delta\omega_{A,B}$) is given by the projection of the incident magnetic field on the magnetic moment of the NV$^-$ ($2g\mu_{A,B} \cdot \vec{B}$). The contrast of each dip has an inverted Lorentzian shape with a minimum line width limited by the dephasing time due to the environment ($T^*$). c. Illustration of NV$^-$ centers in a static magnetic field, each having field splitting frequencies corresponding to their uniquely oriented magnetic moments relative to the magnetic field. To measure ESR spectrums across a wide field of view, the average fluorescence intensity is measured across hundreds of diffraction limited sites (dotted circle) while microwave frequencies are swept. d. Schematic diagram of the method for resolving a switchable emitter by taking the difference between two images where a uniquely addressed emitter is and is not dimmed by resonant microwave excitation, $I(\Omega_{ZFS} \pm \Delta\omega)$ and $I(\Omega_o)$, respectively.
spin transitions uniquely associated with individually oriented NV\(^{-}\) centers. The number of uniquely addressable centers depends on the number of non-overlapping Lorentzian resonances over the maximum frequency splitting due to an applied magnetic field. Given that each center has a splitting of approximately 2.8 MHz/G for the magnetic field magnitude parallel to the NV\(^{-}\) axis, we estimate that for an applied field of 200 Gauss, it is possible to resolve up to \(\sim 55\) uniquely addressable classes of NV\(^{-}\) centers within a diffraction limited spot. With much stronger applied fields along the perpendicular plane to the NV\(^{-}\) axis, the ESR spectrum contrast can decrease due to electron spin mixing of the sub-levels.\(^{15}\)

DESM achieves sub-optical resolution by multi-spectral imaging in the microwave domain. The basic procedure is to individually dim each of the NV\(^{-}\) within a diffraction limited spot by resonantly driving only one ground-state spin transition at a time. As shown in Fig. 1d, only the dimmed fluorescence from a single addressed center remains after subtracting an image acquired with resonant microwave excitation from an image obtained without resonant excitation. The fundamental limit of the signal-to-noise ratio of this subtracted image is approximated by:

\[
\frac{N}{\sigma} \sim \frac{\eta \tau \Gamma(I_{\text{laser}})C}{\sqrt{\eta \tau \Gamma(I_{\text{laser}})(M - 1) + \eta \tau \Gamma(I_{\text{laser}})(1 - C) + AI_{\text{laser}} + B}}
\]

where \(N\) is the number of collected signal photons, \(\sigma\) is the noise, \(\eta\) is the collection efficiency, \(\tau\) is the acquisition time, \(\Gamma(I_{\text{laser}})\) is the fluorescence rate as a function of laser intensity \(I_{\text{laser}}\), \(C\) is the fractional decrease of the total fluorescence on resonance, and \(M\) is the total number of emitters in the collection volume. \(A\) accounts for linearly increasing background with laser intensity, and \(B\) is a constant background noise. As seen in this equation, for other kinds of emitters with a larger switching contrast, \(C\), the DESM technique can resolve a greater number of emitters per site (See Supplemental). For imaging with a 2D CCD array, the ideal shot-noise-limited measurement error for estimating the center of a two-dimensional Gaussian spot is given by:\(^{16}\)

\[
\langle(\Delta x)^2 \rangle = \frac{s^2}{N} \left[1 + \frac{1}{12} \left(\frac{a}{s}\right)^2 + \frac{8\pi}{(a/s)^2} \frac{M - C}{C} \right] + O(N^{-2})
\]
where $s$ is the standard deviation of a point spread Gaussian distribution, and $a$ is the camera pixel size divided by the magnification. We note that this analytical result is known to underestimate the actual error by $\sim 30\%$.\textsuperscript{17}

![Figure 2](image)

**Figure 2:** a. ESR spectrum of two NV$^-$ centers of different orientations in a diffraction limited site under a static magnetic field. The red curve is an inverted Lorentzian fit to the data. b. Three $14 \times 14$ confocal scans over a $0.8 \times 0.8 \mu m^2$ area, with each scan taken at three different applied microwave excitation frequencies: $(\Omega_{ZFS} - \Delta \omega_A, \Omega_o, \Omega_{ZFS} - \Delta \omega_B)$ c. Subtraction of images where emitters are resonantly and not resonantly excited. d. Symmetric Gaussian fits to the subtracted images in (c). e. Reconstruction of the two NV$^-$ centers within a diffraction-limited spot in accordance to the subtraction and fitting method described in the text. The full-width half-maximum of the top and bottom gaussians are $11 \text{ nm}$ and $16 \text{ nm}$, respectively.

We explored DESM in two imaging modalities: confocal imaging, which allows for optimal optical resolution and contrast, and wide-field imaging, which enables sub-diffraction limited imaging of hundreds of NV$^-$ centers simultaneously. For an NV$^-$ site on a bulk sample, we obtained an ESR spectrum of exactly two emitters, A and B (Fig. 2a), and verified the number of emitters by autocorrelation measurements. The spectrum obtained at the position of maximum intensity showed that emitters A and B had different splittings of $\Delta \omega_A = 13 \text{ MHz}$ and $\Delta \omega_B = 87 \text{ MHz}$, respectively, indicating different NV$^-$ orientations. For super-resolution imaging, we used only the $|m_s = -1\rangle$ ground state resonances and acquired the fluorescence at three microwave frequencies, with two being resonant with the two centers and a third being off-resonant from both: $\Omega_{ZFS} - \Delta \omega_A$, $\Omega_o$, and $\Omega_{ZFS} - \Delta \omega_B$ (Fig. 2b). Fitting the difference plots, $\Delta I_{A,B}(\vec{r}) = I(\vec{r}, \Omega_o) - I(\vec{r}, \Omega_{ZFS} - \Delta \omega_{A,B})$, with symmetric Gaussian functions by a least-squares method (Fig. 2c,d) produced the reconstructed image in Fig. 2e, which indicates emitter localization to $11 \text{ nm}$ with an $\sim 80\%$ coefficient of determination and a separation of $195 \text{ nm}$. Each
additional emitter ‘k’ with $|m_s = 0⟩ \rightarrow |m_s = \pm 1⟩$ transition frequencies $\Omega_{ZFS} \pm \Delta \omega_k$ can be localized by acquiring additional images, $I(\vec{r}, \Omega_{ZFS} \pm \Delta \omega_k)$, and following the same image subtraction and Gaussian fitting routine.

Figure 3: 

- **a.** Fluorescence image of nanodiamonds over a 35×35 µm$^2$ area. The gold ‘+’ markings indicate sites that show ESR modulation while the blue ‘x’s do not show modulation. 
- **b.** A reconstructed region from (a) after applying the DESM technique with the blue circles indicating a lack of ESR modulation. Using the ESR spectrum at each site and the DESM technique, multi-emitter sites are reconstructed over a 7×9 µm$^2$ field of view. The numbers correspond to the resonance frequencies of each NV$^-$ in the site. 
- **c.** ESR spectrum of a multi-spectral site from (b). Colored Lorentzian fits correspond to the resonances of each of the four NV$^-$ centers in the site. 
- **d.** Sub-diffraction limited reconstruction of four NV$^-$ centers in (b) and (c). Full-width half-maximum from left to right are: 26 nm, 15 nm, 12 nm, 46 nm, respectively. 
- **e.** A full ESR spectrum of a site containing two centers. Colored lines indicate Lorentzian fits for each center with the arrows indicating the resonance frequencies at which images were taken for monitoring mode reconstruction with a total acquisition time of 1.44 s (further details in the text). 
- **f.** Monitoring mode reconstruction of the site in (b) and (e). The full-width half-maxima of the centers from top to bottom are 53 nm and 24 nm.

To increase the acquisition speed, we investigated the DESM protocol using an emCCD camera for super-resolution imaging over a 35×35 µm$^2$ field of view. A magnification of $\sim$190× projects diffraction limited spots across 5 pixels on the emCCD, which maximizes the signal-to-noise ratio according to Eq. 2. We captured 90 images at microwave frequencies from 2.71 GHz to 2.88 GHz. A spot-finding algorithm selected 116 candidate fluorescence sites based on a fluorescence intensity threshold; 95 showed fluorescence modulation due to the applied microwave field (Fig. 3a). The reconstructed image contains several diffraction-limited sites with multiple NV$^-$ centers, that are each spectrally distinguishable in Fig. 3b. Despite the partially overlapping Lorentzian reso-
nances of the multi-spectral site depicted in Fig. 3c, the DESM reconstruction algorithm spatially resolved the NV$^-$ centers. The resulting reconstruction of that site is shown in Fig. 3d where four centers are identified. The localization of individual centers ranges from 12 to 46 nm and is indicated by the distributions as shown in Fig. 3d. The color of each center corresponds to a frequency in the microwave regime. This illustrates the multicolor aspect of this technique, allowing for multi-spectral labeling with sub-diffraction resolution. With a total measurement time of $\sim 90$ seconds over the entire $35 \times 35 \, \mu m^2$ field of view, NV$^-$ centers were localized with an average uncertainty of 27 nm and a minimum uncertainty of 12 nm. Since a full ESR spectrum was acquired across the entire field of view, a site containing more than four more NV$^-$s does not require additional images. Total acquisition time can be reduced at the expense of a resolution that scales roughly as the inverse square root of the total exposure time.

While acquiring the full ESR spectrum allows us to image all NV$^-$ classes across the entire field of view, in certain applications, such as molecular tracking, it is desirable to focus on a subset of classes for higher acquisition rates. Specifically, we acquire images $I(x,y,\Omega_i)$ only at the microwave transition frequencies $\Omega_i$ of the emitters to be tracked, in addition to one off-resonant image, $I(x,y,\Omega_0)$. Using this high-frame rate monitoring technique, two NV$^-$ centers were resolved to be 55 nm apart with 25 nm resolution in an acquisition time of 1.44 seconds (Fig. 3f). The resolution of DESM can be further improved by increasing ESR visibility. Several factors that impact visibility include laser polarization, power, and the orientation of the microwave field. We estimate that by increasing laser power to $\sim 250 \, kW/cm^2$, it is possible to improve the average spatial resolution down to $\sim 8$ nm.

In conclusion, we have introduced a deterministic emitter switching technique to pinpoint the position of NV$^-$ centers below the diffraction limit with resolution comparable to super-resolution stochastic methods. Several other techniques developed in recent years employ multiple optically distinguishable emitters for super-resolution single-molecule tracking. As DESM can potentially distinguish up to 55 different emitters in a spot, it offers the largest number of spectral channels reported to date for multispectral fluorescence microscopy. Pulsed electron spin tech-
niques\textsuperscript{13,20} can improve the contrast ratio and greatly reduce the effective line-width, resulting in more uniquely resolvable centers within a diffraction volume. Besides such pulsed techniques, higher quality nanodiamonds with long spin-coherence times can also be used for improving super-resolution images.\textsuperscript{21} Super-resolution imaging using fluorescent nanodiamonds holds several advantages over other fluorescent markers for biological applications,\textsuperscript{22–28} including photostability, cytocompatibility, and high-resolution magnetic\textsuperscript{3} and electric\textsuperscript{29} field sensitivity. Furthermore, DESM allows for detecting a high fluorescence intensity exceeding $1.5 \times 10^6$ photons per second for a single NV\textsuperscript{−} center at saturation. In experiments involving biological tissue, we expect two factors that can diminish the signal-to-noise ratio and potentially reduce the number of resolvable emitters: higher background counts and possible rotational diffusion\textsuperscript{30} of the nanodiamonds depending on their location\textsuperscript{24}(See Supplemental). Finally, this technique may also be implemented on other emitters exhibiting ODMR such as the silicon defect center in silicon carbide,\textsuperscript{31} and single organic molecules.\textsuperscript{32} The high frame rate of up to 0.7 Hz, sub-wavelength localization down to 12 nm, and ability for uninterrupted monitoring of individual emitters makes DESM an attractive tool for a range of imaging applications.

**Methods**

**Experimental Setup:** Confocal experiments were performed using a home-built microscope with a Nikon 100X objective (NA = 1.3), an avalanche photon detector (APD), and a 532 nm pump laser at a power of 1,500 $\mu$W. Wide field measurements were performed on a commercial microscope (Zeiss Observor.Z1m) outfitted with a ProEM-512K CCD, using a 532 nm laser with 500 mW of power. Using a sample containing a nano-fabricated array, we calibrated each 16 $\mu$m square pixel on the emCCD to correspond to 82 nm in size. To acquire the ESR spectrum for every NV\textsuperscript{−} centre in the field of view, the emCCD captured one frame, $I(x,y,\Omega)$, for each step in the microwave frequency sweep with an applied static magnetic field of $\sim$55 G. Microwaves were applied through a local wire located 15-30 $\mu$m from the sample surface. A KC1-T-PZ piezoelectric
mount oscillated the defocused pump laser over a 1-2 µm area at \( \sim 110 \) Hz to reduce laser speckle on the sample. The vibrations of the sample were minimized using a rigid sample holder, a closed-loop PI-545 piezoelectric stage with resolution < 1 nm, and also a liquid-cooled camera to avoid vibrations from the camera fan.

**Curve Fitting:** An Airy point spread function was fit to difference plots, \( \Delta I(x, y, \Omega) \), using non-linear least square curve fitting tools in MATLAB by a symmetric Gaussian fitting with five free parameters: \( \hat{I}_o \) for the total area under the Gaussian, \( \hat{b}_g \) for the background counts, \( \hat{\sigma} \) for the standard deviation, and \( (\hat{x}, \hat{y}) \) for the centre of the Gaussian function. Term \( P \) is the length of the sample corresponding to each pixel on the CCD. The centers were fit using:

\[
\hat{I} = \hat{b}_g + \frac{\pi \hat{I}_o \hat{\sigma}^2}{4} [\text{Erf}(\frac{x-\hat{x}+\frac{P}{2}}{\hat{\sigma} \sqrt{2}}) - \text{Erf}(\frac{x-\hat{x}-\frac{P}{2}}{\hat{\sigma} \sqrt{2}})] [\text{Erf}(\frac{y-\hat{y}+\frac{P}{2}}{\hat{\sigma} \sqrt{2}}) - \text{Erf}(\frac{y-\hat{y}-\frac{P}{2}}{\hat{\sigma} \sqrt{2}})] \tag{3}
\]

where \( \text{Erf} \), the error function, is used for binning of the Gaussian distribution due to a pixelated CCD array.

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**Supporting Information Available**

Autocorrelation of 2-NV\(^{-}\) site from Fig. 2, and saturation curve of single NV\(^{-}\) center with APD and emCCD. Also an analysis of the signal-to-noise ratio given a higher density of NV\(^{-}\)s.
material is available free of charge via the Internet at http://pubs.acs.org/.

References


