Single-pixel fluorescent imaging with temporally labeled illumination patterns: supplementary material

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This document provides supplementary information to “Single-pixel fluorescent imaging with temporally labeled illumination patterns,” <http://dx.doi.org/10.1364/optica.3.000971>. Details of the CHIRPT microscope are provided, including a description of the image reconstruction and propagation processes. An expression for the depth of field (DOF) in CHIRPT is derived, and experimental data is presented to confirm the results. Images acquired at higher speeds are also presented. © 2016 Optical Society of America

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1. EXPERIMENTAL METHODS

Two continuous wave diode-pumped solid state (DPSS) lasers were used in these experiments, both of which operate at a nominal wavelength of 532 nm. Data in Fig. 4 and Fig. S4 were acquired with a 100 mW source (Coherent Inc., Compass 315M-100). All other data was collected with a Sprout 5G pump laser (Lighthouse Photonics).

Regardless of the source, the lasers were spatially filtered with a 0.25 NA objective lens (Zeiss A-Plan, UIS) and a 10- μ m pinhole (ThorLabs, P10S). The beam emerging from the spatial filter was collimated with an achromatic lens with a focal length of 80-mm (ThorLabs, ACH254-080-A-ML). The resulting illumination beam was measured to be 4.95 mm wide between the 20% and 80% peak intensity points.

For data collected with the Compass laser, the spatially-filtered beam was focused with a 300-mm focal length cylindrical lens, creating a line focus that extended laterally across the modulation mask, with a vertical height (full-width at the 20%-80% measurement points) of 15 μ m. After passing through the modulation mask, the modulated line focused beam was re-imaged and demagnified with a 250-mm tube lens (ThorLabs ACH-254-250-A-ML), and an objective lens. The objective lens used for collecting experimental data depended on the particular experiment. Data in Fig. 4 were collected with a 20 $\times$ /0.45 NA Zeiss A-Plan UIS objective lens, resulting in a system magnification of 30.4 $\times$. High speed data presented in Fig. S4 was collected

with a 10 $\times$ /0.25 NA Zeiss A-Plan UIS objective lens, providing a system magnification of 15.2 $\times$.

To increase the NA of the CHIRPT imaging system, the microscope was redesigned and rebuilt to allow for more precise control and alignment of the imaging system. In the redesigned CHIRPT system, we used the Lighthouse Photonics laser, as it has a relative intensity noise (RIN) significantly lower than the Compass laser. This substantially reduced the background noise on our CHIRPT data. This configuration was used to collect data displayed in all figures aside from those noted above.

Since the exit pupil of high NA objective lenses often lies inside the objective case, the horizontal slit was used to spatially filter the diffracted orders from the modulator could not be located in the pupil plane in the initial CHIRPT microscope design (Fig. S1). To overcome this limitation we designed the microscope with dual image relay systems to allow unrestricted access to a conjugate pupil plane. First, the illumination beam was brought to a line focus on the modulator by a 150 mm focal length cylindrical lens (ThorLabs, ACY254-150-A-ML). A 125-mm focal length lens (ThorLabs, ACH-254-125-ML) was placed one focal length away from the modulator. One focal length further downstream we placed a horizontal slit to reject one of the diffracted beams. The plane in which the slit was oriented was a conjugate plane to the pupil of the objective lens. A pair of lenses in a 4f configuration were used to image relay this plane to the pupil plane of the objective lens. The first lens

Fig. S1. Schematic of the CHIRPT setup. (a) A constant diffraction angle in the vertical dimension results in vertical offset of the diffracted beam from the undiffracted beam [1]. A horizontal slit is placed near the pupil plane of the objective lens to select a portion of the undiffracted beam and one diffracted order. (b) The objective is oriented such that the illumination beams propagate along $y = 0$. (c) The diffracted and undiffracted beams interfere in the object region to generate a unique modulation intensity pattern for each point in the (x, z) plane. The emitted fluorescence, represented by the orange sphere in the object region, is collected in the epi-direction.

had a focal length of 100-mm (ThorLabs, ACH-254-100-ML) and the second had a focal length of 250-mm (ThorLabs, ACH-254-250-ML). Finally, a $50\times/0.8$ NA objective lens was used (Zeiss, N-Achroplan $50\times/0.8$ NA Pol) to bring the illumination beams together in the object plane. The overall magnification of the microscope from the modulator mask to the object plane was $95\times$. The chirp parameter in the object plane was measured by translating an object in the x dimension and recording the centroid modulation frequency.

The modulation mask contains the pattern: $m(R, \varphi) = 1/2 \{1 + \text{sgn}[\cos(\Delta k R \varphi)]\}$, where (R, φ) represent polar coordinates relative to the center of the modulation pattern. The spatial modulation pattern was lithographically printed onto an aluminum coating applied to a glass substrate (Projection Technologies, Dallas, Texas) at 3600 DPI. A Faulhaber motor (2057S012BK1155) and speed controller (MCBL3006) were used to spin the modulation disk. All data presented in this work used mask designs with $\Delta k = 70/\text{mm}$. For high-speed data in Fig. S4, a disk with four masks was utilized to increase imaging speed.

Photomultiplier tubes (PMT) were used to collect fluorescent light. Data in Fig. S4 were recorded with a Hamamatsu H9305-03. All other fluorescent images were measured with a Hamamatsu H7422P-40. For the high-speed data in Fig. S4, the

signal from the PMT was high-pass filtered by a current preamplifier (Stanford Research Systems, SR570) with 10 kHz corner frequency and 12 dB/octave rolloff. All other data were collected with a low-noise current amplifier (DHPCA-100, Femto Messtechnik GmbH, Berlin, Germany). The transmitted laser intensity presented in Fig. 4 was measured with a large-area photodiode (ThorLabs, DET100A). The signals were digitized with a PCI data acquisition card (National Instruments, PCI-6110 with BNC-2090A breakout box). A custom C# Windows application controlled data acquisition and storage.

For the phase data presented in Fig. 2, $10\ \mu\text{L}$ of fluorescent nanodiamond (FND) slurry (ND-400NV-100nm-10mL, Adámas Nanotechnologies, Raleigh, NC) was dropped onto a microscope slide and allowed to dry before placing a cover slip over the FNDs. The FNDs were 100 nm in diameter, below the diffraction-limited resolution of the CHIRPT microscope (0.8 NA). A single $15\text{-}\mu\text{m}$ -diameter, shell-stained fluorescent microsphere in a prepared slide (LifeTechnologies, FocalCheckTM Slide 1, Well A1) was used for CHIRPT data presented in Fig. 3. Although the slide contains fluorescent microspheres with a variety of fluorophores, the orange fluorescent spheres were most visible with the illumination wavelength of 532 nm.

2. CHIRPT RECONSTRUCTION

Over a single rotation of the modulation mask, a time trace is collected from the photodetector that represents the intensity of the fluorescent light as the illumination pattern is varied. A 1D image of the object is encoded in this measured temporal trace, which is recovered by standard Fourier methods. Here we describe the process for isolating the 1D image from measured data, and how this data is propagated in the axial dimension.

A flow chart for reconstructing and propagating a single 1D image is shown in Fig. S2. In this example, we used simulated data traces to improve the clarity of the CHIRPT numerical processing steps. The signal simulating a signal measured from the photodetector, $S(t)$, was computed assuming a separable contrast function defined as: $C(x, z) = \text{rect}(x/d) \delta(z)$, where $\text{rect}(x/d) = 1$ for $|x| \leq d/2$ and is zero elsewhere, and d is the width of the distribution. $\delta(z)$ is the Dirac-delta function, which we used to represent a thin object in the focal plane of the microscope. The analytic data trace (Fig. S2a) was computed using the parameters of the experimental setup, i.e., 0.8 NA, 532-nm illumination light, $\Delta k = 70/\text{mm}$ and $95\times$ magnification. The width of the contrast function was set to $d = 5 \mu\text{m}$. The illumination intensity was computed using the results of Ref. [1]. The analytic expression for the measured time signal was:

$$S(t) = \frac{d}{4} + \frac{d}{\pi^2} w^2(t) + \frac{d}{\pi} w(t) \cos(2\pi \nu_c t) \text{sinc} \left[\frac{\Delta k_x(t) d}{2} \right] \quad (\text{S1})$$

where $w(t)$ is the temporal window function describing the amplitude of the diffracted order in the object plane with scan time [1, 2], $\nu_c = \omega_c / (2\pi)$ is the carrier frequency of the illumination intensity, and $\Delta k_x(t)$ is the spatial frequency difference between the illumination beams. We selected a Gaussian temporal window function where the $1/e$ width corresponded to the maximum spatial frequency supported by the objective lens.

Because the time signal measured from the photodetector represents the spatial frequency content of the object, the spatial image is encoded in the Fourier transform of $S(t)$. Figure S2b shows the fast Fourier transform (FFT) of the measured signal, which we denote as $\tilde{S}(\nu)$. Since $S(t)$ is real-valued, $\tilde{S}(\nu)$ displays conjugate symmetry in the modulation frequency domain. The carrier frequency separates the redundant image information encoded in $\tilde{S}(\nu)$ by isolating the two encoded images at $\pm \nu_c$. In practice, we isolate the positive sideband, as shown in Fig. S2c. We denote the filtered data as $\tilde{S}_{1+}(\nu)$.

The complex sideband is then inverse Fourier transformed to the temporal domain, where the carrier frequency appears as a linear temporal phase ramp. This complex data in the temporal domain is denoted as $S_{1+}(t)$ (Fig. S2d). The phase ramp can be removed to demodulate $S_{1+}(t)$, which is equivalent to re-centering the sideband in $\tilde{S}_{1+}(\nu)$ about zero modulation frequency. We demodulate by computing the carrier frequency at the centroid of the positive frequency sideband, ν_c , and demodulating by applying the inverse linear phase ramp, i.e., $\tilde{S}_{1+}(t) \exp(-i 2\pi \nu_c t)$. Since the spatial frequency is directly proportional to the scan time, $\Delta f_x(t) = \Delta k_x(t) / (2\pi) = \kappa t$, the demodulated time trace can be calibrated to obtain the frequency-space representation of the 1D image, $\tilde{S}[\Delta f_x(t)]$ (Fig. S2e).

The 1D image can be propagated axially by applying the defocus phase to $\tilde{S}(f_x)$. Specifically, the propagation phase is defined by the axial phase difference between the two illumination

beams at a defocus value Δz , as noted in the manuscript:

$$\Phi(\Delta k_x(t), \Delta z) = k \Delta z \left\{ \sqrt{1 - [\Delta k_x(t)/k]^2} - 1 \right\} \quad (\text{S2})$$

A propagated image is computed in the lateral spatial frequency domain via: $\tilde{S}[\Delta k_x(t)] \exp[i \Phi(\Delta k_x(t), \Delta z)]$ (Fig. S2f), and the propagated image in the spatial domain, $S(x, \Delta z)$, is computed with an FFT of the propagated frequency space data. Two 1D images of the object are presented in Fig. S2g: one unpropagated and the other propagated by $\Delta z = -10 \mu\text{m}$.

Data presented in Fig. S4 was further processed by multitaper power spectral density (PSD) analysis using the function `pmtm()` in MATLAB with a time-bandwidth product of 5/2. The processed image was computed by taking the square-root of the PSD estimate.

Data displayed in the main text were formed by averaging multiple images. Table S1 summarizes the acquisition parameters for each image, where ν_r is the frame rate of 1D data acquisition (2D after CHIRPT reconstruction), N_{scans} is the number of 1D data scans averaged for each position that the object was translated, N_{pix} is the number of positions at which data was collected, t_{tot} is the total image acquisition time, and $\mathcal{D}$ is the dimensionality of the reconstructed CHIRPT data.

Table S1. Data Acquisition Parameters

Figure	ν_r (s^{-1})	N_{scans}	N_{pix}	t_{total} (s)	$\mathcal{D}$
2	17	20	101	119	2D
3	17	15	101	89.3	3D
4	31	1	100	3.23	2D

3. DEPTH OF FIELD IN CHIRPT

One important advantage of CHIRPT imaging is an increased depth of field (DOF) as compared to conventional linear imaging. In tightly-focused imaging techniques, such as laser-scanning microscopy, the depth of field and lateral spatial resolution are both set by the numerical aperture (NA) of the illumination objective, and the DOF varies inversely as the square of the NA. CHIRPT does not suffer this same limitation because collimated beams with varied propagation angles are utilized to sequentially measure the spatial frequency content of an object. Since the CHIRPT signal is generated by the temporally varying part of the illumination intensity, $I_1(x, z; t)$, the depth of focus is dictated by the region over which the intensity modulation is non-zero. This region is formed by the interference of the re-imaged diffracted orders.

Let us assume that the illumination beam incident on the modulation mask has a Gaussian intensity profile in the lateral dimension and is collimated in the axial dimension. We may write the incident illumination field propagating along the optic axis as

$$E_{\text{inc}}(x, z) = u(x) e^{ik_0 z} = e^{-(x-x_0)^2/w^2} e^{ik_0 z} \quad (\text{S3})$$

where w is the width of the Gaussian distribution.

Due to diffraction from the modulation mask, the amplitude distribution propagates at an angle with respect to the optic axis in the object region (Fig. S1). We denote the amplitude

Fig. S2. The reconstruction procedure for CHIRPT images, demonstrated with simulated data. (a) A single temporal scan collected from the photodetector, $S(t)$. (b) The spectral density, $\tilde{S}(v)$, displays conjugate symmetry about DC modulation frequency. (c) Only one sideband is needed for image reconstruction. We select the positive sideband, which we denote as $\tilde{S}_{1+}(v)$. (d) The inverse Fourier transform of the filtered sideband, $S_{1+}(t)$, encodes the spatial phase difference between the illuminating beams. (e) The complex temporal trace is demodulated by the carrier frequency to remove the linear phase. Other phase disturbances, i.e., systematic aberration phase, or pupil phase, and the disk aberration phase are also removed to compensate for known aberrations to the system [3]. The temporal domain is also calibrated to the lateral spatial frequency domain, $f_x \propto \kappa t$. This step gives the 1D image in the lateral spatial frequency domain, $\tilde{S}(f_x)$. (f) $\tilde{S}(f_x)$ is propagated by applying the defocus phase. (g) A one-dimensional FFT with respect to the lateral spatial frequency domain reveals the propagated 1D image in the spatial domain, $S(x)$.

of a diffracted order j in the object region as $u_j(x, z; t)$. The amplitude of the undiffracted beam in the object region is simply given by the illumination amplitude scaled by the magnification of the illumination system:

$$u_0(x, z; t) = u_0(x/M) = e^{-M^2(x-x_0)^2/w^2}. \quad (\text{S4})$$

The diffracted order, $j = 1$, propagates at an angle with respect to the optic axis that depends on the rotational position of the mask and therefore depends on time. To simplify the following computations, we apply the approximation that upon diffraction from the mask the amplitude distribution simply rotates by the diffraction angle, i.e., there are no distortions to the amplitude distribution. In the rotated coordinate basis (x', z') , where z' is the optic axis of the diffracted beam in the object region, the spatial distribution of the diffracted order is

$$u_1(x'/M) = e^{-M^2(x'-x'_0)^2/w^2} \quad (\text{S5})$$

The DOF is dictated by the region of overlap of the diffracted and undiffracted spatial distributions:

$$\Lambda(x, z; t) = u_0(x, z; t) u_1(x, z; t) = u_0(x) u_1(x') \quad (\text{S6})$$

The diffracted order distribution can be written in the unrotated coordinate basis, (x, z) , via the coordinate transformation:

$$x' = x \cos \theta_2(t) - z \sin \theta_2(t) \quad (\text{S7})$$

Assigning the centroid of each distribution to the origin, $x_0 = x'_0 = 0$, we can write:

$$\begin{aligned} \Lambda(x, z, t) = & \exp \left[-\frac{x^2}{w^2} \right] \exp \left[-\frac{x^2 \cos^2 \theta_2(t)}{w^2} \right. \\ & - \frac{z^2 \sin^2 \theta_2(t)}{w^2} \\ & \left. + \frac{2xz \sin \theta_2(t) \cos \theta_2(t)}{w^2} \right] \end{aligned} \quad (\text{S8})$$

Using the shorthand notation $\Gamma \equiv \lambda M \kappa_1 t$, the overlap distribution in the object region is:

$$\begin{aligned} \Lambda(x, z, t) = & \exp \left[-\frac{x^2}{w^2} \right] \exp \left[-\frac{(1 - \Gamma^2) x^2}{w^2} \right. \\ & \left. - \frac{\Gamma^2 z^2}{w^2} + \frac{2\Gamma \sqrt{1 - \Gamma^2} xz}{w^2} \right] \end{aligned} \quad (\text{S9})$$

Although the DOF varies slightly with position in the object region, it is instructive to examine the DOF along the optic axis, i.e., $x = 0$:

$$\begin{aligned} \Lambda(x = 0, z, t) = & \exp \left[-\left(\frac{\lambda M \kappa_1 t}{w} \right)^2 z^2 \right] \\ = & \exp \left[-\left(\frac{\lambda f_{x,2}(t)}{w} \right)^2 z^2 \right] \end{aligned} \quad (\text{S10})$$

where $f_{x,2}(t)$ is the lateral spatial frequency in the object region. We define the DOF along the optic axis, DOF_z , as the full-width of the overlap distribution at the $1/e$ -point, which we can extract from Eq. (S10):

$$\text{DOF}_z(t) = \frac{2w}{\lambda M \kappa_1 t} = \frac{2w}{\lambda f_{x,2}(t)} \quad (\text{S11})$$

Since the highest spatial frequency that contributes to the measurement corresponds to the maximal scan times, $t = \pm T/2$, Eq. (S11) can be recast in terms of the numerical aperture of the imaging system to find the DOF for the full numerical aperture of the imaging system.

$$\text{DOF}_z = \frac{2w}{\lambda M \kappa_1 T/2} = \frac{4w}{\lambda M \Delta k v_r T} = \frac{4w}{\lambda M \Delta k} = \frac{2w}{\text{NA}} \quad (\text{S12})$$

Unlike conventional light focusing, where the DOF varies as $1/\text{NA}^2$, the DOF in CHIRPT at the full numerical aperture of the system varies as $1/\text{NA}$. This allows for high resolution imaging from a much broader depth.

We measured the DOF in CHIRPT by collecting an image of the illumination laser transmitted through a 20 line/mm Ronchi ruling as the ruling was defocused (Fig. 4). A 0.45 NA objective lens was used to resolve the object. The working distance of the objective lens is 510 μm , yet images were reliably formed though a scan range of 14 mm, or >27 times the working distance of the lens. Note that far away from the focal plane of the lens there is a loss of lateral spatial frequency content, made clear in Fig. 4b by the odd harmonics of the binary, 50% duty cycle Ronchi ruling have a depth-dependent amplitude. The yellow line indicates the on-axis depth of field as a function of lateral spatial frequency, $\text{DOF}_z = 2w/(\lambda f_x)$. The beam size, w , was estimated from the image in panel Fig. 4a to be 100 μm . Since the largest spatial frequency passed by the system is related to the numerical aperture by $\text{NA} = \lambda f_{x,\text{max}}$, the on-axis DOF for the full resolution of the lens can be recast as: $\text{DOF}_z = 2w/\text{NA}$.

We calculated the axial depth over which the full lateral spatial frequency support of the imaging system was retained to be approximately 440 μm , in good agreement with the data in Fig. 4. This is approximately $83\times$ larger than the predicted value of 5.3 μm for a conventional imaging system, defined as $\text{DOF} \approx 2n\lambda/\text{NA}^2$ [4].

4. HIGH-SPEED CHIRPT

For the majority of data presented here, we chose to display averaged images to improve the signal-to-noise ratio (SNR) (Table S1). We note however that CHIRPT reliably formed images with single-shot data acquisition. An example of this can be found in Fig. S3. While Fig. S3a shows a 2D image in the (x, z) plane reconstructed from data collected in a single rotation of the modulation mask, Fig. S3b shows the average of 20 measured 2D images. The single shot image was collected in 0.07 s while the averaged image was acquired in 141.4 s, not including time added by stop-and-hold stage scanning. While the SNR of Fig. S3b is indeed better than the single-shot image in Fig. S3a, the single-shot image was acquired over 2,000 times faster. We note that if only a single image were acquired by scanning axially in Fig. S3b, this speed increase would be approximately $100\times$.

Another example of high-speed single-shot imaging with CHIRPT is shown in Fig. S4, where we imaged a fluorescent object with an acquisition time of 2.48 ms for each 1D image. The full 3D image volume was acquired in approximately one second, ignoring the hold time of the linear positioning stage that translated the specimen vertically.

A. Increasing the imaging speed in CHIRPT

Since excited fluorescent molecules emit light spontaneously, there is a delay between excitation of a fluorophore and emission of a fluorescent photon. This delay is characterized by

Fig. S3. Single-shot vs. averaged image acquisition of fluorescence from an FND. (a) A 2D image reconstructed from a single measured 1D image at 14.3 Hz. (b) An image of the same region in the (x, z) plane formed by translating the FND and averaging twenty 1D images at each defocus location. Total acquisition times for (a) and (b) were 0.07 s and 141.4 s respectively.

the fluorescent lifetime, τ_f , of the molecule. A requirement of CHIRPT imaging is that emitted fluorescent light must follow the temporally-modulated illumination intensity pattern. This requirement is met when the modulation frequency in the illumination intensity is well below the inverse lifetime of the molecule, i.e., $\nu \ll \tau_f^{-1}$. In all work presented here, the CHIRPT modulation frequencies were well below this limit. Assuming a typical fluorescent lifetime of 10 ns [5], this modulation frequency limit is 100 MHz. In the experiments described in this work, the typical carrier frequency was approximately 25 kHz. The exception is the high-speed data presented in Fig. S4, where the carrier frequency was approximately 800 kHz, still well below the inverse lifetime of most fluorescent molecules.

As noted above, the high speed data was acquired by multiplexing four modulation patterns on one disk. Unfortunately, the spatial resolution of the lithographic mask printing process caused aliasing in the modulation pattern. This caused a reduction in the amplitude of the first-order diffracted beam by directing energy into additional diffracted orders, imposing limits on the signal-to-noise ratio (SNR) of the extracted images in Fig. S4. With higher resolution mask printing, it should be possible to achieve much faster line scanning rates. While we show acquisition at 403 lines per second (lps) in Fig. S4, similar imaging schemes have achieved frame rates in excess of 4,000 lps [6]. With appropriate design of the modulation system, we anticipate scan rates on the order of 5,000 lps. By scanning the line cursor in the vertical dimension with a 1D galvonometric mirror, the repositioning time can be fast enough to be considered negligible. In this case, the limitation for volumetric imaging would be the number of pixels in the vertical dimension. Assuming 500 pixels, this still permits an unprecedented 10 volumes per second.

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Fig. S4. High-speed epi-fluorescent CHIRPT images of lens tissue stained with highlighter ink. Scan times were measured by (a) using repetitive features in the temporal scan, and (b) adjusting the trigger delay of the DAQ card to collect two successive central peaks located near the center of the scan time, i.e., $t = 0$. Scan periods were found to be 2.48 ms in each case, corresponding to data acquisition for a full 2D (x, z) plane at 403 lines per second. Each image is composed of 401 vertical scans for a total acquisition time of 995 ms for the full volume. In both (a) and (b) the temporal waveforms are the mean of 401 scans at varied vertical positions. (c),(d) Propagated images of the lens tissue, with the amplitude of each image estimated with a multi taper analysis. The propagation distances were $-42 \mu\text{m}$ and $+20 \mu\text{m}$ in panels (c) and (d) respectively. Scale bar: $100 \mu\text{m}$.