Fast live simultaneous multiwavelength four-dimensional optical microscopy

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This article is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2009.

Edited by Joseph G. Gall, Carnegie Institute of Washington, Baltimore, MD, and approved June 1, 2010 (received for review April 5, 2010)

Live fluorescence microscopy has the unique capability to probe dynamic processes, linking molecular components and their localization with function. A key goal of microscopy is to increase spatial and temporal resolution while simultaneously permitting identification of multiple specific components. We demonstrate a new microscope platform, OMX, that enables subsecond, multicolor four-dimensional data acquisition and also provides access to sub-diffraction structured illumination imaging. Using this platform to image chromosome movement during a complete yeast cell cycle at one 3D image stack per second reveals an unexpected degree of phototoxicity of fluorophore-containing cells. To avoid perturbation of cell division, excitation levels had to be attenuated between 100 and 10,000-fold below the level normally used for imaging. We show that an image denoising algorithm that exploits redundancy in the image sequence over space and time allows recovery of biological information from the low light level noisy images while maintaining full cell viability with no fading.

OMX | phototoxicity | image processing | denoising | yeast

The ability to collect live biological image information in three dimensions as a function of time, four-dimensional imaging, is a powerful use of optical microscopy. It has led to the discovery of new phenomena, and in combination with analysis of mutations or other perturbations, can link biological functions to molecular mechanisms. The dynamic information gained from four-dimensional data also allows the accurate measurement of quantitative physical parameters, such as diffusion constants or velocity of active movement.

Though a powerful technique, live fluorescence imaging imposes constraints, which can severely impede its use. Biological processes within a cell are sensitive to the excitation light used for fluorescence imaging (for a review, see ref. 1, chap. 19). This may be evidenced by a failure or delay of cell division, morphological changes, or perturbation of other biological processes. The phototoxicity resulting from excitation light is in part caused by the long-lived triplet state present in all fluorescent processes interacting with molecular oxygen, generating very reactive intermediates such as free radicals. High levels of free radicals kill cells (2). In addition, excitation light can damage the fluorochrome, leading to the well-known phenomenon of photobleaching. Both phototoxicity and bleaching are directly proportional to the excitation light intensity. In general, one reduces the excitation intensity to minimize the photodamage. However, this has the undesirable consequence of lowering the signal-to-noise ratio of the image, resulting in a dim and therefore noisy image. These two competing considerations make information retrieval from live image sequences a challenging problem.

Using a newly devised fast multidimensional image acquisition platform (OMX) (SI Text), we address here the problem of sample damage due to excitation light and demonstrate that reduction of excitation light by several orders of magnitude, in combination with the appropriate use of image denoising algorithms, can allow informative four-dimensional imaging at previously impracticable rates without phototoxicity or fading.

Results

Preservation of Live Cell Viability Requires Reduction of Light Intensity

In the course of imaging experiments on yeast chromosome dynamics, we observed that yeast cells that had been imaged under what is normally considered to be a low-light dose failed to divide when left overnight, whereas their nonimaged neighbors divided normally. This prompted us to quantitatively measure the phototoxicity of our experiments. A yeast strain containing a Lac repressor::GFP fusion (YDB271) binding to a specific amplified Lac operator (3) was used to study phototoxicity during and after four-dimensional data collection. Three-dimensional images (25 Z sections) were acquired at 23 °C every 15 s over a period of 20 min, covering roughly 20% of a yeast cell cycle. This imaging regime is hereafter referred to as “sparse” (Fig. 1). Initially, we performed imaging under an excitation light intensity that resulted in an image intensity sufficient to allow direct automated tracking of the Lac repressor::GFP spots after deconvolution of the data. For these and subsequent experiments, this excitation intensity, 4.8 × 10⁻⁵ W/µm², is referred to as I₀, as shown in SI Text.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

See Commentary on page 16005.


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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1004037107/-/DCSupplemental.
Fig. 1. Although cells imaged under these conditions showed no defects during the actual imaging period, they were observed the next day arrested with the large dumbbell shapes characteristic of lethal DNA damage (4). To assess the overall sensitivity of yeast to light intensity, four-dimensional images were collected in the same sparse regime at $I_0$ and four lower light levels, reducing light intensity at each step by approximately a factor of ten (see Fig. 1, and in more detail in Table 1). After data collection at each excitation intensity, we monitored cell viability as described in Materials and Methods.

A plot of yeast viability as a function of excitation light intensity (Fig. 2) shows that the $I_0$ excitation light arrests or kills the yeast cells with little or no cell division occurring after time-lapse data collection. The excitation light at $I = I_0 - 10^{-1}I_0$, one log down, appears to arrest the cells at a checkpoint with a protruding bud and eventually resume dividing. Only at excitation $I = 10^{-2}I_0$, two logs down in intensity, were the cells observed to divide normally compared to the no-excitation control. In summary, the yeast cells as a representative in vivo sample are very sensitive to excitation light, necessitating the reduction of normal excitation intensity by two orders of magnitude for unperturbed viability in the sparse imaging regime. The photon flux with 488 nm light at our imaging regime, is $480 \text{nW/\mu m}^2$-sec or $1.2 \times 10^{12} \text{photons/\mu m}^2$-sec. Under our standard experimental conditions of 10 msec exposure time in each of 25 Z sections, this translates into $1.64 \times 10^{12}$ photons/3D image. We measured typical light exposure of yeast under room light during the daytime at $\sim 1 \text{pW/\mu m}^2$, 5 orders of magnitude less than the light intensity that starts affecting viability. Therefore, the conditions of even low-light fluorescence imaging are significantly brighter in comparison to the unimaged state.

**Denoising Recovers Information from Dim Images.** To preserve the ability of a cell to divide, the light intensity had to be reduced by at least two orders of magnitude in the sparse imaging regime. The consequence is that the images became very noisy (Fig. 3) and were no longer suitable for spatial or other quantitative analysis. When excitation light was reduced even further, the...
dominance of noise in the $I = 10^{-4}I_0$ series required time-averaging of the four-dimensional data even to be certain that cell image information was present. Maintaining both viability and sufficient image information is therefore a considerable technical hurdle in live imaging.

Remarkably, it is possible to computationally recover useful information from the extremely low-dose images without making any assumptions about sample structure. The approach is to use generalized denoising strategies that seek to remove statistical noise while preserving relevant sample intensity and spatial information. A promising denoising algorithm suitable for 2, 3, 4, and 5 (space, time, and wavelength) dimensional imaging has been recently published (5, 6) and implemented on several computer platforms. A diagram depicting the functioning of this algorithm is illustrated in Fig. 4. In essence, the denoising procedure typically first analyzes the image data for statistical signatures of Poisson and Gaussian noise originating from the limited number of photons (Poisson) and the dark current inherent to electronic imaging detectors (Gaussian). The overall strategy is to find regions of the image, which by virtue of similar statistical behavior, are likely to have the same underlying intensity distribution and then average them to reduce noise. The challenge is to correctly identify appropriate regions to average. More specifically, for each point of the image sequence, a set of pixels, termed a “patch,” of predefined size (e.g., $3 \times 3 \times 3$ or $5 \times 5 \times 5$ pixels) is considered. A local space and/or time neighborhood (a subset of the entire image centered on the current point) is scanned for patches showing similar intensity statistics. These patches are then averaged using weights defined as an increasing function of the similarity with the reference patch. This procedure is iterated several times, always averaging the original data but using the result of the previous step to perform patch comparisons. At each step, space-time neighborhoods are increased in size alternately in space and in time until a statistical control procedure (a “bias-variance” tradeoff) locally stops the growth in space or in time, whereas it may be continued in other locations of the image sequence. As a result, the shape of space-time neighborhoods used for denoising is locally adapted to the image content. Finally, the algorithm has been shown to preserve edges and image intensities over a wide variety of test cases (5, 6).

We applied this denoising strategy to the images in Fig. 3. Inspection of the intensity series of images shows that the yeast fluorescent chromosome site is clearly visible even in the $I = 10^{-4}I_0$ series. Line plots through the center of the fluorescent chromosome site dramatically demonstrates the rescue of the cell image data from the noise. In addition, in many cases it is possible to find the outline of the cell, the boundaries of the nucleus, and possibly other cellular structures from the faint GFP background fluorescence once the noise has been removed (see Fig. 7). This is important as it indicates the ability to recover more general shapes and not just point-like objects. We conclude that denoising is a useful tool for studying live 3D structure at light excitation levels that preserve cell viability.

To test for possible distortion of image information by the denoising algorithm, 100 nm fluorescent beads were imaged in 3D at different levels of excitation intensity, and compared with and without denoising (Fig. 5). Inspection of the fluorescent signal’s full width at half maximum (FWHM) from $I = 10^{-3}I_0$ to $I = 10^{-4}I_0$ shows no significant difference. A slight broadening of the FWHM at $I = 3 \times 10^{-6}I_0$ is seen, although the overall bell-shaped profile was unchanged, in contrast to the completely noise-dominated profile of the raw image. Peak intensity is largely preserved, but shows a slight reduction after denoising. The magnitude of the intensity decrease correlates with the amount of noise present. When corrected for excitation intensity, the measured intensity of the fluorescent bead signal after denoising (Fig. 5C) is level within error, but increases in inverse proportion to excitation in the absence of denoising, reflecting the fact that noise makes up a larger proportion of the recorded image at low excitation intensities. This gives reasonable confidence in quantitative image features after denoising.

**Reduction of Excitation Light Intensity Greatly Reduces Fading.** The use of low excitation light levels consistent with viability, made possible by denoising, benefits imaging in other areas as well. A major problem for all live time-lapse microscopy is fluorophore fading. Fluorescent molecules, both small molecule dyes and fluorescent proteins such as GFP, are susceptible to light-induced chemical alteration. In many cases only a few time points can be collected before the signal approaches the noise floor, or becomes swamped by autofluorescence. Computational bleach correction can retain the brightness, but this cannot prevent the loss of signal to noise, and eventually extracting quantitative information becomes impossible. Fading can be eliminated, however, by reducing the excitation light. A time-lapse intensity series (from Fig. 3) displays reduced bleaching curves as the excitation intensity is progressively reduced to $I = 10^{-4}I_0$ as seen in Fig. 6A. The $I_0$ curve fades very quickly into the noise, and even the $I = 10^{-4}I_0$ series falls off rapidly. The signal at $I = 10^{-3}I_0$ increases slightly, possibly due to weak photoactivation of EGFP (7), and the emission levels at $I = 10^{-3}I_0$ and $I = 10^{-4}I_0$ are essentially unchanging as a function of time, indicating a negligible amount of fading.
Fast Four-Dimensional Live Imaging and Fading Trade-Offs. A major goal of the OMX microscope is very fast live four-dimensional image collection. This capability is documented in Fig. 6B for yeast strain YDB271. Four-dimensional images were collected with a 10 msec exposure time in one stack of 13 sections, four stacks of 10 sections, or ten stacks of 8 sections, every second for 30 s (Fig. 6B) at an excitation light intensity of $I = 10^{-2}I_0$ that was previously shown to be compatible with complete long-term viability in the sparse imaging regime. As the rate of three-dimensional imaging is increased, the samples receive more total light over the 30 s interval: 3.9 s of excitation at 1 Hz, 12 s of excitation at 4 Hz, and 23.7 s of excitation at 10 Hz. Fading therefore occurs to a greater extent for the faster time series, a serious problem for in vivo imaging of rapid processes. If no fading at all is desired, it is necessary to reduce the excitation light intensity to $I = 10^{-3}I_0$ or lower. Even in cases of fading, denoising allows the image information to be recovered well into the fading region (see SI Text), whereas without denoising, the information is buried in the noise at the end of the fast data collection.

Because excitation intensity, density of time sampling, fading and cell viability are interconnected, we analyzed cell division (as an assay for cell viability) as a function of intensity. To capture at least one yeast cell cycle at 30°C (estimated to be approximately 90 min), we extended the time length for four-dimensional data collection to 2 h. We recorded one three-dimensional image (6 μm stack height at 0.25 μm spacing) per second, in the time-lapse regime we term “dense” (Fig. 1). The results are summarized in Table 1. The yeast LacI/O GFP sample (strain YDB271) had no cell divisions in the dense regime at $I = 10^{-3}I_0$ or greater. At $I = 5 \times 10^{-4}I_0$ (or lower) the three-dimensional data (at one 3D stack/sec) for the full 2 h showed unperturbed cell divisions (as well as no fading). A control strain (W1588-4C) with no GFP, also imaged at one 3D image/second for 2 h, failed to undergo cell division at $I = 10^{-2}I_0$. Even at

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**Fig. 5.** Quantification of denoising effects on low-light images of fluorescent latex beads. (A) The same bead is measured in 3D at four different excitation intensities ($10^{-3}I_0$, $10^{-4}I_0$, $10^{-5}I_0$, and $4 \times 10^{-6}I_0$). Single Z sections through the 3D stack are shown and analyzed. The raw images (Left) lose signal-to-noise as excitation intensity decreases, whereas this is mostly recovered in the denoised images (Right). (B) Line profiles through the beads demonstrate overall maintenance of peak width measured by Gaussian fitting, until the noisiest condition (bottom), in which the raw image does not give a fit at all, and the denoised image shows peak broadening. (C) Peak intensities corrected for excitation intensity display sensitivity to signal-to-noise ratio. A field of fluorescent beads was imaged 60 times, subjected to denoising, and both raw and denoised images were time-averaged to enable comparisons. (Left) Excitation-corrected raw peak intensities increase as excitation decreases, whereas denoised peak intensities are more stable. Error bars show variation (±1 standard deviation) in individual bead intensities. (Right) Ratios of denoised to raw peak intensities are plotted as mean ± standard deviation (n = 19 fluorescent beads).

**Fig. 6.** Fading plots of Lac repressor::GFP foci during dense time sampling. (Upper) Yeast cells are imaged at five successively lower excitation levels, and peak intensities in each 3D stack, normalized to the intensity at the first time-point, are plotted as a function of time. Photobleaching is visible at both $I = I_0$ and $I = 10^{-1}I_0$. At $I = 10^{-2}I_0$, intensity is seen to increase, which may be due to weak photoactivation of GFP. Lower intensities remain flat for the entire 30 s of imaging. (Lower) Fading increases at a given intensity level ($I = 10^{-2}I_0$) as the rate of imaging increases. In total, the samples receive 3.9 s of excitation at 1 Hz, 12 s of excitation at 4 Hz, and 23.7 s of excitation at 10 Hz.
Table 1. Viability in dense imaging regime

<table>
<thead>
<tr>
<th>Strain</th>
<th>W1588-4C</th>
<th>YDB271</th>
<th>SO992a</th>
<th>SO992b</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP:</td>
<td>None</td>
<td>GFP:Lac</td>
<td>None</td>
<td>GFP:FYVEEEA</td>
</tr>
<tr>
<td>$I = 1.0I_0$</td>
<td>−</td>
<td>−</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>$I = 0.1I_0$</td>
<td>−</td>
<td>−</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>$I = 10^{-3}I_0$</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>$I = 10^{-4}I_0$</td>
<td>+*</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>$I = 5 \times 10^{-4}I_0$</td>
<td>+</td>
<td>+</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>$I = 10^{-4}I_0$</td>
<td>+</td>
<td>+</td>
<td>N/D</td>
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</tr>
</tbody>
</table>

*Growth resumed after 3 h delay.
†Viable at exposures below 250 msec per second.
‡Insufficient signal to track spots.

$I = 10^{-3}I_0$, the control strain took approximately 3 h to resume normal cell division and growth after the 2 h data collection, presumably reflecting a repair-induced delay.

Another yeast strain (strain SO992), containing a GFP fusion to FYVEEEA, an endocytic pathway component, (8) was similarly imaged (SI Text). At $I = 10^{-3}I_0$, this strain did not divide after 2 h of dense data collection, indicating phototoxicity. An isogenic control with no GFP did not divide at $I = 10^{-3}I_0$ but did divide at $I = 10^{-3}I_0$ intensity, similar to the LacI/O control (strain W1588-4C). Because the strain without GFP can tolerate more excitation light than the strain with GFP, and the strains are otherwise isogenic, the interaction between light and GFP itself may be responsible for the increase in phototoxicity. However, even strains without GFP are sensitive to excitation light.

Given the capability to image at low light levels in the dense time regime, it became possible to image an entire cell cycle in yeast at a rate of one 3D image per second. Whereas strain YDB271 was completely viable at $I = 10^{-3}I_0$, the images obtained after denoising did not allow us to reliably detect spots at every single timepoint. We therefore tested viability at the intermediate level of $I = 5 \times 10^{-4}I_0$ (Fig. 7). To maintain full viability at this intensity level, we found that the exposure time had to be reduced to a total of 160 msec out of each second over a 2 h period. This meant that we could not collect Z stacks divided into 25 sections every 0.25 μm as before, as this would require exposing the sample to excitation light for 250 msec out of every second. To record an entire 6 μm stack in this short of a timespan required the use of stereoscopic projection imaging (see SI Text). In projection imaging, the stage is swept through the Z stack during the entire time in which the shutter is open, 80 msec in this case. Two such stage sweeps are performed every second: the first moving the stage up, and the second moving the stage both down and 2.5 μm to the right. After this second image, the stage is moved 2.5 μm back to the left. LacI::GFP foci are computationally detected in the resulting images and Gaussian profiles are fit to their centers with subpixel accuracy. The disparity in position along the X-axis (stereoscopic parallax) between every pair of points taken at successive time intervals is then used to calculate the Z position of the focus. Fig. 7 shows individual projection images taken at this speed and the entire time series depicted as a kymograph. Intensity plots as function of time (SI Text) show no discernable fading at this intensity level during dense time domain imaging. In the kymograph, the intensity of the brightest point can be seen to increase during the cell cycle, indicating DNA synthesis during S phase and recruitment of more LacI::GFP protein to the LacI operator array.

Because of the fast dynamics of chromosome movement in living cells (9), it is desirable to collect several 3D images per second. We therefore wished to test the viability of yeast under such extremely fast imaging conditions, in which the excitation light is activated for almost the entire imaging period. To test this, strain YDB271 was imaged at $I = 10^{-4}I_0$ for 5 min (at 30°C) with a time domain sampling increased to 10 three-dimensional data stacks/sec (defined as “fast,” see Fig. 1). With a 10 msec exposure time and single images captured at 91 Hz, the sample is exposed to excitation light for over 90% of the entire imaging process. After data collection, the sample showed no delay or lack of cell division, suggesting little photon damage at this fast speed for this brief time period.

In summary, using low excitation light levels and denoising on the OMX platform makes it possible to image fluorescent reporters at dense (1 3D stack/sec) temporal resolution over an entire cell cycle or more, at full viability as monitored by cell division, with the resulting images suitable for quantitative analysis.

Fast Live Four-Dimensional Data and Denoising in General. To ascertain whether denoising was applicable to only point-like data, or is generally applicable to many kinds of biological samples, we studied two additional systems. The first example is a GFP fusion to the Drosophila male differentiation pathway X chromosome specific complex component MSL3 (10), kindly provided by Mitzi Kuroda. It specifically binds the X chromosome at all points in the cell cycle. The rest of the chromosomes are visualized by a red fluorescent protein (RFP) fusion to histone variant H2AvD. As seen in Fig. 8A, reduction of the excitation light intensity by a factor of 10 or 100 gives rise to very noisy data, but denoising recovers the biological information for the two labels.

The second example (Fig. 8B) is a fusion of GFP to ZYG-12 (11), important for centrosome attachment to the nucleus, and chromosome movement in meiotic prophase, in a live Caenorhabditis
elegans" worm. The original image is very noisy, whereas denoising recovers several image features. In particular, foci of ZYG-12 are clearly distinguishable against the background in the denoised images, and can be tracked in three dimensions. A line profile through two patches (Left) displays the retention of image intensity after denoising. The peak widths are changed slightly (the left peak is broadened, the right is narrowed).

**Discussion**

Live imaging, the centerpiece of modern optical microscopy, requires a number of components to come together to work effectively. The samples must be unperturbed by the excitation light; little or no photobleaching should occur; the specifically labeled biomolecules must be discernable from the imaging noise; and finally the imaging hardware must be able to acquire the time sampled three-dimensional data at a fast (in principle oversampled) rate, ideally at multiple simultaneous wavelengths. This paper documents that all these components have come together to accomplish live imaging in a general fashion.

One of the main challenges of live fluorescence imaging is to avoid phototoxicity in the cells under observation, while at the same time obtaining enough emitted light to generate informative images from the raw CCD data. To avoid studying a system that is perturbed by photodamage, live imaging requires careful consideration of the dose (intensity and total time) of excitation light. For example, certain techniques, such as fluorescence recovery after photobleaching (FRAP), employ very intense light, approximately two orders of magnitude above our maximum \( I_0 \), four orders of magnitude above the cutoff for viability in the dense regime of \( I = 10^{-2} I_0 \), and six orders of magnitude above the cutoff for imaging a whole cell cycle in the dense regime of \( I = 5 \times 10^{-4} I_0 \). The potential for severe phototoxicity suggests that every live imaging study, regardless of the technique used, should contain controls for viability, preferably one that includes cell division.

Whereas it is likely that different cell types will differ in their sensitivity to excitation light intensity, we chose the LacI/LacO system in yeast as a representative live GFP fluorescent biological sample whose fast division time and ease of imaging facilitates the observation of phototoxicity. Our conditions for successful imaging were (1) the ability to track in three dimensions the center of the signal obtained from the GFP::Lac repressor fusion, and (2) the unperturbed viability of the cells, compared to nearby nonimaged cells, after imaging. For cells that take much longer to divide or do not divide at all, other controls must be devised.

The level of excitation required to detect fluorescence signals depends on the sensitivity and the efficiency of the imaging system, on the fluorophore density, and most strongly on the number of fluorescence photons emitted at each exposure. In a fully viable yeast OMX high-resolution imaging experiment in the dense regime, with an objective of NA = 1.4, the number of EGFP per point spread function (PSF) volume is approximately 30 (see SI Text), and the most intense exposure that can be used is \( I = 5 \times 10^{-4} I_0 \) for 10 ms. With this low light condition, the number of photoelectrons generated per electron multiplying charge coupled device (EMCCD) pixel is 5 (12), with a signal-to-noise ratio of 1.6 (see SI Text). To overcome this low S/N problem, the denoising algorithm assembles the signal from the resolution limited spot of 3 × 3 pixels, effectively reaching a signal-to-noise ratio of 4.8 enough to computationally boost the signal beyond the noise floor to reconstruct the true image.

Whereas this study emphasized cell division as a viability assay, for numerous reasons many biological systems are not amenable to this test. A number of other assays, such as quantitative measurement of the unperturbed long-term motility of biomolecules, or detection of indicators of damage such as DNA repair enzymes, can be used in addition (1). The amount of light reduction will in most cases be a compromise between signal recovery and phototoxicity. There are potentially two viability-enhancing strategies: (1) reducing excitation light, and (2) protecting the cell from light. One may be able to reduce phototoxicity by increasing expression of free radical scavenging enzymes, or targeting them to the nucleus, for example. Other methods could include removing oxygen from the environment of cells that do not require it, or adding high concentrations of molecules that react with singlet oxygen to form harmless species that do not interfere with fluorescence.

The denoising method dramatically recovers biological image information from the noisy images taken at low excitation light intensity. Whereas we could attenuate to \( I = 10^{-4} I_0 \) and still recover some information from our yeast GFP samples, it may be possible to even go down one order of magnitude more, depending on the brightness of the signal. The lower light intensities used in this study, combined with the particular GFP system under observation, approaches the limits of the denoising algorithm’s ability to retrieve information.

The lack of fading over long time periods made possible by low excitation light is a crucial step forward for fast 3D imaging, because the data are of a constant signal-to-noise level from the beginning to the end of the imaging period. This allows reliable measurement of dynamic information across long time spans, such as throughout an entire cell cycle. The observation of the two-fold increase in intensity in the LacI::GFP signal during the cell cycle in Fig. 7, reflecting the synthesis of DNA and recruitment of new protein, is an example of the kind of imaging result only possible with low excitation light that does not cause fading.

The ability to track the dynamic behavior of subcellular components at high temporal resolution through an entire cell cycle is an important facet of live imaging for many reasons. Rare events
that may only occur once per cell cycle will always be captured, and biologically relevant differences in movement as a function of the cell cycle are possible to discern. Another advantage is that instead of starting and stopping at arbitrary points, the trajectory of the signal throughout an entire cell cycle is available for analysis. The unbiased nature of the dense imaging regime allows true comparisons of dynamics between one cell and another without needing to artificially synchronize them; instead, trajectories can be registered with each other at a defined timepoint (such as the separation of two fluorescent signals at anaphase).

Whereas we have performed these experiments on a single microscope system (OMX), the question is raised whether the relationship between excitation power and sensitivity would hold on other microscopes. A careful study of 100 nm fluorescent beads in both OMX and a DeltaVision microscope showed that the detected fluorescence counts per watt of excitation light were within 6.7% of each other, thus demonstrating the equivalent sensitivity and light throughput of both systems. Most modern microscopy systems have excellent light throughput and are not likely to appreciably vary from each other; therefore, our results are likely to be generalizable to all in vivo imaging. We have demonstrated here that fast imaging of live cells involves a surprising amount of phototoxicity, but that with the proper image processing algorithms, excitation light may be attenuated in compensation, and useful information can be retrieved.

Materials and Methods

Strains. **Yeast.** YDB271 ho HMLa hml∥pro::lacO(256)-LEU2 MATa HMRa-BarnH1 ura3 ade1 ade2::GAL::HO leu2 trp1::hisG ura3-52 Spc29-RFP-(kan::

SO992a \(\Delta\) hmlprox::lacO(256)-LEU2 MATa

W1588-4C MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 ssd1-1 d RAD5 bud4 ybp1-1

Drosophila. Imaged fly tissue genotype: msl3::GFP, H2AvD::mRFP/CyO; msl3::GFP C. elegans. ZYG-12::GFP worms were strain WH223: oj19 yzg-12ABC::GFP unc-119(ed53); unc-119(ed3)

OMX Microscope. See SI Text for a complete description of the microscope and its operation.

Data Acquisition. Cells were grown overnight in synthetic dextrose (SD) medium (yeast minimal medium + glucose) at 30 °C in 5 mL cultures on a rotary shaker. SD medium with 2% agarose was liquified in a microwave and poured into a glass-bottomed Petri dish (Bioptechs Delta-T) to a depth of 5 mm. The bottom of the dish is coated with indium tin oxide, allowing heat to be generated by the application of current. Solidified agarose pads were removed from the dishes; 10 μL of cells were placed in the center of the dish, and covered with the pads. Dishes were placed on the OMX microscope via a custom-built adaptor connected to a power source (Biotechs, Inc) that provided a current to keep the dish at 30 °C. The objective used for imaging was kept constantly heated to 30 °C by a thermal ribbon and microcontroller (Minco, Inc) using a custom-built copper collar, 3 mm thick, coupling the thermal ribbon to the objective. Cells were imaged with room light only for the initial 30 min, to distinguish growing from nongrowing cells by the appearance of a bud. The positions of newly budded cells were saved in the microscope control program, and random subsets of these cells were selected for imaging. For bead imaging, 100 nm red-emitting fluorescent latex beads (Molecular Probes, Inc) were diluted 1:10,000 in ethanol. A 1 μL drop of diluted beads was placed in the center of a plasma-cleaned coverslip, allowed to spread out and air-dry, then mounted on a slide with 5 μL of glycerol. Laser light at 488 nm and 532 nm was used for excitation of yeast cells and fluorescent latex beads, respectively. Images were acquired on Andor Ixon EMCCD cameras set at their highest gain level. Dark current was calculated by averaging 512 frames taken with no excitation light, and subtracted from images before further processing.

Viability Measurement. After fluorescent imaging was completed, the dish containing the cells was retained in position on the microscope for overnight monitoring with brightfield imaging using ambient room light. Three-dimensional brightfield images were acquired every 30 s or 5 min for 12 or more hours during the overnight period, to measure cell division. Maximum-intensity projection images of these images were assembled into movies (see SI Text) and visually inspected to assess viability.

Image Processing. 4D datasets were processed with the denoising algorithm “ndsaif” (5) modified to accept files in our data format. The command typically used was: ndsaif_piram $INFILE $OUTFILE -sampling=2 -iter=5 -p=3 -noise=gaussian -adapt=0 -island=5 -usetmp, indicating the patches are calculated every 2 pixels, the number of iterations (increasing the patch size) was set to 5, the initial patch size was set at 3 pixels, the noise model was presumed to be Gaussian, the adaptivity parameter was set at 0, the island threshold parameter was set at 4, and intermediate steps were saved as temporary files. For point tracking, the FindPoints program of the Prisim software suite (http://msg.ucsf.edu/IVE) was used. 3D Gaussian fitting was performed on detected peaks using a search box of 5 × 5 × 3 pixels in X, Y, and Z. For 2D projection imaging, Gaussian peaks were located in each pair of images; the X coordinate distance between peak pairs, multiplied by the ratio between the Z and X stage movements, was used as the inferred Z coordinate.

ACKNOWLEDGMENTS. We thank Mitzi Kuroda and T. Sural for providing the Drosophila strain, Abby Dernburg for providing the C. elegans strain, Wendell Lim and Jessica Walter for providing yeast strain SO992, Yuri Strukov for providing and imaging the Drosophila sample preparation, Eric Branlund for technical support and advice, and Jason Swedlow and Orion Weiner for comments on the manuscript.


Supporting Information

Carlton et al. 10.1073/pnas.1004037107

**SI Text**

**SI Materials and Methods** OMX: a fresh approach for wide-field microscopy.

**Overall concept.** It became clear that questions about dynamic chromosome motion in the nucleus required much higher temporal sampling (and resolution) than what the conventional microscope platform could deliver. Therefore, a fresh top-to-bottom microscope design and implementation was undertaken.

A recent quantitative review of all modern fluorescent microscope techniques (1) has shown that wide-field microscopy is the most efficient in photon collection, with spinning disk confocal microscopy at approximately 0.25 its sensitivity, followed by standard confocal microscopes at a factor of 0.01 – 0.005 the sensitivity of wide-field. Wide-field microscopy, using only an objective and a tube lens, and precise sequential positioning of the focal plane within the sample along the optical axis, collects a three-dimensional image (a Z stack) distorted by out-of-focus light. Constrained deconvolution returns out-of-focus light to its correct location (2). Four-dimensional in vivo microscopy must be able to operate under a large range of timescales, from minutes to milliseconds, corresponding to the wide variation in timescales of biological processes. Many interesting biological problems fall in the extremely rapid end of this range. For example, interphase chromosomes (3) and actin waves (4) exhibit motion in the sub-second range that is very difficult to acquire and study on current microscopes, usually limited, at best, to approximately one dimensional image/sec. In addition, multiple colors (wavelengths) are usually acquired sequentially, making it difficult to quantitatively compare the position of differently labeled subcellular components at the same timepoint. Thus, the very fast four-dimensional platform we describe, capable of simultaneous recording of different wavelengths, will provide an important imaging modality well-suited for fast biological processes.

OMX addresses the shortcomings common to most commercial systems, and enables the implementation of many new features. With OMX live imaging we are able to collect ten three-dimensional images every second at four simultaneous wavelengths. In addition, this platform implements three-dimensional structured illumination (3D-SIM), allowing subdiffraction 100 nm xy and 200–300 nm z resolution (5, 6), as well as single-molecule localization microscopy capability (PALM, STORM, etc.).

There were several design considerations. First, we wanted to use a new microscope design to incorporate features that might be especially important to future cell biology, especially the imaging of whole organisms or tissues. Tissues contain many potentially identifiable cell types in a 3-dimensional arrangement. A commonly encountered problem is rapidly finding small pieces of tissue scattered over the cover-slip, or finding one sample of interest out of hundreds of similar tissue pieces on the cover-slip. Secondly, the new microscope platform must collect very fast subsecond 3-dimensional data cubes at multiple simultaneous wavelengths for sustained periods of time. Thirdly, the microscope had to have high-sensitivity electron multiplying (EM) CCDs so that every possible photon could be counted. Coupled with these features, all the optics had to be designed with minimization of stray-light to achieve the highest signal-to-noise data. Fourthly, the microscope had to be extremely stable, drifting only tens of nanometers per minute, using precision technology.

An entire room, approximately 200 ft² (in our case 10 x 20) comfortably houses this microscope system (Fig. S3). Broadly speaking, OMX consists of three parts:

First, there is a separate low magnification microscope station used for sample location, the LMX microscope. This subsystem is built around a Kramer M2, a Zeiss upright dissecting microscope body with Kramer fluorescence capability and metallurgical objectives, producing high resolution images at very high working distances. A CCD is provided for continuous scanning. The motorized xy-stage is encoded with submicron capability and these positions are cross-indexed with OMX itself. We wrote custom software to allow building an image mosaic covering a whole 22 x 22 mm cover slip (Fig. S4). Extended sensitivity and resolution by averaging and/or integrating over times are possible. This auxiliary microscope can then find objects of interest for further data analysis by OMX.

The second subsystem is the laser light source. Solid-state laser sources are very bright (typically 100 + mW), with high stability (better than 1 part in 1000 intensity stability) over the short and long term, long-lived, and easy to launch into fiber optic cables leading to the OMX microscope itself. Currently we are using 403 nm, 488 nm, 532 nm, 560 nm, and 642 nm lasers. These are gated by laser shutters (also beam traps when shutter is closed) for fast 150 Hz shuttering (nmLaser LST200) followed by a computer driven filter wheel (Thorlabs FW902) with a series of dielectric intensity stable neutral density filters (CVI laser). Then the light is parsed by extremely stable pop-up mirrors (New Focus 8892), under computer control to send the light to either conventional wide-field illumination or structured illumination, or a complex closed-loop piezo-aligned single-mode laser launch device (Thorlabs NanoTrak/BNT001) so that all laser lines can be optimally launched into the single-mode fiber for TIRF. Finally the coherence of the laser light is scrambled by a rapidly rotating light shaping diffuser (Luminit, formerly Physical Optics, model LSD5GL1) to minimize laser speckle.

The third subsystem is OMX itself, which consists of several components. The first component is a clean room enclosure approximately 5 x 5 x 8 ft. The enclosure is light tight, class 100 (when purged and people free for a few hr), prefiltered with charcoal filters to remove coarse and fume/odor laden air, with acoustic dampening/isolation. The enclosure exists in a conventional air-conditioned room (22.3 °C, with -0.5 °C temperature stability), but the HEPA fan unit, providing positive pressure, buffers the temperature fluctuation to -0.01–0.03 °C over many hours. Whereas the enclosure was designed to throttle the fan motor down to reduce vibration and use PID solid-state air conditioner for ultimate temperature stability, we have not used these design features. The enclosure is designed for users to open the door, (automatically turning on the room light) place the sample on OMX, and close the door (turning off the light). Subsequent interaction with the sample, through the use of CCDs, takes place at the console/visualization station.

Inside the enclosure, OMX sits on a Newport vibration isolation 4 inch damped (SG series) breadboard-table. OMX is machined out of a solid block of 7075 stressed-relieved aluminum, solidly bolted to the table, and purged with positive pressure filtered (0.45 μ) dry nitrogen for moisture control of the dichroic and emission filters. Extensive care in the OMX design was made for minimizing stray-light and for utmost stability. Zerodur, a highly temperature invariant and insulating ceramic, was used for kinematic mounting of the single objective and the stage. Movement of the stage over a 1-inch cube in x, y, z uses a Newport 462 series interferometer grade stage, encoded microstepper stage motors and incorporated closed-loop piezoelectric motors (Piezosystem Jena) in each axis. An alignment laser is
permanently embedded into the OMX system to be able to align the optics and to periodically check for alignment drift at several places in the optics layout.

All the essential beam-splitting dichroic mirrors are housed in a kinematically mounted removable drawer module. Each dichroic mirror is alignable in two degrees of freedom from the front of the drawer allowing precise coregistration of the different wavelength images. Again careful attention to stray-light suppression was made.

The computer hardware for OMX is distributed over nine Windows based computers. The computers are connected to a local network switch for fast easy-to-implement communication. Each CCD camera has its own computer with the capability to perform a “gain and offset” correction of the CCD image on-the-fly, and normalizes the baseline so that image intensity is stable as a function of time. Another computer handles low-resolution x, y, z position, DIC z position, multiple temperature readout, TIRF angle position, and some diagnostic features. LMX and the single-mode TIRF fiber system each has its own computer. The master computer runs the acquisition and display software plus data storage. The overall control is a DSP computer that supplies analog voltage modulation for the piezo control, TTL lines for all cameras, shutters etc., leading to a precise sequencing of image acquisition events.

The last aspect of OMX itself is the software. An early part of the software design was the incorporation of a flexible scripting language (Python) that would be fast, well-supported (heavily used in the astronomy community), and easily modified and maintained. We also wanted extensive documentation, a worldwide network database for bug fixes, and local expertise (the UCSF computer graphics group of Tom Ferrin). We also wanted the software to be open and freely distributable so that all users would modify and extend the software. We wanted the software for image acquisition to have a series of “instruments,” under software control, so that the user could navigate in 3 and 4D space as the data was collected. The user could see what was being collected at all wavelengths with every image displayed and the ability to scale these dynamically on-the-fly. There were to be auto-focus software instruments, as well as intuitive position reporting in x, y, z. In addition, there was to be extensive mosaic image capability that would be synchronized with LMX.

The four modalities of OMX.

OMX can be used in any of four imaging modalities. All four modalities make use of OMX’s stability, fast timing and precision sequencing of image acquisition.

Fast live.

The first OMX modality is its fast live imaging capability. Several technological advances make it possible for OMX to collect very fast three-dimensional data. At the top of the list is the master timer, the digital signal processor (DSP). This computer board, in a regular PC, allows for deterministic events to be sequenced to a series of buffered TTL lines going to the cameras, shutters, and lasers. In addition the DSP has a number of 16-bit digital to analog voltage lines that modulate the piezo channels. The DSP is programmed in a template format so that it is very easy to modify the acquisition process to add features or tune the data collection process. The next important feature, crucial to fast data collection, is the closed-loop piezo device that moves the 3 axes of the stage. This fast, stable, precise, smooth motion device moves the Z stage in a triangular ramp for focus, utilizing both the up and down aspects of the triangular wave motion. The piezo axis control also allows for the possibility of biological sample drift control. The capabilities of the fast live modality are featured in the accompanying paper.

3D structured illumination microscopy (3D-SIM).

The second OMX microscope modality is 3D-SIM, described in (5). Its main advantages are increased spatial resolution, up to 100 nm laterally and 250 nm axially, and its ability to be routinely used on diverse biological samples.

3D TIRF.

The third OMX modality is total internal reflection fluorescence microscopy (TIRFM). Laser light at multiple wavelengths is launched into a single-mode fiber by use of a Thorlabs piezoelectric tracking and feedback device (BT001/Nanotrac). The other end of the single-mode fiber, at the OMX microscope, is coupled to a stable closed-loop motorized stage (Newport 462 XYZ) together with a Physik Instrumente 40 μm closed-loop piezo stage so the emitted single-mode light reproducibly enters the back focal plane of the objective at a precisely defined point, and emerges at the critical angle for total internal reflection. With precise control of the fiber position, a range of TIRF angles are possible, allowing depth modulation of the evanescent wave.

PALM.

PALM and related techniques (8–13) have enabled 20 nm resolution of specific biological substructures. PALM is based on wide-field microscopy and thus can be successfully applied to our OMX platform, and represents the fourth modality. PALM needs specialized software, and an important feature of our OMX software is its customizability. The software is now modified to allow excitation/activation cycles for stochastic activation/switching of fluorophores. The programmed shutter and camera sequence is sent to the DSP control for fast execution. An acquisition series consisting of 20,000 512 × 512-pixel images taken at 30 msec exposure time with multiple wavelengths takes about 13–30 min to finish depending on the number of activation images. The activation and excitation can be multiple wavelengths and the duration of any of these can be progressively changed during data acquisition if necessary.

The design of the dichroic mirror drawer system in OMX greatly facilitates PALM 3D reconstruction. Multiplane PALM is possible using combinations of four cameras, additional lenses and beam splitters. Astigmatism is induced by installing cylindrical lenses in the drawer, or by flexing the dichroic mirrors with tightening screws. Users of the microscope can simply switch drawers when needed. Whereas both multiplane and astigmatism PALM methods have 50–100 nm axial precision for bright organic dyes (12, 13), dimmer fluorescent proteins are not successfully reconstructed with such accuracy. In that case, piezoelectric control of the OMX stage is far more accurate, as long as fluorescent proteins continue to fluoresce.

The stage has very low drift, allowing short data acquisition (<3 min) without drift correction. For longer acquisition, we use another channel for drift correction (e.g. DAPI staining of chromatin) because multiple wavelengths can be simultaneously collected. The drift is measured by cross correlation and corrected by our PALM reconstruction program. Lateral stage drift is usually within 80 nm/10 min.

PALM is essentially single-molecule imaging within biological samples. Therefore, unless the sample is an ideal material for single-molecule imaging, background noise diminishes Gaussian peaks, resulting in low-resolution reconstruction (~100 nm accuracy is common even with 1,000 photon yield in the presence of lots of background). Thus, removing noise is the most important aspect in performing PALM with real biological structure.

TIRF has been a preferred mode to remove out-of-focus light if the object of interest is within the evanescent field (~100 nm from the cover slip in conventional TIRF mode). As already described above, our TIRF settings allow the depth of the evanescent wave to be precisely set up to ~500 nm. The illumination angle can go lower than the critical angle, resulting in another
optical modality of highly inclined laminated sheet optical microscopy, which also has an advantage in removing out-of-focus light (14). We found that high NA (1.40), high magnification objective lenses (×100, ×150) have a rather shallow depth of field, and thus as long as refractory index mismatch is low (i.e. sample is close to the cover slip), standard wide-field microscopy without TIRF is often good enough for single-molecule analysis. Lateral background fluorescence is removed by computer-controlled field apertures.

The denoising algorithm reported here has been successfully used for cleaning up the raw single-molecule images. This speeds up the processing time of PALM reconstruction up to 100 times depending on the noise level. The image is further high-pass filtered to remove low frequency background noise and for individual molecules to stand out. We use 2D iterative deconvolution for this high-pass filter. Because localization accuracy depends both on noise and width of the point spread function (PSF) (and other factors), denoising and deconvolution improves localization accuracy. Denoising and deconvolution also improves final point density (AM, DAA, JWS, manuscript in preparation) by helping PSFs to stand out. The combined effect of these processing steps with our usual chromosome samples results in approximately fourfold improvement in resolution by increasing localization accuracy and point density.

**Photon detection and photo-electron shot-noise.** Cell viability is inversely related to the dosage of excitation light exposure. On the other hand, a low dosage of excitation light can cause low signal-to-noise in the raw image data, and can thus severely affect the quality of the microscopy image processing. In this supplement, we discuss this trade-off due to the limitation coming from the quality of the microscopy image processing. To-noise in the raw image data, and can thus severely affect the other hand, a low dosage of excitation light can cause low signal-

Fig. S1. 3D tracking over time in low-light-level images in dense-regime imaging. (A) Individual frames from image series selected from indicated timepoints. (B) Kymograph of denoised images over the entire 2 h period. Region highlighted in green, corresponding to the left side of A, is contained in Movie S4. (C) The intensity of one tracked point continuously present in the mother cell (at bottom in kymograph) is shown. Note drop in intensity at the point of cell division (arrow) and subsequent increase to predivision level. (D) The X, Y, and inferred Z (see Fig. S2) coordinates, in microns, of each point. (E) Tracked points displayed in 3D demonstrate the extent of movement.
**Fig. S2.** Diagram of stereo-projection imaging for 3D tracking of point-like objects. (Left) Conventional Z stack imaging of two points in the dense-regime. The shutter is closed in between each image acquisition, and the stage moves while the camera is being read out. Collecting 25 sections with 10 msec exposures and 12 msec readout time requires 538 msec to collect an entire Z stack. The next Z stack begins 462 msec after the last acquisition of the previous Z stack, at $T = 1$ sec. The 3D positions of the spots are temporally blurred over a period of 538 msec, but are directly available in the Z stack. In contrast, with projection imaging, the shutter opens for 80 msec, during which time the stage is moved through the entire Z stack, resulting in an average-projection of the 3D image. The shutter is then closed while the camera is read out and the stage moves to the right. Another projected Z stack is immediately acquired with the stage moving in the opposite direction in Z, as well as to the left. Acquisition of both images requires 172 msec. The second Z projection image is slightly spread out in the X direction, causing the recorded X position of imaged foci to depend on their location in Z: higher foci will be shifted to the right, and lower foci will be shifted to the left. The distance offset between the same point in both directions is measured by Gaussian fitting, and multiplied by the ratio between the Z and X stage movements to obtain the Z position.
Fig. S3. OMX room figure. The layout of the room containing the OMX microscope and associated hardware.
**Fig. S4.** Mosaic images from the auxiliary microscope LMX. A representative mosaic view of fixed 3T3 cells stained with DAPI. (A) Entire coverslip view (blue box is a square of 21 × 21 mm) at zoom 0.04. (B) Magnified view of boxed region in (A) at zoom 0.33. Individual 1024 × 1024 pixel images are highlighted with gray grids. (C) Magnified view of the boxed region in (B) at zoom 1.0 (pixel size 0.6152 μm). (D) An optical section from 3D stack of a metaphase chromosomes boxed in (C) taken with OMX (pixel size 0.0792 μm). Bar is 50 μm in (B) and (C), and 5 μm in (D).

**Movie S1.** Yeast cells imaged with brightfield (fluorescent room light) every 15 min for an 8.5 h period after undergoing sparse imaging at $I = 10^{-2}I_0$ (Left), $I = 10^{-1}I_0$ (Center), or $I = I_0$ (Right). All cells were located on the same agarose pad within 1 mm of each other. The cells at left show unperturbed growth; the middle cell shows limited growth after a delay; whereas the cells at right do not grow at all.

**Movie S1**
Movie S2. Average-projection images of strain S0992b containing GFP: FYVE<sub>EEA1</sub> is imaged in the dense regime at $I = 10^{-3}I_0$. Whereas wild-type cells imaged at this intensity survive, GFP-containing cells failed to divide after imaging.

Movie S3. Yeast cells (strain YDB271) from still image in Fig. 7, imaged in projection mode in the Dense-regime (1 3D image per second) at $I = 5 \times 10^{-4}I_0$. Each frame is a time-average of 30 s.

Movie S4. Yeast cells (strain YDB271) under 2.5D stereographic parallax imaging mode at $I = 5 \times 10^{-4}I_0$. Raw single projections with constant X stage position are shown at left, whereas the denoised version of the same image is shown at right. The span of this movie over the full 2 h of recording is indicated in by the transparent green overlay on the kymograph in Fig. S1(B).
Table S1. Excitation light attenuation values

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*Power values were measured at the objective back focal plane; intensity values calculated for the sample plane.