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A portable laser photostimulation and imaging microscope

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Abstract

We describe a compact microscope that uses a Spatial Light Modulator (SLM) to control the excitation laser light. The flexibility of SLMs, which can mimic virtually any optical transfer function, enables the experimenter to create, in software, arbitrary spatio-temporal light patterns, including focusing and beam scanning, simply by calculating the appropriate phase mask. Our prototype, a scan-less device with no moving parts, can be used for laser imaging or photostimulation, supplanting the need for an elaborate optical setup. As a proof of principle, we generate complex excitation patterns on fluorescent samples and also perform functional imaging of neuronal activity in living brain slices.

Keywords

SLM; laser; light field; calcium; uncaging

Introduction

The advent of laser scanning microscopy revolutionized imaging in the life sciences, especially in neuroscience (Yuste, 2000, 2005). For instance, two-photon microscopy has enabled, for the first time, long-term imaging of live brain tissue *in vivo* and *in vitro* (Denk et al., 1990), and when combined with photostimulation techniques with caged compounds or light activated channels, give the experimenter the ability to control the biological elements of the system, such as neurons in a fast and highly efficient manner (Callaway and Yuste, 2002).

Traditionally, laser scanning microscopes employ devices such as galvanometer mirrors or acousto-optical deflectors to direct the laser beam onto a desired point on the sample, either for photostimulation, or for acquiring optical signals (usually fluorescence). Typically, laser microscopes operate with a single beam and direct the entire beam to a single point in space. This is an inherently serial way of illuminating the sample, which fundamentally limits the scanning speed, and suffers from many practical constraints. Because the complete optical power of the incoming beam is necessarily directed to a single point in space, in many cases the input power needs to be greatly attenuated to avoid phototoxicity or chromophore saturation at the target. As a result, the full power of the excitation laser cannot be utilized (Pawley, 2006). Several multi-beam techniques have been proposed for imaging and/or photostimulation that overcome these limitations, for example spinning disk confocal systems (Bewersdorf et al 1998)), and also strategies based on extensions of standard

microscopic imaging (Hell and Andresen, 2001; Niesner, et al. 2007), with some utilizing novel computational imaging strategies such as light field microscopy (Levoy, 2006 and 2009). In our previous work we described a system for single-photon and two-photon photostimulation and imaging that takes advantage of diffractive (phase-only) spatial light modulators (SLM), as a simple addition to existing galvanometer-based laser scanning systems (Nikolenko et al., 2008). Others have also independently used diffractive SLMs have for one-photon uncaging of glutamate (Lutz et al., 2008). Here we generalize these ideas and demonstrate that an SLM with minimal additional optics, or even by itself, can perform many of the functions of laser scanning microscope. In effect, this greatly simplifies the overall optical design and leads to creation of portable and lightweight imaging system, one that is robust, compact, easily portable, and could potentially be further miniaturized for medical applications and used in in-vivo (Deisseroth et al., 2006).

SLM-microscopy

The use of phase modulation in microscopy is not new, in fact, it was explicitly stated in the pioneering work of Denis Gabor on holography (Gabor, 1948). Spatial light modulators are modern holographic devices; in our case based on liquid crystal arrays, which pattern the incoming lightfield by modifying its phase, its amplitude, or both, as a function of position on the array. We prefer to rely on using "phase-only" modulation, because this preserves the maximal optical power of the incoming beam; however, this requires that the source be coherent, but that is a property that comes with laser illumination. In the far field, the action of the SLM can be viewed as modifying the light field such that one generates the desired excitation pattern in the spatial Fourier plane, and because of this they can be viewed as a universal optic (Figure 1).

The basic idea behind using the spatial light modulators in microscopy is that many complex optical systems, and more specifically, the excitation path of laser-scanning microscopes commonly used in biological imaging, can be considered as wavefront modulators. This is accomplished with a combination of lenses and mirrors. In a traditional scanning approach, the excitation pathway defines the point on the sample that is excited. In the simplest case a microscope consists only of an objective, which transforms the incoming collimated beam from the laser source (e.g., – ideally a plane or gaussian electromagnetic wave) into a spherical electromagnetic wave that converges onto the focal point and thus generates an optical signal from that point (Figure 1A). A scanning system, such one provided by galvanometer mirrors, changes the propagation direction of the excitation beam, which results in a translation of the focus point. With this motion, the system is now able to gather optical signals from different points of the sample. In most cases, this signal is also an electromagnetic wave (light), which is consequently detected by additional imaging optics such as photomultiplier tubes or camera.

This simple optical transformation, the conversion of a plane wave into a spherical one by the objective, is just one of a more general class of operations that can be described in the language of optical Fourier transforms. The lens maps the incoming beam's spatial frequencies in the object plane onto positions in the image plane (Figure 1B). We stress here that the spatial variation generated from the incoming beam need not formed by intensity

modulation, but can be generated by imprinting spatial phase variations (for additional information and examples of optical Fourier transformation, see Lehar). Using this knowledge, we can then imagine that nearly everything in the excitation pathway of the microscope, or at least the dynamic part that actively modifies the beam (apertures, lenses, etc.), could be replaced by a single element, a programmable diffractive optical modulator that can dynamically modify the incoming beam.

Diffractive spatial light modulators (SLM) are such modulators, capable of almost universal transformation of optical wavefronts. The ones used in our microscopes are based on a two-dimensional addressable nematic liquid crystals array, in which each pixel can be controlled to locally retard the wavefront (0 to 2π retardation; Figure 1C). One important feature of the SLM is that it does not have any moving parts, with the exception of the realignment of the liquid crystal molecules themselves – therefore beam “scanning” is done by simply sending different patterns to the modulator. It is also uniquely suited for non-linear imaging because the dispersion of the ultrafast pulses (80–200 fs) typically used in biological imaging is negligible. Finally, because phase only devices redistribute the available laser power, rather than simply block it, it has advantages for light patterning in non-linear microscopies, or any other form of microscopy that require high power densities at the sample.

In this manuscript we present results obtained with single photon imaging excitation. This was done for demonstrational purposes, and to show the performance of a microscopes built using inexpensive, “off-the-shelf” lasers, outputting visible wavelengths. In a different study, with a similar SLM, we have performed two-photon imaging and photostimulation (Nikolenko et al., 2008), and are in the process of modifying this microscope for non-linear microscopies by using photonic bandgap fibers that transmits the ultrafast pulse with minimal dispersion.

The Pocketscope: a portable SLM microscope

We have implemented the idea of using an SLM as the basis of a small versatile microscope prototype, a “Pocketscope” (Figure 2). The principle focus was to create a small, capable microscope with a simple optical design and relatively low cost. As a result, we used parts from generic manufacturers, and have only a few components that are “scientific grade.” With the exception of the home-built SLM holder and dichroic mount, all of the mechanics are from Thorlabs. In this manuscript we present results obtained with a solid state 473 nm CW laser (BLM-300 from Extreme Lasers, Seabrook TX) that provides up to 300 mW of power. We also used a small 532 nm 10 mW DPSS CW laser (LDCU3/3663 from Power Technology Inc., Little Rock AR) when imaging chromophores such as sulphorhodamine 101 (not shown). The power of both these lasers can be controlled using an analog input. Moreover, we achieved similar results (not shown) for light patterning presented on Figure 3, using standard 532 nm 5mW laser pointers from several generic manufacturers.

The central element is a diffractive SLM that phase modulates the collimated incoming coherent light beam. We used a transmissive phase-only SLM (LC 2002, Holoeye Inc) that has SVGA resolution (800×600), a 60 Hz refresh rate, and a fill factor of 55%. The pixel pitch is 32 microns, and each pixel allows independent phase control from 0– 2π with 8 bit

resolution. Coupled with the fill factor, this allows for a total efficiency of almost 50% into a single diffractive order. The reported damage threshold for the SLM is 50 mJ/cm^2 (nanosecond pulses), with the active area of the device being 5.5 cm^2 . In our experiments, we have seen no damage with continuous power densities of 50 mW/cm^2 for CW lasers, (blue and green), and no apparent damage after prolonged illumination with $> 200\text{ mW/cm}^2$ of NIR light from a 80 MHz pulsed femtosecond laser.

We currently couple the laser beam into the SLM either through a multi-mode optical fiber (Ocean Optics OP-600-2-VIS/NR; transmitting more than 80% in 260–1000 nm range, 0.22 NA) and a collimating lens and a polarizer, or via free space coupling through a beam-expanding telescope consisting of an old microscope objective lens (various manufacturers) and a plano-convex lens (Thorlabs). In both cases the magnification is chosen so that the SLM is nominally filled in its short aspect. Although the SLM alone could also serve as the objective lens with the simple application of a phase lens mask, the physical size and pixel pitch of the current SLM limits the light cone, hence the numerical aperture, to low values, hence limiting the resolution produced by the system. With addition of a short telescope and objective lens, the overall NA is governed by the objective lens rather than the SLM.

Another telescope, between the SLM and objective lens provides a 1:3 angular magnification (Thorlabs plano-convex lenses, LA1608-A and LA1951-A) that allows the small deflection angles produced by our phase-only SLM (± 1 degree) into larger movements/positions of the focused beamlets on the sample plane. This optical design also allows for convenient placement of a dichroic mirror and emission path filter (Chroma Technology, 545nm long-pass, and 570/140 band-pass, respectively) so the Pocketscope can perform imaging in the traditional epifluorescence mode.

The camera used in this prototype was an inexpensive monochrome security CCD with VGA resolution (640×480). It has a $1/3''$ CCD chip, auto-iris and composite video analogue output. The signal was digitized by a NI PCI-1410 frame grabber (30 fps, interlaced 640×480 pixels VGA resolution) from National Instruments (Austin TX). When the system runs from a laptop, we used a consumer-grade multimedia video capture device connected via USB interface (USB Instant Video from ADS Technologies, Cerritos CA). The simple CCD that we used is certainly not an optimal scientific-grade imaging detector, so the results of Ca-imaging presented in the Figure 4 represent perhaps, one of the worst experimental scenarios, but still clearly show the system is sufficient to record biologically relevant imaging data, captured at a video frame rate, with remarkable signal to noise ratio. Current, high end EM-CCD devices have a quantum efficiency of greater than 90%, higher than nearly all photomultiplier tubes and avalanche photodiodes, with readout noise sufficiently low for a single photon to be detected above the noise floor. While photomultiplier tubes and avalanche photodiodes remain the detector of choice for very low photon count rates or for precise time-resolved detection, in most cases, high-end scientific CCDs provide ample sensitivity for single photon functional imaging of neurons and give the added advantage of simultaneous, multiplexed detection. For the results presented in this paper, even a very low-end CCD device was clearly sufficient. Imaging was performed using a variety of air and water-immersion objective lenses from Olympus: a $20\times$, 0.5NA or $10\times$, 0.3NA water immersion objectives were used for multi-spot imaging of neurons. We also used a $40\times$, 0.8

NA water immersion and air 10 $\times$, 0.3 NA or air 4 $\times$, 0.1 NA objectives for calibration and testing purposes. For most of the experiments described in this manuscript, we used the 20 $\times$, 0.5 NA objective lens, which, using the Rayleigh criteria, yields a resolution of ~ 600 nm laterally. For our optical system, the total effective magnification was roughly half of that given by the objective, e.g, the real image given by the 20 $\times$ objective on the camera chip was 10 $\times$ larger than the object itself, and the depth of field was $\sim 3.5\mu\text{m}$. Additionally, we sacrificed some of the advantages gained by using infinity corrected objectives and coupled the camera directly to the objective for simplicity. This introduces some aberrations in the imaging path, but none significant for these experiments. For maximum resolution, the addition of a simple single tube lens front of the camera would restore full infinity correction, and allow for easy insertions of multiple filters or analyzers without altering the magnification, or introducing extra chromatic aberrations.

In our current “typical” configuration with the LC2002 SLM, 3:1 relay telescope, Olympus 20 $\times$, 0.5NA objective and the small 1/3” CCD, the field of view captured by the camera is $300\mu\text{m} \times 220\mu\text{m}$, sampled at 460 nm per pixel (effective optical magnification of $\sim 10\times$). For the 0.5 NA objective, and our detected wavelengths, this is slightly undersampling given the Rayleigh criteria, but more than sufficient to image neuronal somata. The SLM allows for electronic lateral control of the beam over a $\sim 220\mu\text{m} \times 220\mu\text{m}$ subfield of the imaged area, and $\pm 120\mu\text{m}$ in the axial direction,

Currently we are using an “on-center” configuration of the SLM wherein the non-diffracted beam is present in the field of view. This “zero-order” beam is always present, and the simplest solution to remove it is to employ a small beam-stop between the SLM and objective to remove it. For thin samples or multiphoton microscopies, an alternative way to effectively remove the zero order is by using the SLM to apply a lens function such that the focus of the zero order beam and the patterned beam are axially separated (Nikolenko et al., 2008). It is also possible to generate the appropriate phase mask to effectively cancel the zero order beam and redirect its energy outside the image field. Alternatively, the SLM can be arranged for use in the “off-center” configuration, in which only one side of angular space is directed to the sample, and the non-diffracted beam is outside of the field of view.

The SLM “head” – the main unit with the SLM, objective and a detector (camera) that performs all functions described above was designed to be portable, but no significant efforts were made to make it particularly small (Figure 2B). Already it is small enough for “field applications” of imaging and photostimulation as the entire microscope and all accessory components, including lasers, power supplies and a laptop PC for control and acquisition can be easily packed into a briefcase. In its present form, it is clearly too large for head mounting to most animals for freely moving experimental studies. At the same time ours is just an initial prototype and there are many different approaches to make it significantly more compact and reduce the size of the unit to the point that would be needed to be physically attached to a living sample. The generic optical components and distances can easily be made smaller - the limiting device is the SLM itself. In our system, the SLM and driver electronics are housed together, increasing the size. The SLM chip itself is about $2\times 3\times 0.2$ cm, and can be physically separated from the rest of the electronics, connected via fine wires. The chip size is limited by the desired resolution and pixel pitch. Manufacturing

improvements have already shrunk the pixel pitch of commercially available liquid crystal based SLMs down a factor of 4 from the device used in this study, and other phase modulation technologies may become available that shrink this even further. Because of this, we believe this is just the first of a new class of small, flexible, advanced microscopes.

Light patterning with the Pocketscope

As one of the first demonstrations of the whole system and in order to show the flexibility of the SLM, we projected a non-trivial pattern using the SLM (Figure 3). The desired pattern (“HHMI” logo; Figure 3a), was used to generate a phase mask (Figure 3b), through an iterative Fourier algorithm (Fienup 1986), which was uploaded to the SLM, and projected onto a fluorescence target (fluorescent dye coated plastic slide, yellow highlighter (Sanford Sharpie)), generating the fluorescence imaged captured by the camera (Figure 3C). In addition, by applying simple transformations to the phase mask, the Pocketscope can scale the desired pattern, translate and rotate it on the sample, and alter the axial focal position.

Calcium imaging with the Pocketscope

Finally, we demonstrate that our portable SLM microscope can be used for functional biological imaging at video rates (30Hz). Animal handling and experimentation was done according to NIH and local IACUUC guidelines, and coronal slices prepared following our standard procedure (Nikolenko et al, 2007)}. We imaged calcium transients in living mouse neocortical brain slices that were bulk-loaded with the calcium indicator Oregon Green 488 BAPTA-1 AM (Figure 4), in a similar manner as described in the two-photon variant of this system (Nikolenko et al., 2008). First, an epifluorescent image was acquired with the camera using defocused light and no pattern applied to the SLM to uniformly illuminate the field of view (Figure 4A). We then used this image to select a subset of individual neurons and generated an SLM mask pattern that targeted the somata of these selected cells (Figure 4B, C and D). The beamlets generated by SLM thus illuminated the cell bodies of pre-selected cells while avoiding the spaces in between them. The camera then acquired images of the whole field, and functional signals at video rate. In these movies, one can reliably detect calcium signals, induced by action –potential activity (Smetters et al., 1999; Figure 4E). To ensure spontaneous activity, we enhanced the circuit activity, and therefore the frequency and amplitude of spontaneous calcium transients, by blocking synaptic inhibition with 20–30 μM of gabazine (Cat No. 1262, Tocris Ellisville, MO), a GABA_A receptor antagonist that enhances neuronal activity detected by calcium imaging (Badea et al., 2001). Although photobleaching was significant, as is expected from 1-photon fluorescent imaging, functional signals were still readily detectable after many minutes of continuous illumination.

Summary

In conclusion, we have built a simple and compact laser microscope that uses an SLM to shape the incoming light field into any arbitrary spatio-temporal patterns. This prototype can be used effectively to image and optically manipulate the sample, and even to focus and spatially translate the illuminating light, without any moving parts. Such sophisticated optical performance has so far only been possible with more substantial laser microscopes

with many complicated elements. Based on our experience, we foresee great potential in the future of such diffractive optics in simple, flexible, miniaturized laser SLM microscopes. These systems could make optical stimulation techniques more accessible for neuroengineering, and be used in-vivo, either for research, therapy, or even for brain-machine interfaces.

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Figure 1. Optical phase patterning with SLMs

A. Representation of a typical complex microscope as a simple device which transforms the incoming light field into a converging spherical wave.

B. Example images of optical Fourier transform relationships. On the left is the incoming (object) light beam's spatial profile, on the right is the corresponding light field in the image plane. Low spatial frequencies are mapped near the origin (DC component) in the image plane, while high frequencies are mapped further out. The patterns and resultant images show reflection symmetry – the patterns need not be symmetric, and single spot or other asymmetric patterns can be generated.

C. Example of sinusoidal phase imprinting with phase masks. The incoming beam has a planar wavefront, and the phase mask retards the beam yielding the outgoing sinusoidal wave. If this beam was directed through a lens, the image would consist of two spots, as shown in 1 B.

Figure 2. The Pocketscope: a portable SLM microscope

A. Optical scheme of the “PocketScope” using a minimal number of passive optical elements (see text for details) and does not have any moving parts. The light from the source (laser) could be delivered to the transmissive SLM either through optical fiber (as shown here and panel C) or via direct coupling of the laser to the system (panel D).

The diagram shows the detection (imaging) optical path configured for fluorescent imaging using epi-illumination (i.e. the objective used for both illumination and detection). The dichroic mirror separates excitation light and fluorescent image of the sample. The image is acquired using a miniature camera: in all our experiments we used a low-cost analog camera designed for security circuit TV applications, the video signal was digitized using 8-bit frame grabber at 30 frames per second.

B. 3-D rendering of the Pocketscope photostimulation imaging head. This concept illustrates that the system is very simple and could potentially be miniaturized up to the point of being small enough to be used in awake-behaving animal experiments or medical purposes as

head-mounted device. For scale, the upright tube holding the camera is ~1" in diameter.

Drawing of human brain is for illustration purposes and not to scale.

C. The actual photo of the system configured to be used with an optical fiber.

D. The photo of the table-top version of the system with direct laser coupling that was used for imaging of the acute brain slices (Figure 4). For the sake of convenience of working with in-vitro preparations, we attached a small XYZ translator to move the sample.

Figure 3. Light patterning using a portable SLM microscope

A. The pattern used for the test (HHMI logo).

B. The phase mask uploaded to the SLM – it is obtained using iterative Fourier transformation algorithm of the image in panel A. Different shades of blue indicate different phase delays introduced by SLM in corresponding pixels. This pattern is tiled across the SLM.

C. Fluorescence induced from the sample (a cover glass coated with dye from a yellow highlighter (Sanford Sharpie)) when it is illuminated by the diffraction pattern produced by

the SLM. The 0-diffraction order beam can be either mechanically blocked, deflected away, or defocused from the sample plane. The inhomogeneity in the fluorescent image has two main sources, first, as a stationary sample illuminated from a coherent source, there is speckle. Second, the dye deposition was not completely homogeneous. Scale 25 μm .

Figure 4. Calcium imaging using a portable SLM microscope

Example of experiment using the Pocktscope to acquire 1-photon fluorescent images of acute brain slices bulk loaded with Ca indicator (Oregon Green 488 BAPTA-1 AM).

A. Wide field image of the sample used for identifying and targeting neurons. In this case SLM was not activated, and the beam was defocused to produce a relatively uniform illumination pattern (as well as not have 0-order diffraction beam focused). The image is comparable to the quality of epifluorescent images obtained using full size commercial

fluorescent microscopes and high-end cameras. Individual cell bodies are easily identifiable. Scale 25 μm .

B. 25 neurons were targeted to be continuously illuminated using the diffraction pattern generated by the SLM (the full power of the laser will be split and directed only to those cells).

C. The pattern used as the command image in SLM software.

D. The phase pattern calculated to generate desired illumination pattern of the panel C. Different shades of blue indicate different phase delays introduced by SLM in corresponding pixels.

E. The actual image of the sample while it is continuously illuminated by the SLM pattern. Only selected neurons are illuminated and therefore imaged. 473 nm laser is used for excitation, total ~4mW of power under the objective in the 1st diffractive order split approximately equally among 25 spots.

F. Time-lapse traces of Ca signals acquired from neurons (normalized for photobleaching). ~30 μM gabazine was added to the preparation to induce activity. Trace 1: Ca signals that are characteristic for neurons. 3 large events are observed during ~110 s of continuous imaging – they presumably correspond to group activity of multiple neurons. Trace 2: much slower signals recorded from another neuron. The kinetics of these Ca signals is consistent with Ca waves observed in astrocytes.

G. One more trace from a different experiment; in this case a particular neuron not only participated in group activity, but also experienced many much smaller Ca events, that are consistent with continuous firing of short bursts of a few action potentials.