ACTIVE ILLUMINATION SOLUTIONS

OBSERVE, PERTURB, MEASURE
Photonic Instruments was founded in 1996 and grew to be a market leader in Active Illumination and laser ablation systems for confocal and widefield microscopy. Andor has continued to enhance product performance and features, as well as expanding the range of available products.

Since the acquisition of Photonic Instruments in 2010 Andor has built on an existing FRAPPA offering to provide a comprehensive Active Illumination Portfolio.
Active Illumination, or AI, describes a rapidly evolving range of optical techniques with an increasing impact on scientific enquiry and experimentation. AI has developed over the last two decades alongside the revolution of fluorescent proteins in biology (ref 7, 8), the instrumental and technological developments of confocal laser scanning microscopy (CLSM), solid state light sources (lasers and LEDs), fast galvo and optical MEMS technology, and of course the ubiquitous personal computer.

We include among these techniques:

- Photoactivation and switching 1, 10, 15
- Constrained or adaptive illumination 2
- FRAP 1, 12, 14
- Ablation, cutting and marking 4, 5
- Unicaging of caged compounds
- Optogenetics e.g. channelrhodopsin 9, 10, 11

Many of these techniques were first envisaged or implemented in CLSM, but because they are core to the study of living specimens, such as cells, tissues and embryos, there has been a drive to enable their use in systems better suited to observing live specimens, such as spinning disk confocal microscopy. AI techniques target optical radiation at user-defined region(s) of the specimen and use optical imaging to observe, and often to measure, the impact of the “perturbation”. This AI portfolio, enables the user to easily observe, Perturb and Measure. Andor has established the most powerful and flexible portfolio of products for these technologies and provides them in both systems and component format. To this end we work with third parties to ensure high levels of support across multiple hardware and software environments.

USER’S REFERENCES

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Mayer M., Delpian M., Bals J., Jäckle F., Grill S. Nature, 467, 617-621

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Moos J., Magdison V. Khodjakov A., Cooper J. Current Biology 19, 2006-2009

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The green fluorescent protein

Constructing and exploiting the fluorescent protein paintbox (Nobel Lecture)

Millisecond-timescale, genetically targeted optical control of neural activity
Boydin, E.S., Zhang, F., Bambarg, E., Nagel, G., and Deisseroth, K. Nature Neuroscience. 8, 1263–1268

A light-gated, potassium-selective glutamate receptor for the optical inhibition of neuronal firing
Janovjak, H., Szobota, S., Wyart, C., Trauner, D., Isacoff E.Y. Nature Neuroscience 10

Spatiotemporal control of cell signalling using a light-switchable protein interaction

Drosophila neuroblasts retain the daughter centrosome
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An inverse relationship to germline transcription defines centromeric chromatin in C. elegans
Gassmann, A, Rechtssteiner, KW Yuan, A, Muroyama et al. Nature 484, 537-537

Local Oxidative Stress Expansion through Endothelial Cells – A Key Role for Gap Junction Intercellular
Fahime I, Pirknas I, Salomon Y, Sicher A. PLoS ONE 7(7) 2012

Propagation of cell death following confined oxidative insult (COI).
Endothelial bEnd.3 monolayers were subjected to COI through photoactivation of the bacteriochlorophyll based photosensitizer WST11. Cell death was monitored by changes in the fluorescence of membrane viability probes, CaMg and Propidium Iodide. Dead cells are defined as CaMg negative (green), PI positive (red). Time zero represents termination of COI. 15
WHAT IS ACTIVE ILLUMINATION?

**Uncaging Of Caged Compounds**
Caged compounds are light-sensitive probes that functionally encapsulate biomolecules to render them inactive. Targeted illumination releases the biomolecule enabling localized perturbation of biological processes. Caged compounds are commonly released with UV light and when used with fluorescence microscopy provide a powerful tool for observation, perturbation and measurement.

**Photobleaching (FRAP)**
Repeated excitation of a fluorophore can result in its transition from excited singlet state to excited triplet state and so to irreversible chemical modification. This reduces the pool of fluorescent molecules known as photobleaching. This can be used to study the kinetics of a targeted mobile pool of molecules in embryos, cells and organelles using FRAP or FLIP protocols. Photobleaching is also used in FRET to elucidate interaction between molecules on the 10-50 angstrom scale.

**Optogenetics**
The development of GFP as a tool for biological imaging provided a rich legacy of tools for transfection of genes across species. These techniques are now used to produce a wide range of optically active cell lines and transgenic animals. The proteins expressed in these engineered cells and organisms respond in precise ways to specific, targeted optical radiation. This capability is the dawn of optogenetics and promises a new chapter in life science research.

**Photoactivation**
Photoactivatable fluorescent proteins can undergo dramatic changes in their properties in response to absorption of specific illumination. They can be converted, for example, from a dark state to bright fluorescence. Targeted photoactivation provides unique possibilities for optical labelling and tracking of living cells, organelles and intracellular molecules where their general abundance can prohibit the understanding of a local mechanism.

**Photoablation**
When short pulses (ps, ns) of highly focused laser light are incident upon materials (e.g. cells and tissues) photoablation can occur as a result of localized plasma formation. Molecular bonds break quicker than they can reform and the material decomposes. MicroPoint's pulsed dye laser lends unique flexibility to ablation allowing use of UV photons for surface surgery of cells or embryos while longer wavelengths provide access to material deeper in the specimen.
MicroPoint provides a flexible and field-proven tool for photo-stimulation. Supplied with a patented compact, pulsed nitrogen pumped tunable dye laser it is capable of ablation, bleaching and uncaging over a wavelength range of 365 to 656 nm. Broad wavelength range and energy control allow MicroPoint to be optimized for a wide range of scenarios. More than 20 wavelengths can be utilized with available dye resonator cells, while appropriate dichroic filter sets enable simultaneous imaging and photo-stimulation of the specimen. MicroPoint is supplied with a UV-Vis imaging quality Epi illumination adapter for both current and previous generation microscopes from Leica, Nikon, Olympus and Zeiss.

MicroPoint is used in our research for precisely targeted uncaging in single cells. This capability allowed us to design new experiments to identify the functional role of calcium signalling in the NF-κB signalling pathway and cell division. MicroPoint is a valuable tool for targeted photostimulation.

MicroPoint systems consist of a wavelength tunable pulsed laser, coupling optics, beam steering optics, a microscope adapter, a selection of beam splitters and interference filters, and a motorized or manually driven optical attenuator to adjust spot size and power. There are three MicroPoint versions available:

### MicroPoint Manual
Angular and spatial alignment of the illumination at the sample target is manually controlled via a 2-axis joystick. This manual version can be upgraded in-situ to provide galvo or Bluetooth control.

### MicroPoint Galvo
Galvanometer based beam steering is provided through PC control, enables precise and repeatable laser ablation and/or illumination in synchronization with other experiment parameters.

### MicroPoint Bluetooth
In laser marking or circuit isolation applications a computer is often unnecessary, the advanced and automated features of the MicroPoint can be controlled through the handheld PDA interface.

**FEATURES AND BENEFITS**

- Simultaneous laser delivery, microscope viewing and image acquisition
- Low maintenance with fiber optic delivery that maintains alignment when system is moved
- Quick set-up with manual beam positioning or automatic pattern generation
- User control of ablation and illumination plane provided by z-axis telescope

**SPECSIFICATIONS SUMMARY**

- **Wavelength**: 365 nm to 656 nm
- **Spectral bandwidth**: 4 nm FWHM
- **Transmission variation**: 0.1%-100%
- **Resolvable spot size**: Near diffraction limited
- **Average power**: 750 μW, 15 Hz / 50 μJ
- **Peak power**: 12 kW
- **Pulse width**: 3 to 5 nsec
- **Pulse repetition rate**: 0 to 15 Hz
- **Certification**: CDRH IIIb

**KEY APPLICATIONS**

- Laser Ablation
- Photobleaching
- Uncaging
- Photoswitching
- Semi-Conductor Marking
- Circuit Isolation

MicroPoint manual angular and spatial alignment of the illumination at the sample target is manually controlled via a 2-axis joystick. This manual version can be upgraded in-situ to provide galvo or Bluetooth control.

MicroPoint galvo Galvanometer based beam steering is provided through PC control, enables precise and repeatable laser ablation and/or illumination in synchronization with other experiment parameters.

MicroPoint Bluetooth In laser marking or circuit isolation applications a computer is often unnecessary, the advanced and automated features of the MicroPoint can be controlled through the handheld PDA interface.

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MicroPoint is used in our research for precisely targeted uncaging in single cells. This capability allowed us to design new experiments to identify the functional role of calcium signalling in the NF-κB signalling pathway and cell division. MicroPoint is a valuable tool for targeted photostimulation.
**CONFIGURING MICROPOINT TO YOUR EXACT NEEDS**

MicroPoint is a highly versatile illumination source, compatible with all leading microscopes and most legacy systems. There are a selection of control interfaces, filter and laser/lamp attachment options.

### Microscopy

<table>
<thead>
<tr>
<th>Microscope</th>
<th>Olympus</th>
<th>Nikon</th>
<th>Zeiss</th>
<th>Leica</th>
<th>Other</th>
</tr>
</thead>
</table>

### Control Interface

<table>
<thead>
<tr>
<th>Interface</th>
<th>Galvo</th>
<th>Bluetooth</th>
<th>Manual</th>
</tr>
</thead>
</table>

### Laser

**Pulsed Nitrogen Laser System**
- Includes 2M delivery fibre, Microscope specific interlock and Microscope Epi cube with Eyesafe 505 nm longpass laser filter and alignment tools. Pulsed Nitrogen Laser w/interlock and safety kit.

### Dye Cells and Laser Dye

- **UV - 365 nm Dye Cell** (Supplied with)
- **Multiline Dye Cell**
- **High Power 435 nm Dye Cell** (Supplied with)
- **365 nm Dye 50 ml**
- **388 - 665 nm Dye 50 ml**
- **435 nm Dye 50 ml**
- **100% epi Plug**
- **0% epi (blank) Plug**

### Beamsplitters

- **30 Epi : 70 Laser Beamsplitter Plug**
- **50 Epi : 50 Laser Beamsplitter Plug**
- **70 Epi : 50 Laser Beamsplitter Plug**
- **Shortpass Laser 435 nm Beamsplitter Plug**
- **Short reflect / long reflect Laser Notch**
- **435 nm Beamsplitter Plug**
- **100% Epi Plug**
- **0% Epi (Blank) Plug**

### Epi Illumination Lamp

- **Microscope Specific HG Lamp**
- **DG4**
- **Andor AMH**
- **Other**

- N.B. Any default lamp house flange will that of the microscope specified in Step 1.

### Optional Epi Excitation Filters

- **FITC exciter filter**
- **GFP exciter filter**
- **Rhodamine exciter filter**
- **Blank Filter holder**

### Optional Epi Excitation Filters (choose one or more)

- **Laser Pulsed nitrogen laser system**
- Includes 2M delivery fibre, Microscope specific interlock and Microscope Epi cube with Eyesafe 505 nm longpass laser filter and alignment tools. Pulsed Nitrogen Laser w/interlock and safety kit.

### Optional Accessories

- **Microscope Accessories**
  - Analyzer for Zeiss Axioskop
  - Analyzer and Barrier 25 nm Laser delivery filter set for 365/405/445/confocal
- **Microscope Specific Epi Cubes**
- **Spare**
  - Laser Dye bottle [50ml]
  - First Surface Mirror microscope slide
- **Dye Cell Cleaning Kit**

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Page 10

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Page 11
**Typical Applications**

Due to the broad range of wavelengths MicroPoint can be tuned to, it is used for a broad variety of applications, popular with core facilities. The more frequent applications are ablation (365 nm) in fields such as developmental biology and DNA damage. Both of these applications take advantage of the diffraction limited spot to target single cells, or subcellular components.

### Release of Caged Compounds

Uncaging of rhodamine fluorophores is often used to study hematopoiesis (blood cell development) in zebrafish embryos.

1. CD41:eGFP+ cells (green) were injected into wild type 72 hpf embryos and appear in the pronephric tubules one day later.

2. Similar cells in CD41:eGFP embryos previously injected with caged rhodamine were targeted 40 hpf with MicroPoint 365 nm pulses for 10-15 seconds each. Ten cells were targeted in each embryo.

3. Their fate was tracked and four days later they can be seen to invade the thymus and become rag2:eGFP thymocytes. (Bertrand J Y et al. Development 2008;135:1853-1862)

### Diffraction Limited FRAP

Yeast cells are labeled with Nuf2p: GFP to mark the spindle pole bodies and Ase1p: GFP in the mitotic spindle mid-zone. They are observed in DIC and fluorescence microscopy.

1. Metaphase spindle before elongation.

2. Telophase spindle at the end of anaphase. Note that Ase1p: GFP remains localized to the spindle midzone throughout mitosis.

3. Ase1p: GFP was photobleached on the metaphase spindle.

4. Ase1p: GFP completely recovers within 20 minutes as the spindle elongates.

### Ablation

Ablation is the vaporisation of material. Typically in biology single cells are targeted for studies such as cell lineage, or tissue in damage and repair mechanism research. MicroPoint’s 365 nm option, with a diffraction limited spot, is perfect for ablation.

1. Ablation of spiral ganglion neuron.

2. Related apoptotic death of its sensory inner hair cell in 6 day old organotypic culture of the newborn mouse cochlea 18 hours after single pulse laser beam injury.

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**MicroPoint**

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FRAPPA is a galvo scanning instrument, named by conjoining acronyms for fluorescence recovery after photobleaching (FRAP) and photoactivation (PA). FRAPPA has a unique switching design that allows it to be configured in the imaging path. In bypass mode it acts as a relay optic, projecting an image to the detector; while in scanning mode it acts as a laser scanner, targeting user-defined regions of the specimen. This “in-line” configuration allows it to utilize the same wavelength for imaging and photo-stimulation.

The cost of “in-line” operation is sequential execution of imaging and photo-stimulation, but the switching speed is optimized at 20 ms inter-image switching time to minimize its impact. FRAPPA utilizes galvo technology, has a single laser input and is designed to deliver a diffraction limited spot from CW and pulsed lasers in the wavelength range 400 - 800 nm.

These features make it an attractive option for photobleaching, switching and activation, as well as DNA damage studies.

**KEY APPLICATIONS**

**FRAP**

**Photoactivation**

**Photoactivation**

**FEATURES AND BENEFITS**

- “Bypass” mode provides 1:1 relay imaging for “in-line” configuration
- “FRAPPA” mode performs laser scanning via imaging C-port
- Diffraction limited spot size ~0.6 μm @ 488 nm FWHM
- “In-line” operation enables use of all laser lines for FRAPPA actions
- Mode switching in < 10 ms
- Integrated control with iQ² software provides “point-and-shoot” and protocol modes
- Arbitrary multi-region scanning of points, rectangles and polygons
- Share imaging lasers with Andor’s unique multi-port adapter
- Active blanking output for ALC integration

**SPECIFICATIONS SUMMARY**

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>400 - 800 nm</td>
</tr>
<tr>
<td>Resolvable spot size</td>
<td>Near diffraction limited</td>
</tr>
<tr>
<td>Laser input</td>
<td>Single mode fiber FC</td>
</tr>
<tr>
<td>Beam power</td>
<td>Up to 2 Watts optical</td>
</tr>
<tr>
<td>Intensity Control</td>
<td>ADIF 0.1 - 100%</td>
</tr>
<tr>
<td>Laser Compatibility</td>
<td>Pulsed or CW</td>
</tr>
<tr>
<td>Pixel Dwell Time (minimum)</td>
<td>20 μs</td>
</tr>
<tr>
<td>Certification</td>
<td>CDRH IIIb</td>
</tr>
</tbody>
</table>

In bypass mode the FRAPPA acts as a 1:1 relay imaging system. As shown by the yellow beam path the X galvo mirror is positioned at the instrument pupil and completes a 4f imaging path.

In FRAPPA mode, the laser beam is injected from the laser input port and is steered to the Y galvo. The X galvo is positioned to scan the laser beam into the microscope to execute the defined photo-stimulation.

Dr. Eran Meshorer
Institute of Life Sciences
Hebrew University Jerusalem, Israel
**PRODUCT COMPONENTS**

FRAPPA is available in a single model for “in-line” or dichroic configuration. With C-mount input and output, FRAPPA is easy to configure with the “in-line” setup, FRAPPA uses the bypass mode for imaging and the scanning mode for photo-stimulation. Its output can be coupled directly to a camera or a confocal scanner e.g. CSUX.

FRAPPA can be used for simultaneous photo-stimulation and imaging when configured on a separate microscope C-port and used with a dichroic mirror. FRAPPA is compatible with visible to near IR lasers operating in CW or pulsed mode (400 - 800 nm) for photo-stimulation.

A typical FRAPPA system setup is shown below, here the FRAPPA unit is connected to the microscope through a side port and confocal imaging is performed using an EMCCD / Confocal Spinning Disk combination. The Andor Laser Combiner can provide up to six laser wavelengths and the MPU can rapidly switch these laser lines between the FRAPPA, CSU or TIRF illuminator.

**TYPICAL ANDOR REVOLUTION XD SYSTEM COMPRISING FROM LEFT:**
- High performance detector or camera; CSUX spinning disk (SD) confocal; FRAPPA FL device; microscope; TIRF and Epi fluorescence illumination; Andor multi-line laser combiner (ALC);
- Multi-port unit with 3 fiber outputs.
TYPICAL APPLICATIONS
FRAPPA is popular as it allows you to bleach at the wavelength you image at, therefore optimal absorption of the fluorophore. More importantly it is not limited to bleaching at short wavelengths, which can cause concerns of UV-related damage in live cell imaging studies. Most popular applications are using bleaching for studying protein dynamics (e.g. actin) and cell compartment continuity, or photoactivation, in which sub-cellular components (e.g. centrosomes and vesicles) can be tracked.

Photobleaching
Photobleaching of sub-regions of endosomal tubules illustrate the relative diffusion rates of B2AR and TIR. FRAPPA’s precise interactive capability enabled this experiment from a recent paper by Puthenveeda et al in Cell (Cell 143, 761-773, November 24 2010, Elsevier Inc.). Even visually you can observe that B2AR fluorescence recovers more slowly than TIR. This experiment endorsed the researchers’ hypothesis that recycling of these proteins is supported by actin-stabilized endosomal microdomains.

DNA Damage
Asynchronously growing HCT116 cells were transfected with three GFP-tagged proteins. PARP1 (Poly-ADP Ribose Polymerase 1) and NBS1 (Nijmegen Breakage Syndrome 1 or Nibrin) are markers for single- and double-strand DNA breaks respectively while PCNA (Proliferating Cell Nuclear Antigen) anchors DNA polymerase onto the template DNA and highlights active DNA replication. 24-hours post-transfection cells expressing the proteins were point irradiated in the nucleus either with FRAPPA and a CW 405 nm laser (PARP1 and PCNA), or with MicroPoint and 365 nm pulses (NBS1). Immediately after damage, time-lapse recordings were made and two time points selected for presentation.

1) PARP1 was recruited in a few milliseconds, 2) PCNA within a minute, while, 3) NBS1 takes up to two minutes for detectable recruitment. Note the characteristic distribution of PCNA in replication foci within the S-phase nucleus.

Photoswitching
Microtubules in this cell are labeled with Dendra, a monomeric Photoactivatable Fluorescent Protein (PaFP). The orange region shows the result of a single FRAPPA scan with 405 nm illumination, which has locally converted Dendra to its red fluorescent state. Subsequent time-lapse imaging allows tracking of converted tubulin monomers.
MOSAIC3
Simultaneous illumination of multiple regions of interest
in real time and with zero delta acquisition time

Mosaic is a patented instrument platform built around MEMS Digital Mirror Devices (DMD). DMDs were developed at Texas Instruments in 1987 and are now in widespread use in digital display devices. The DMD comprises an array of individually addressable micro-mirrors that can be switched “on and off” (tilted). DMD arrays contain hundreds of thousands to millions of micro-mirrors.

Mosaic exploits DMD in a proprietary programmable platform, integrated with scientific light sources including lasers, LEDs and arc lamps, and operates from 360 to 800 nm.

It is offered with a range of high performance microscope adapter optics and can be integrated with CLSM, spinning disk and wide field imaging modalities.

Mosaic allows you to continuously illuminate an arbitrary mask in the imaging field. You simply can’t do this using laser-scanning microscopes, which never provide true simultaneous illumination. This important feature, combined with ease of use and direct interfacing with software, makes the Mosaic a unique and valuable tool for the optogenetic community.

KEY APPLICATIONS
- Optogenetics
- Parallel FRAP
- Photoswitching
- Unzapping
- FRET

FEATURES AND BENEFITS
- Unlimited flexibility in shape, size, complexity of illumination mask
- Simultaneous illumination of multiple regions of interest
- Precise illumination of areas of interest that protects target specimen and fluorophore
- Zero delta acquisition time for true digital excitation
- Complementary illumination option enables on and off control in optogenetics studies
- Longest lifetime and lowest maintenance with rugged semiconductor device

High speed frame switching (up to 5,000 frames/sec) makes Mosaic suitable for many dynamic applications including optogenetics, bleaching, unzapping, photoswitching, and constrained illumination.

Mosaic has a unique capability to illuminate in parallel an arbitrary number of complex regions (sometimes called “zero delta t”) that sets it apart from galvo-based devices and makes it especially attractive for unzapping, photoswitching and light activation. It is a unique tool for the study of optically stimulated intra- and inter-cellular activity in neuroscience and physiology, as well as for function structure studies with photoswitching fluorescent proteins.

Mosaic can be used in conjunction with other experimental hardware systems, such as those used in electrophysiology, with a range of trigger in/out options for synchronised use. Please enquire about your potential application needs for an appropriate solution.

Mosaic uses dichroic coupling to the microscope light path and is therefore capable of simultaneous stimulation and imaging.

SPECIFICATIONS SUMMARY
- Wavelength: 360 nm - 800 nm
- Intensity stability: Absolute (light source dependent)
- Extinction ratio: > 800:1
- Minimum resolvable spot: Diffraction limited with 100x objective
- Trigger to exposure delay: < 200 μs
- Minimum exposure time: 50 μs - 200 μs
- Trigger mode dependent
- Maximum frame repetition rate: up to 5,000 frame/sec
- On-head RAM capacity: 139 binary images (800 x 600 pixels)
- Maximum Number of Sequence Frames: 256
- Maximum Number of Sequence Loops: 65,536
- Certification: CDRH IIIb (if fitted with a laser source)

See page 34 for application note: Precise optical inhibition of individual neurons
The Mosaic can be tailored to suit your needs, there are a number of options including mosaic illumination sources, Epi illumination sources, digital mirror array magnification and filtering.

### Product Components

- **Microscope Options**
  - Mosaic Illumination input shutter with controller and BNC cable
  - Laser delivery / safety filters
  - Three channel TTL synchronization module with delay and duration inputs / outputs

- **Input Excitation Filter** (choose one)
  - Large GFP filter in holder (480x40 nm)
  - Large blank filter holder

- **Epi Light Source**
  - AMH
  - DG4
  - Hg
  - MicroPoint
  - Other

- **Epi / Mosaic Beamsplitters**
  - 30 Epi : 70 Laser Beamsplitter Plug
  - 50 Epi : 50 Laser Beamsplitter Plug
  - 70 Epi : 50 Laser Beamsplitter Plug
  - 100% Epi Plug
  - 0% Epi Plug
  - 0% Epi (blank) Plug

- **Secondary Light Source** (8206 only)
  - 30 Epi : 70 Laser Beamsplitter Plug
  - 50 Epi : 50 Laser Beamsplitter Plug
  - 70 Epi : 50 Laser Beamsplitter Plug
  - 100% Epi Plug
  - 0% Epi (blank) Plug

- **Optional Epi Excitation Filters** (choose one or more) (8206 only)
  - RITG exciter filter
  - GFP exciter filter
  - Rhodamine exciter filter
  - Blank filter holder

- **Multiplex with MicroPoint**
  - For applications requiring both ablation and zero delta time fluorescence excitation of multiple regions with complex geometries, the Mosaic’s Epi Illumination port can be fitted with a MicroPoint system.
TYPICAL APPLICATIONS
Mosaic’s strength is in high-speed simultaneous multi-region targeting of light. Key applications are therefore centered around highly dynamic processes such as protein translocation (bleaching and activation), ion imaging (uncaging), and most recently it has become very popular for optogenetics. The latest functionality means that high speed pattern generation, used with optogenetics, can mimic cell signalling such as in neuroscience.

Release of Caged Compounds
Mosaic can be used with UV sources including arc lamps, LED and lasers to uncage biomolecules and render them active. In the images shown below:
1) Two cells are shown in resting state and pseudo-colored.
2) Caged calcium is released in the cytoplasm of the left hand cell and in the nucleus of the right hand cell.
3) Calcium fluctuations do not propagate throughout these cells, although propagation has been reported in hippocampal neurons (Eder and Bading, BMC Neurosci. 2007; 8: 57).

Photoactivation
Photoactivation of pa:GFP-histone in U2OS nuclei lights up histones green in a linear region. This technique can be used to study histone mobility within the nucleus, in response to agonists and relative to other nuclear features such as nucleoli or other compartments.

Fluorescence Recovery after Photobleaching (FRAP)
Mosaic allows simultaneous illumination of multiple regions, for FRAP as shown here. This can enable multiple measurements in a single experiment. Here the mitotic spindle of a dividing cell has been bleached in preparation for observing fluorescence recovery and estimation of tubulin motion properties.

DMD Technology
The core of Mosaic is the Digital Micromirror Device (DMD), a high speed and highly efficient semiconductor-based “light switch” array of hundreds of thousands hinge-mounted, addressable, tiltable, microscopic mirrors. When a DMD chip is coordinated with a digital video or graphic signal, a light source, and beam delivery optics its mirrors reflect a digital image of the illumination mask onto the sample.
Some photosensitive proteins can be activated with one wavelength of light and de-activated, or “silenced”, with another. This property is exploited in the Mosaic Duet e.g. to sharpen control over protein localization. A second light source is coupled into the DMD at a complementary angle to the primary. In this configuration pixels NOT selected for activation define an inverse mask which can be illuminated to “silence” molecules outside of the activation region(s) or those diffusing away.

**MOSAIC DUET**

Complementary multicolor illumination

Mosaic is uniquely suited to optogenetics, because it enables simultaneous multi-region illumination and interaction. Mosaic Duet is especially powerful because it enables simultaneous activation and silencing of photo-sensitive proteins in complementary regions of the cell. Thus precise targeting of protein complexes is achieved with diffusing molecules being silenced as they drift away from the activation site.

**KEY APPLICATIONS**

- Optogenetics
- Parallel FRAP
- FRET
- Uncaging
- Multi-plexed optical control

**Microscope Options**

- Mosaic Illumination input shutter with controller and BNC cable
- Laser delivery / safety filters
- Three channel TTL synchronization module with delay and duration inputs / outputs

**Microscope**

- Olympus
- Nikon
- Zeiss
- Leica

**Field of Illumination**

- 5.4 x 4.1 mm

**Primary Left Sided Light Source**

- Diode laser
- aLC
- AMH
- DG4
- Hg
- Other
- N.B. If laser chosen, then laser kit (containing fluorescent target, fiber coupler, homogenizer, fiber collimator and interlock) is required

**Primary Right Sided Light Source**

- Diode laser
- AMH
- DG4
- Hg
- Other
- N.B. If laser chosen, then laser kit (containing fluorescent target, fiber coupler, homogenizer, fiber collimator and interlock) is required

**Epi Light Source**

- AMH
- DG4
- Hg
- MicroPoint
- Other

**Epi/Mosaic Beamsplitters**

- 30 Epi : 70 Laser Beamsplitter Plug
- 50 Epi : 50 Laser Beamsplitter Plug
- 70 Epi : 50 Laser Beamsplitter Plug
- 100 Epi Plug
- 6% Epi (Blank) Plug

**Secondary Light Left Source**

**Secondary Light Right Source**

**Beamsplitters**

- 30 Epi : 70 Laser Beamsplitter Plug
- 50 Epi : 50 Laser Beamsplitter Plug
- 70 Epi : 30 Laser Beamsplitter Plug
- 100% Epi Plug
- 0% Epi (Blank) Plug

**Left Side Excitation Filter (choose one)**

- Large GFP filter in holder (480x40 nm)
- Large blank filter holder

**Right Side Excitation Filter (choose one)**

- Large GFP filter in holder (480x40 nm)
- Large blank filter holder

**Optical Epi Excitation Filters (choose one or more if required)**

- rGFP Exciter Filter
- aGFP Exciter Filter
- Rhodamine Exciter Filter
- Blank Filter Holder

**Microscope**

**Field of Illumination**

**Primary Left Sided Light Source**

**Primary Right Sided Light Source**

**Epi Light Source**

**Epi/Mosaic Beamsplitters**

**Secondary Light Left Source**

**Secondary Light Right Source**

**Beamsplitters**

**Left Side Excitation Filter (choose one)**

- Large GFP filter in holder (480x40 nm)
- Large blank filter holder

**Right Side Excitation Filter (choose one)**

- Large GFP filter in holder (480x40 nm)
- Large blank filter holder

**Optical Epi Excitation Filters (choose one or more if required)**

- rGFP Exciter Filter
- aGFP Exciter Filter
- Rhodamine Exciter Filter
- Blank Filter Holder

Mosaic Duet Provides a Flexible AI Tool

With its complementary illumination masks, when Duet is combined with fast switching source(s) the masks can be quickly updated to provide arbitrary forms for combined delivery of activation, silencing and fluorescence imaging.
LIGHT SOURCES

Light sources are chosen for high brightness and efficient coupling to AI instruments. FRAPPA uses CW and pulsed lasers in the range 400 - 800 nm and powers up to 1W. MicroPoint is designed for pulsed dye-laser operation, but can use other lasers (on request) in the range 365 - 660 nm. Mosaic is compatible with lasers, arc lamps and super-luminous sources according to the application. Uncaging, photoactivation, photoswitching and optogenetics need lower power density so incoherent sources function well and are cost effective.

### ACTIVE ILLUMINATION SOURCE

<table>
<thead>
<tr>
<th>Source</th>
<th>XLED1</th>
<th>ALC/MPU Multi Laser System</th>
<th>Dg4 Xe Arc Lamp</th>
<th>405nm High Power Diode</th>
<th>Hg Lamp</th>
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### EPI / WIDEFIELD ILLUMINATION

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### XLED1

LED light source for fluorescence microscopes

The XLED1 light source from Lumen Dynamics is the perfect solution for live cell applications. With high speed on/off cycling, fine intensity control and the ability to synchronize with other image capture devices for multiparameter imaging, you can be sure of protecting your live samples from phototoxicity and bleaching. The XLED1 is the ideal partner for the Andor Mosaic device used for applications such as optogenetics, photoactivation, switching/conversion.

### FEATURES

- **Wavelength range**: 360 – 750 nm
- **Switching time**: TTL 10 μs, USB 1 ms
- **Intensity control**: 0 - 100% - 1% resolution
- Optional touch screen controller
- Easy switch LED/Dichroic for additional wavelengths

### 405 nm Highpower Laser

475 mW, with direct modulation

This high-power laser diode delivers a CW output of approximately 475 mW. The laser can be directly modulated at MHz rates, making it highly effective for both sustained and pulse mode operation. To ensure uniform illumination of Mosaic’s DMD, the optical fiber, which delivers the laser output into the Mosaic, is subjected to mode mixing or phase shearing. This homogenizes the optical intensity profile, achieving typical uniformity better than 5%.

### FEATURES

- **Wavelength**: 405 ±1 nm
- **Output Power**: approximately 475 mW
- **Processing**: mode-mixing or phase via homogenizer
- **Maximum modulation rate**: 20 MHz
- **External modulation**: TTL
- **Uniformity**: ~ 5% typical
- **Applications**: include activation and bleaching

### Andor Laser Combiner (ALC)

**with optional Multi Port Unit (MPU)**

Compact / Flexible / Robust

ALC is an established market leader with up to six solid state laser lines in a single 19” rack enclosure and integral AOIF for rapid switching and blanking. The MPU provides unique flexibility with millisecond switching to one of three fiber ports for confocal, FRAPPA and/or TIRF.

### FEATURES

- **Integral AOIF for microsecond laser selection and modulation**
- **Fast laser blanking minimizes specimen exposure**
- **Long life solid state lasers with excellent stability (typical ± 2% over 8 hour period peak to peak)**
- **Choose up to six solid state lasers from 405, 445, 488, 491, 515, 532, 594, 640 nm**
- **Unique MPU provides ms switching to one of three fiber ports, FRAPPA, CSU and/or TIRF illumination**
- **Compact 4u 19” rack mount enclosure**
- **Quiet, cool with low power consumption**
- **Laser powers from 25 – 250 mW depending on wavelength**
EPi fluorescence microscopes have traditionally used Mercury or Xenon arc lamp housings. These are still supplied with a large number of new microscopes and can be used to illuminate Mosaic for AI. These broadband light sources deliver wavelengths from UV to IR and are adapted for use with Mosaic using an electro-mechanical shutter and excitation filter to select the AI wavelength range. They can also be used for their original purpose of EPi illumination in Mosaic and MicroPoint. When used with Mosaic the rich UV output can be used for localized release of caged compounds and other wavelengths can be used for activation and bleaching.

**AMH-200 Series (Metal Halide)**

_Bright / Stable / Long Life Lamp No Alignment Required / Optional Filter Wheel_

The AMH-200 is a 200 Watt DC stabilized metal halide light source with 2200 hours lamp life. Support from Andor IQ and third party software make this a flexible and efficient source for a broad range of applications. The easily replaced lamp and liquid light guide provide broadband excitation (UV-Vis-NIR) with no alignment and offer outstanding uniformity. AMH-200-F6S includes a fast 6-position internal filter wheel providing a cost effective, integrated EPi illumination package.

**FEATURES**

- Wavelength range 380 - 700 nm
- 0.0 - 100% motorized intensity / shutter
- 10 ms switching time and 500 μs shutter
- Synchronized operation with Fast LZ imaging
- UV-Vis liquid light guide coupling
- Choice for fast Calcium ion imaging
- TTL, RS232, parallel control interfaces

**Lamp Housing**

_Use your existing arc to illuminate Mosaic_

Epi fluorescence microscopes have traditionally used Mercury or Xenon arc lamp housings. These are still supplied with a large number of new microscopes and can be used to illuminate Mosaic for AI. These broadband light sources deliver wavelengths from UV to IR and are adapted for use with Mosaic using an electro-mechanical shutter and excitation filter to select the AI wavelength range. They can also be used for their original purpose of EPi illumination in Mosaic and MicroPoint. When used with Mosaic the rich UV output can be used for localized release of caged compounds and other wavelengths can be used for activation and bleaching.

**FEATURES**

- Flexible - use for Active or EPi Illumination
- Usable wavelengths - 360 - 800 nm
- Mechanical shutter open-close - 20 - 30 ms
- Mercury or Xenon lamp
- Direct coupling - heat filter supplied
- Cost-effective
- User-defined imaging (AI wavelength ranges)
- Optional integration with filter wheel
- Uncaging, activation, bleaching
Research imaging and AI techniques require software to enable the interactions between researcher and specimen and the software must adapt to each AI approach.

Andor AI devices are supported in a number of software packages, but there are many that do not support it. We have designed Andor iQ2 software to support numerous standard protocols, and a “virtual camera” module that allows the user to control the AI device in combination with the image generated from the core imaging platform. This can run on the host PC.

We also provide a Python extension to allow motivated users to adapt processing to their needs.

**ANDOR’S AI HARDWARE SUPPORTS**

- Diffraction-limited point photoablation, uncaging, photoactivation and photobleaching
- Complex scanned multi-region uncaging, photoactivation and photobleaching
- Complex simultaneous multi-region uncaging, photoactivation and/or photobleaching

AI sources provide pulsed, scanned and variable duration and rate exposure of selected regions. These capabilities provide industry-leading power, ease of use and flexibility.

**FEATURES**

- Interactive tools for region definition on image surface
- Supports different photo-stimulation modes for different region types
- Protocol and interactive photo-stimulation
- Dual camera acquisition - 30 full frames per-second (iXon3 897)
- Extensive hardware support for quality third party products
- Integrated Python environment for user programming
- Smooth integration with Imaris for deep analysis

**THIRD PARTY SOFTWARE**

- MetaMorph
- Nikon Elements
Andor’s AI solutions have been on the market for over 15 years, and were historically sold by Photonic Instruments, which is now part of Andor Technology.

The application notes here are based on scholarly articles chosen to illustrate the diversity of scientific questions that can be addressed with Andor’s AI products. AI has been a powerful tool for scientific research to date, but recent advances in “optogenetic” tools promise a new era in life science research not unlike the revolution brought about by GFP for which Shimomura, Chalfie and Tsien received a Nobel prize in 2008.

**Innovative Techniques and Cutting Edge Research**

- Uncaging reveals calcium’s role in cell cycle progression
- Laser ablation of microtubules during cell division progression
- Using frappa to study the dynamics of chromatin binding proteins in embryonic stem cells
- Precise optical inhibition of individual neurons
- Spatiotemporal control of cell signalling with a light-switchable protein pair
- Matching field of illumination with field of view

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[Application and Learning Centre andor.com/learning](#)
D-type cyclins such as CD1 are known to play an important role in cell cycle progression. Its level varies during cell cycle, and the amount of CD1 protein is directly dependent on the rate of CD1 gene transcription. CD1 gene transcription is regulated by multiple transcription factors, including nuclear factor kappa B (NF-κB), which can control cell growth and differentiation through transcriptional regulation of CD1.

Even though NF-κB appears to play a role in cell cycle progression, the intracellular events leading to this transcription factor’s activation upon growth factor stimulation are unknown. Stimulation of non dividing cells with growth factors or mitogens causes a transient rise in intracellular free calcium concentration. Intracellular calcium has been shown to play a role in the G1/S and G2/M phases of the cell cycle, but the University of Liverpool researchers wanted to find out more about its role in the G0–G1 transition.

Using targeted photolysis to uncage a calcium chelator and a calcium donor in living cells allowed them to observe how the targeted cells. These facts suggested that NF-κB appears to signal the downstream events.

The researchers used the MicroPoint system to uncage Diaz-2 in a cell of interest by exposing it to a train of 4 s flashes of 360 nm light over 10 s. They performed simultaneous imaging and photo-stimulation of the specimen, and could therefore follow the targeted cells and normal cells with time-lapse confocal microscopy. In this way they directly monitored the effects of uncaging on intracellular signaling and cell fate. As Violaine Sée explains, they did not detect NF-κ translocation into the nucleus or NF-κ-dependent gene transcription from the NF-κ consensus and CD1 promoters in the targeted cells. These facts suggested that the serum-induced calcium peak appears to signal the down-stream events.

To study the long-term role of the serum-induced intracellular calcium increase, the researchers needed a noninvasive method to eliminate this transient increase. They used the caged calcium scavenger Diaz-2. Its normally low affinity to calcium increases 30-fold when illuminated at 360 nm. To eliminate the serum-induced calcium peak, they applied the illumination to Diaz-2-loaded cells just after serum induction. The Diaz-2 photolysis inhibited the serum-induced calcium peak without effecting subsequent intracellular calcium levels.

The researchers used the MicroPoint system to uncage Diaz-2 in a cell of interest by exposing it to a train of 4 s flashes of 360 nm light over 10 s. They performed simultaneous imaging and photo-stimulation of the specimen, and could therefore follow the targeted cells and normal cells with time-lapse confocal microscopy. In this way they directly monitored the effects of uncaging on intracellular signaling and cell fate.

Figure 1. Single cell illumination of Swiss 3T3 cells loaded simultaneously with NP-EGTA (caged calcium) and Fluo-4 (calcium dye). The arrow indicates the illuminated cell (A) before illumination and (B) during illumination. The increase of fluorescence indicates calcium release in the illuminated cell. (C) Thirty seconds after illumination, calcium levels are back to basal levels. Image courtesy of Dr. Violaine Sée, University of Liverpool.

The result of suppressing and replicating serum-induced calcium peaks using caged molecules suggested that NF-κ translocation and downstream regulation of gene transcription depends on a transient increase of intracellular calcium, but other serum-dependent signals are required to activate NF-κ. These results, combined with other experiments, led the researchers to propose that the G0–G1 transition depends on mitogenic kinase activity as well as calcium signaling and that NF-κ activation has a critical role in the transduction of these mitogenic signals to the nucleus.

“The work showed that calcium has a lead role in cell cycle. Calcium ions have been implicated in many aspects of cell function, but its role in the cell cycle was not well described,” said Violaine Sée. “We found that calcium can initiate the cell cycle through activation of the mitogen-activated protein kinase-NF-κ pathway.”

**Equipment List**

Zeiss Laser scanning confocal microscope was used for time-lapse imaging

MicroPoint with 365 nm dye cell was used for uncaging.

**Acknowledgement**

Appreciation is gratefully extended to Professor Mike White, Dr. Violaine Sée and Dr. Dave Spiller, University of Liverpool

Research Paper:

Violaine Sée, Nina K.M. Rajala, David G. Spiller, and Michael R.H. White, Calcium-dependent regulation of the cell cycle via a novel MAPK-NF-κB pathway in Swiss 3T3 cells, The Journal of Cell Biology, Volume 166, Number 5, August 30, 2004 661–672.
APPLICATION NOTE 2
MICROPOINT

LASER ABLATION OF MICROTUBULES DURING CELL DIVISION PROGRESSION

The organization of a cell is critical for its function and understanding how organization affects function is a major goal of cell biology. Researchers led by Dr. John A. Cooper at the University of Washington in St. Louis and Dr Alexey Khodjakov at the Wadsworth Center, Albany, New York used microtubule ablation to learn more about how cells use the cytoskeleton to integrate spatial information into cell cycle regulation.

Budding yeast cells are one model organism used to study cell division. During division in the budding yeast, Saccharomyces cerevisiae, one end of the mitotic spindle is drawn through the bud neck to deliver a genome to the daughter cell. Cytoplasmic microtubules projecting from the spindle pole bodies carry out this process by interacting with the cell cortex to orient the spindle along the mother-bud axis and then move one spindle pole body through the neck and into the bud.

The cell has quality control mechanisms in place in case this process goes wrong. For example, mutations can cause a delay in the movement of the spindle. In this case mitosis proceeds in the mother cell, but if things have not corrected themselves by anaphase a cell cycle checkpoint mechanism known as the spindle position checkpoint will stop mitosis. Scientists have some understanding of how this checkpoint halts mitosis, but it isn’t fully known how the checkpoint mechanisms detect that the spindles are not in the correct position to proceed.

Previous work had suggested that dividing yeast prohibit cell cycle progression when the mitotic spindle is not adequately positioned between the nascent mother and daughter cells. This implies that the cell must monitor the position of the spindle, and interpret it relative to some other site, such as a landmark. To test the hypothesis that cytoplasmic microtubules extending from the spindle to the bud neck are important for this process Cooper’s team interrupted microtubule interactions with the bud neck using laser ablation.

The researchers used a MicroPoint pulsed laser system with its emission tuned to 539 nm to perform laser microsurgery of GFP-labeled microtubules in dynein mutant budding yeast cells. Dynamin mutants cannot pull the spindle through the bud neck, and thus mitosis is stopped by the spindle position checkpoint.

They had originally purchased the MicroPoint system to add a photobleaching/photoactivation module to their microscope, but the tunability of the system’s laser gave them the flexibility to also use the system to ablate microtubules in this experiment. MicroPoint allows the user to image the specimen during laser ablation and this allowed the researchers to visually target dynamic microtubules for ablation. Microtubules move quickly through the cell, so being able to observe the cell during ablation was critical for nimble targeting and subsequent verification of microtubule severing.

After using pulses of 539 nm light to sever individual cytoplasmic microtubules between the bud neck and spindle pole bodies, the researchers observed displacement of a distal fragment from the remaining microtubule. The fragment and remaining microtubule depolymerized and then grew back after several minutes. They monitored the spindle of these cells for up to 90 minutes to see if the cells remained arrested in anaphase. For imaging, they took time-lapse 2 series of the cells using an inverted fluorescence microscope with a 100 x, 1.35 N.A. oil objective lens, CSU spinning disk confocal scanner with 488 nm laser excitation and EMCCD camera for detection. The 2 series covered 3 um in depth and were captured at 30 or 60 s intervals.

When cytoplasmic microtubules in the bud neck were ablated most of the cells exited mitosis, showing that the absence of cytoplasmic microtubules in the bud neck activates the mitotic exit network. The researchers next performed experiments to find out if disrupting cytoplasmic microtubules not in the neck would disrupt the checkpoint. In checkpoint-activated cells with microtubules extending from one spindle pole body through the neck they ablated microtubules at the other spindle pole body (microtubules not extending through the neck). Most of these cells remained in mitosis. Finally, they did experiments in which they damaged the spindle pole bodies or severed microtubules near the spindle pole bodies. Neither of these actions prompted mitotic exit.

Collectively these ablation experiments showed that disrupting the microtubules that extend from the spindle to the bud neck cause the cells to fall to prohibit cell cycle progression and thus to exit mitosis. This suggests that the cytoplasmic microtubules are important for reading the position of the spindle upstream of cell cycle regulation. These findings have implications across species. They may be particularly important for the faithful distribution of genomes to proper regions of the cytoplasm during the asymmetric cell divisions that undertake metazoon development and the homeostasis of adult tissues.

**Equipment List**

CSU laser dual spinning disk confocal scanner, equipped with EMCCD detector
Time-lapse imaging software
MicroPoint with 539 nm dye resonator cell
Pulsed ablation targeting software

Acknowledgement
Appreciation is gratefully extended to Dr. John A. Cooper, University of Washington, St. Louis and Dr Alexey Khodjakov, Wadsworth Center, Albany, New York.

Research Paper:
APPLICATION NOTE 3

FRAPPA

USING FRAPPA TO STUDY THE DYNAMICS OF CHROMATIN BINDING PROTEINS IN EMBRYONIC STEM CELLS

As imaging technologies advance, researchers are able to observe more biological events in real time with increasing spatial and temporal resolution.

Recently, Nissim-Rafinia and Meshorer (ref. 1), from the department of genetics in the Alexander Silberman Institute of Life Sciences at the Hebrew University of Jerusalem, used active illumination and the technique known as FRAP (fluorescence recovery after photobleaching) to study the dynamics of chromatin binding proteins in mouse embryonic stem (ES) cells. This is of particular interest because other methods of studying chromatin binding proteins use purified chromatin from populations of cells, or from fixed cells. The ability to observe the real-time dynamics of chromatin binding proteins in single cells allows them to extract biological information that is otherwise unavailable using purified proteins.

Most chromatin binding proteins, such as HP1 (heterochromatin protein 1) and H1 (linker histone H1), are bound to DNA at any given time, but for long. The binding is transient, such that these chromatin binding proteins associate and dissociate with DNA with a half-life in the order of seconds. This high turnover of residence on chromatin is a key factor in the ability to generate plasticity of gene expression. Furthermore, ES cells are known to have an even higher rate of chromatin binding protein exchange than differentiated cells.

Nissim-Rafinia and Meshorer used the system in maximum acquisition speed because we concentrated on the dynamic behavior of chromatin binding proteins, which reveals quantitative information about the binding dynamics of the proteins.

The Revolution XD allowed the researchers to perform photobleaching fast enough to allow accurate measurement of the recovery half-time, while at the same time enabling highly sensitive time-lapse confocal imaging that minimized observation photobleaching. “Combining the high acquisition speed and the low phototoxicity of the spinning disk with photobleaching capacity makes FRAPPA superior to any other system,” says Eran Meshorer, senior lecturer in the department of genetics at the Hebrew University of Jerusalem. “It is unique by allowing rapid acquisition, long-term imaging and photobleaching experiments.”

Nissim-Rafinia and Meshorer found that both of the chromatin binding proteins they studied, HP1 and H1, have a chromatin binding half-life of a few seconds. However, they also were able to differentiate between different types of chromatic: FRAP was slower in heterochromatin than in euchromatin, indicating that chromatin binding proteins are less dynamic in heterochromatin. The authors surmise that this difference is due to a higher concentration of binding sites for HP1 and H1 in heterochromatin, along with molecular crowding. They also used a computer simulation to fit their experimental data with mathematical models. They used a single exponential equation I(t) = A(1-e^-kt), where 1 is t, A is the mobile fraction of chromatin binding proteins, 1-A is the mobile fraction of chromatin binding proteins, and k is the dissociation constant. The authors calculated a direct estimate of the off rate of binding (koff), as well as the parameter A, which can be used to calculate the association rate.

Meshorer says that the chromatin binding proteins move slowly relative to the fast biological events that Andor’s Revolution system equipped with FRAPPA can handle. In future experiments, they look forward to studying faster events that will make full use of FRAPPA’s capacity. “So far we haven’t used the system in maximum acquisition speed because we concentrated on the dynamic behavior of chromatin binding proteins,” Meshorer notes in regard to FRAPPA. “However, I envision that we will investigate some highly dynamic properties of the cell nucleus, which will require faster imaging after photobleaching. In addition, we will perform long time-lapse experiments during which we will introduce photobleaching experiments very so often to monitor gradual changes in the dynamic properties of proteins during cellular differentiation. This can only be achieved using such a system.”

Acknowledgement: Appreciation is gratefully extended to Dr. Malka Nissim-Rafinia and Dr. Eran Meshorer from the department of genetics in the Alexander Silberman Institute of Life Sciences at the Hebrew University of Jerusalem

Reference Material:
APPLICATION NOTE 4

MOSSAIC

PRECISE OPTICAL INHIBITION OF INDIVIDUAL NEURONS

Scientists can learn about the functions of specific neurons in networks, like the brain, using proteins that bind natural or synthetic photoswitches. Light can turn these photoswitches on and off, allowing the control of activity in specific cells and thus observation of how processing and behavior are altered by defined neuronal populations.

Processes happen fast in neurons, so speed can be critical for some studies. The bacteriorhodopsin and Halor families are the only light-activated inhibitory proteins with millisecond resolution. However, they are not without room for improvement. Both require continuous illumination to stay activated, and continuous light can cause problems such as rebound excitation or eventual partial inactivation. Also, Halor can require high light intensities because the magnitude of the current is driven by the constant activation of the pumping cycle.

Thus researchers led by Ehud Y. Isacoff from the University of California, Berkeley and the Lawrence Berkeley National Laboratory designed a ligand-gated ion channel that is K+ selective and controlled with light. Called HyLighter, the ion-channel isn’t active until exposed to light, is more sensitive to light than Halor, and can reach its maximum current over a wide range of light intensities. The light-activated ion channel can convert a light pulse into a stable hyperpolarizing current that stays on with no illumination until it is turned off using a complementary photoswitch. Light can turn these photoswitches on and off, allowing the control of activity in specific cells and thus observation of how processing and behavior are altered by defined neuronal populations.

To create HyLighter, the researchers first engineered chimeric iGlurRs with the ligand-binding domain of iGlur6, the first engineered chimeric iGlurs with the cation channels mediate excitatory neurotransmission in higher organisms and cation channels mediate excitatory glutamate receptor (iGlur). These receptors are permeant to Na+ and K+. They experimented with the various chimeras they created and found that one had all the characteristics they needed. It functioned as a light-gated K+ channel, was maximally activated by low light intensity at wavelengths ~390 nm, maintained activity in the dark, and could be turned off with 500 nm light.

They tested HyLighter by transfecting cultured hippocampal slices from early postnatal rats with HyLighter-GFP using biologic gene transfer. It was expressed well in all regions of the hippocampus and homogenously distributed in all parts of the neuron. They activated HyLighter with 390 nm illumination from a Lambda DG-4 light source coupled to the microscope and projected onto the sample through the Mosaic DMD through a 40X objective.

Among the existing microscopy solutions, only the Mosaic allows you to continuously illuminate an arbitrary mask in the imaging field. You simply can’t do this using laser-scanning microscopes, which never provide true simultaneous illumination. This important feature combined with ease of use and direct interfacing with software makes the Mosaic a unique and valuable tool for the optogenetic community,” said Dr. Harald Janovjak, who was part of the research team and is now at the Institute of Science and Technology Austria (IST Austria). In the hippocampal slices, HyLighter induced strong hyperpolarization that could silence neuron firing until deactivated by 500 nm light. At the sample, the light intensity was approximately 20 mw mm\(^{-2}\) at 390 nm and 40 mw mm\(^{-2}\) at 500 nm.

The researchers also generated transgenic zebrafish expressing HyLighter. When the tails of these fish received 390 nm, the probability of an escape response after a mechanical stimulus was reduced. This was reversed by 500 nm illumination. The illumination had no effect on escape responses in zebrafish not expressing HyLighter.

The researchers say that as a new, light-activated, purely hyperpolarizing ion channel, HyLighter complements existing optogenetic tools. Its push-pull two-wavelength design, low light requirement and unique spectral sensitivity make it a useful way to suppress activity in specific cells in intact neural circuits with temporal precision. In addition, the two-wavelength design means that it can be used in experiments in which neurons are silenced after light exposure and behavioral analysis then performed in ambient light or in the dark without a visual stimulus affecting the effects.

The researchers look forward to HyLighter emerging as an important tool for the optogenetic control of neuronal activity. “When it comes to silencing of nerves cells, bi-stability and the requirement for small amounts of light make HyLighter unique,” said Dr. Janovjak.

Figure 1. An ionotropic glutamate receptor (top left) and a synthetic photoswitch called Maleimide-Azobenzene-Glutamate (MaG, bottom) is the basis for the light-controlled HyLighter ion channel (right). Images courtesy of Dr. Harald Janovjak.

Figure 2. MIP image of overlayed confocal stacks of HyLighter:GFP with non-specific tdTomato. The neuron is the boundary (green bar). The membrane marker is the MIP image of overlayed confocal stacks of HyLighter:GFP with non-specific tdTomato. The neuron is the boundary (green bar). The membrane marker is the MIP image of overlayed confocal stacks of HyLighter:GFP with non-specific tdTomato. The neuron is the boundary (green bar).

Figure 3. Action potentials triggered by current injections are robustly silenced when HyLighter is activated by 390 nm light (violet bar) and no longer inhibited when deactivated with 500 nm light (green bar).

Acknowledgement: Appreciation is gratefully extended to Dr. Harald Janovjak, Institute of Science and Technology, Austria and Dr. Ehud Isacoff, University of California, Berkeley.


Equipment List

- Olympus IX70 for electrophysiology
- Zeiss AxioExaminer for imaging
- Axopatch and P-Clamp (Molecular Devices Inc.) used for patch-clamp voltage recording
- Mosaic DMD and software for targeted inhibition of neuronal firing
- Sutter DG4P light source for Mosaic illumination
- Cool laser spinning disk used to acquire confocal images of neurons
Researchers from the University of California, San Francisco, used the phytochrome signaling network of plants to develop a genetically encoded light-controlled system for fine spatial and temporal control over proteins. The Mosaic Active Illumination System was a key part of the optical setup, allowing them to achieve tight spatial patterns of protein recruitment in mammalian cells.

Phytochromes are photoreceptive signaling proteins that control many light-sensitive processes in plants by detecting red and near infrared light. The researchers optimized phytochrome B (Phyb) and phytochrome interaction factor 3 (PIF3) to produce a photo-sensitive pair, Phyb-PIF, which binds in response to red light (650 nm) and dissociates in response to infrared light (>750 nm). When Phyb is membrane-bound, then fluorescently labelled PIF3 can be seen to translocate to the plasma membrane under 650 nm illumination, where the Phyb-PIF complex forms.

The researchers then developed a fully automated method to expose the cell to both wavelengths of light. “The main challenge for developing the optical setup was simultaneous inverse patterns of red and infrared light,” said Dr. Orion Weiner, a member of the research team. “To combat lateral diffusion of plasma-membrane bound PIF–YFP, we needed a zone of recruitment (red light) surrounded by an inverse zone of infrared light.” To accomplish this, researchers worked with Photonic Instruments to develop a “Complementary Mosaic Active Illumination System” that coupled the red light to the “on” state of the device’s mirrors and coupled a separate infrared light source to the “off” state of the mirrors. In this configuration the device acts to both “target” activity in user-defined regions and “silence” activity outside of those regions.

The Mosaic Active Illumination System contains an array of hundreds of thousands of microscopic semiconductor-based mirrors known as a Digital Micromirror Device. The hinge-mounted mirrors can be individually tilted very quickly and efficiently allowing generation of red pixels and an inverse pattern of infrared pixels at the same time. “There is no other commercial device that can generate simultaneous inverse patterns of red and infrared light,” said Weiner. “This was essential for our ability to generate tight spatial patterns of protein recruitment in mammalian cells with the Phyb-PIF system.”

Using the Mosaic Duet they could even project a simple pixel-based movie onto the cell membrane. TRIF imaging of the cell membrane using an Andor ixon EMCCD camera showed that the illumination pattern produced features as small as 3 µm. The ixon camera allowed them to use as little fluorescent excitation light as possible, which was desirable because the imaging wavelengths also slightly activate the photosactivatable system said Anselm Levskaya, who led the research team.

The researchers could also use software to “dither” the average amount of red light in the target mask, allowing them to smoothly titrate the fraction of active Phy and recruited PIF–YFP. This showed that the technique could be used for “grayscale” control of the chemical potential.

The genetically encoded, light-switchable Phy-PIF interaction module the researchers developed had a titrated and reversible interaction and could potentially be used to control any live cell process that depends on a recruitment event. Because the light can be controlled with high spatial and temporal resolution highly complex spatial or temporal patterns can drive a process using the Phy-PIF module.

The researchers are currently using the system with closed-loop control to automatically tune activation of the photo-modulated signal transduction pathway. This is important for implementing quantitative microscopy experiments with single cells, said Levskaya. They foresee the Phy-PIF module being useful for controlling a wide variety of cell biological processes without requiring case-by-case protein engineering.

“I think one of the biggest applications it will have will be in perturbing gene transcription and signal transduction pathways in developing animals,” said Levskaya. “Being able to alter in time and space what signals and genes are being made should allow us to perform very novel kinds of experiments during development to try to reverse engineer the machinery that establishes the animal body plan.”

Acknowledgement: Appreciation is gratefully extended to Anselm Levskaya, Orion D. Weiner, Wendell A. Lim and Christopher C. Voigt, University of California, San Francisco.

This application note is based on: Spatiotemporal control of cell signalling using a light-switchable protein interaction, Nature 461, 997-100, Oct. 15, 2009, doi:10.1038/nature08446.
**TECHNICAL NOTE**

**MATCHING FIELD OF ILLUMINATION AND FIELD OF VIEW**

Here we define Field of View (FOV) by detector size and microscope objective, and Field of Illumination (FOI) relative to the detector and in the image plane.

**Field of View and Illumination**

In a microscope system, the camera is coupled via a C-mount adapter and located in a primary image plane (PIP). The PIP is our reference for the definition of FOV and FOI. In the PIP, FOV is defined as the extent of the image sensor in X and Y dimensions. For consistency we define FOI as the extent of illumination in the PIP. This is convenient because we can easily calculate overlap between the two.

As mentioned above, it is common to match FOV and FOI, but with active illumination other factors such as Power Density (PD) or Resolution may also be important considerations. Mosaic is equipped a ZK laser zoom collimator so you can trade FOI for PD.

**Imaging Resolution and Detection**

In a fluorescence microscope resolution is dominated by the objective lens, which both illuminates and images the specimen. The objective numerical aperture (NA) and wavelength of detected light (λ) define Resolution, RXY, by the Raleigh criterion as follows:

\[
RXY = \frac{1}{\lambda NA} \quad \text{and} \quad \text{RXY}_m = \frac{1}{\lambda m}\]

In Table 1 below RXY is computed for a range of objective lenses with λ = 550 nm.

<table>
<thead>
<tr>
<th>Magnification (mm/image)</th>
<th>Numerical Aperture</th>
<th>RXY (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 (air)</td>
<td>0.6</td>
<td>0.51</td>
</tr>
<tr>
<td>40 (air)</td>
<td>0.8</td>
<td>0.38</td>
</tr>
<tr>
<td>40 (W)</td>
<td>1.0</td>
<td>0.30</td>
</tr>
<tr>
<td>60 (W)</td>
<td>1.1</td>
<td>0.28</td>
</tr>
<tr>
<td>60 (C)</td>
<td>1.4</td>
<td>0.22</td>
</tr>
<tr>
<td>100 (C)</td>
<td>1.4</td>
<td>0.22</td>
</tr>
</tbody>
</table>

1/RXy is a good approximation of the maximum spatial frequency in the image. To capture all information in the image (e.g. with a CCD detector) we must sample at frequency F to avoid “aliasing errors”. This is known as the Nyquist criterion:

\[
2 \times \text{Px} \leq 2 \times \text{RXY}
\]

Translating into the PIP with objective magnification of MO, we can compute the sensor pixel size required to fulfill the Nyquist criterion:

\[
2 \times \text{Px} \leq 2 \times \text{MO} \times \text{RXY}
\]

Subject to application requirements, Nyquist may or may not be necessary. Using an objective lens of 100X, 1.4 NA we see that Neo, Clara and Luca R are all capable of achieving the Nyquist criterion: 2 * Px ≈ 22 µm. While at 60X 1.4 NA, only Neo and Clara can provide small enough pixels.

**Illumination Resolution**

MicroPoint and FRAPPA use Gaussian laser beams and Gaussian beams remain Gaussian with a theoretical minimum focus spot diameter of ψ, where ψ is the wavelength. This can only be achieved if the collimated laser beam fills the objective aperture. The following variation on the Raleigh criterion provides a definition for spatial resolution SXY of the illumination system:

\[
SXY = 1.22 \sqrt{f/D}; f = \text{focal length of objective}, \quad D = \text{beam diameter.}
\]

This is the size of the smallest object the microscope can resolve, sometimes called the diffraction limit, and is also the diameter of the smallest spot to which a collimated laser beam illuminates. This is the size of the smallest object the microscope is sensitive to, and also the diameter of the smallest spot to which a collimated laser beam illuminates. The shape of the spot is an Airy disk or optical point spread function, PSF, characteristic of the system.

**Power Density and Illumination Time**

Power density, PD in the specimen plane is estimated from the ratio of beam power and area. Spectral transmission and chromatic errors in the microscope objective are critical to performance. To estimate specimen plane PDs, the Mosaic output beam power density is multiplied by the square of the magnification and the systems spectral transmission, T(λ).

\[
PD = \text{PDD} \times \text{MO}^2 \times T(\lambda)
\]

Channalrhodopsin2 (Chrh2) is a light activated cation channel which can be expressed in neurons and used to control behavior in host organisms, including mice, c. elegans and drosophila. Stimulation with blue light (~470 nm), the power density, for photo-activation of Chrh2 is in the range 0.1-1.1 mW mm-2 and has a wide dynamic range.

Figure after Wang et al (www.pnas.org/cgi/doi/10.1073/pnas.0700384104)

![Figure 1](https://example.com/figure1.png)

**CAMERA FIELDS OF VIEW**

<table>
<thead>
<tr>
<th>Model</th>
<th>FOV (Lamp house)</th>
<th>DMD Pixel size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Luca R</td>
<td>12.2 x 11.3</td>
<td>8.6 x 8.0</td>
</tr>
<tr>
<td>Clara</td>
<td>14.2 x 11.3</td>
<td>8.6 x 8.0</td>
</tr>
<tr>
<td>model 8201</td>
<td>15.8 x 11.3</td>
<td>8.6 x 8.0</td>
</tr>
<tr>
<td>model 8206</td>
<td>15.8 x 11.3</td>
<td>8.6 x 8.0</td>
</tr>
<tr>
<td>model mP-2204 +</td>
<td>17.2 x 11.3</td>
<td>8.6 x 8.0</td>
</tr>
<tr>
<td>model mP-2204</td>
<td>17.2 x 11.3</td>
<td>8.6 x 8.0</td>
</tr>
<tr>
<td>model mP-2204 FRAPPA</td>
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<td>8.6 x 8.0</td>
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</tbody>
</table>

**Table 2**

<table>
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<tr>
<th>Magnification (mm/image)</th>
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</tbody>
</table>

Table 2 shows the approximate theoretical smallest spot sizes for a perfect microscope.
Andor products are regularly used in critical applications and we can provide a variety of customer support services to maximise the return on your investment and ensure that your product continues to operate at its optimum performance.

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Andor offers a variety of support under the following formats:

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Training services can be provided on-site or remotely via the Internet.

A testing service to confirm the integrity and optimise the performance of existing equipment in the field is also available on request.

A range of extended warranty packages are available for Andor products giving you the flexibility to choose one appropriate for your needs. These warranties allow you to obtain additional levels of service and include both on-site and remote support options, and may be purchased on a multi-year basis allowing users to fix their support costs over the operating lifecycle of the products.