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Photobleaching setup for the biological end-station of the darmstadt heavy-ion microprobe

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ABSTRACT

We report the upgrade of the epifluorescence microscope of the GSI heavy-ion microprobe with a galvo-scanned, 488 nm laser diode. The laser is focussed into the object plane by the water-immersion objective resulting in a focal spot size of about 1 μm. To increase temporal and spatial resolution a water-immersion objective with a high numerical aperture is integrated into the custom-build microscope. The upgraded system can now be used to bleach GFP-tagged proteins recruited to DNA damage induced by targeted single-ion irradiation. The system is demonstrated on NIH 3T3 cells with Ku80-GFP ion-targeted in heterochromatic and euchromatic DNA. Fluorescence recovery after photobleaching (FRAP) is shown to be significantly slower in heterochromatin.

1. Introduction

The GSI high-energy heavy-ion microprobe [1] is equipped with a compact end-station for radio-biological experiments, where a 500 nm wide ion beam is injected into the liquid environment of cultures of living biological cells [2] via fast beam switching and fast magnetic steering, single ions are directed into cellular compartments selected by online fluorescence microscopy. With the setup optimized for mechanical and thermal stability and with the absolute beam location determined accurately [3], the mean systematic targeting error for a single ion is currently 670 nm with a sigma of 400 nm ($N = 111$).

Local damage induced at and around the sites of individual ion impacts can be assessed using a plethora of biological assays also including visualisation of damaged sites by online fluorescence microscopy of green-fluorescent-protein (GFP) fused to repair proteins. These repair proteins are recruited in so called repair foci around the damage. Here, the local concentration of tagged proteins in form of local fluorescence intensities is monitored as a function of time directly after targeted irradiation of the subcellular compartment of interest. Protein kinetics and dynamics can readily be compared in different compartments of the same cell.

This type of fluorescence microscopy of cells genetically modified to include a GFP transcribing sequence yields a measure for local concentration of the corresponding protein. However, exchange processes with no change in local protein density cannot be detected. As long as the in-flux of tagged molecules into a microscopic volume is equal to the out-flux, both the molecule concentration and the fluorescence emitted from the volume remain constant. Protein turnover with no concentration change can be made visible in a fluorescence microscope by selectively manipulating the fluorescence state of the tagged molecules in the volume of interest: Depending on the chromophore, fluorescence can be switched off by bleaching, photoactivated by a light pulse or absorption, or emission wavelengths changed with an appropriate incoming light pulse [4].

Here, we present the implementation of an intense light source focused into the object plane of our online fluorescence microscope. The focal point can be moved to selected positions to permanently bleach GFP molecules with a brief flash of light in the absorption band of GFP. When recording a sequence of fluorescence images after the bleaching event, the replacement of bleached molecules with fluorescent ones from the vicinity can be evaluated as a measure for local protein turnover even when no change in concentration occurs [5].

2. Photobleaching setup

A schematic of the radiobiology end-station with its fluorescence microscope including the new photobleaching module is de-

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picted in Fig. 1. The stainless-steel cell dish with cells growing on a suitable, few μm thin polymer foil is mounted on the X-Y stage at a distance of less than 50 μm from the 200 nm thin silicon nitride vacuum window. Cells are imaged with the custom-build epifluorescence microscope [2,6], set up on an optical bench that can slide back and forth on precision bearings for focusing as well as cell dish exchange. The microscope is equipped with a LED light source (Colibri, Carl Zeiss Microscopy GmbH, Germany) supplying up to four narrow-band excitation wavelengths with negligible heat production. In the lamp house, the excitation light is shaped for Köhler-illumination of the object plane: While each point in the object plane receives light from every point of the lamp (homogeneity of illumination), the rectangular field aperture limits the illuminated object area to the area that is projected onto the camera sensor (no photobleaching is possible outside the illuminated field of view (FOV)). Additionally, the circular objective aperture is imaged onto the backside entrance lens of the objective. Both the field and the objective apertures block light that would not contribute to the micrograph of the object but instead detrimentally increase the amount of stray light either carrying no information or information that is not registered (contrast enhancement) [7,8]. Excitation light is reflected into the objective by a multi-chromatic beamsplitter and excites fluorescent dye molecules in the FOV. Red-shifted emission light returning from the object is transmitted through filters that block wavelengths other than the dye emission and then imaged onto a CCD camera sensor (Pixelfly XS270, PCO AG, Germany). Using a multi-chromatic beamsplitter and an emission filter (Filter Set 62 HE, Carl Zeiss Microscopy GmbH, Germany) that are both matched to multiple LED excitation and dye emission wavelengths, no mechanical components need to be moved to record multiple dyes in fast succession. The use of mechanically switched emission filters and beamsplitters would not only slow down the measurement, but also introduce minute optical shifts since both splitter and filter are thick, refracting elements. Slight imperfections of the mechanical positioning would thus lead to a shifted image on the CCD chip.

To record image sequences with a good temporal and spatial resolution, the use of a high-numerical aperture immersion objective is imperative. Water is preferable as immersion medium since the cells reside in an aqueous environment and, in contrast to oil-immersion, no refraction mismatch occurs. To keep low-viscosity immersion medium between the objective and the cover glass of the cell dish in our horizontal microbeam setup, water is continuously injected just above the microscope objective and extracted by suction in the lower part (Fig. 2). With the cells attached on the far side of the cell dish, long-working distance objectives are needed to allow for a thick layer of culture medium in the cell dish. A good compromise between high numerical aperture (NA) and long working distance is obtained with a Nikon CFI Apochromat Lambda S LWD 40XW objective (Nikon Corporation, Japan) with a working distance of 600 μm and NA 1.15. Resolution measurements with 200 nm green fluorescent polymer beads show that sub-resolution spheres (point sources of green light) with a distance of 400 nm can easily be resolved [9]. One challenge of immersion objectives in a microprobe setup is to avoid loss of ion-targeting accuracy by uncontrolled movement of the objective being coupled to the moving microscope stage by a viscous liquid. Mean distances between targeted and hit positions have been measured in situ by fast recruiting proteins in cell nuclei. Evaluation of over hundred single ion hits show no significant change of the targeting accuracy of 670 nm with a sigma of 400 nm ($N = 111$). [10]

To bleach GFP, a parallel 488 nm laser beam (iBeam smart, Toptica Photonics AG, Germany) is focused into the FOV by the microscope objective. To make full use of the objective's NA, the laser beam cross-section is adjusted to fill the objective backside entrance lens by means of an infinity-focus telescope (magnification 2.8x) that is folded in a right-angle configuration for compactness. In the lamp house, the scan lens and the 'lamp house exit lens' act as a second beam expander for the laser beam resulting in a total magnification of 4.2x. The scan lens is focusing the laser beam into the plane of the luminous field aperture that is imaged into the FOV by the 'lamp house exit lens' and the microscope objective. Two galvo-scanners (QuantumScan-7, Nutfield Technology Inc., USA) in orthogonal configuration are used to deflect the laser beam into any chosen point in the field aperture plane and thus in the FOV of the microscope. The choice of lenses was aided by means of ray-tracing simulations with ZEMAX (ZEMAX Development Corp., USA). Measurements of the spot size via a suitable fluorescent material (e.g., 50 μm thin Ce:$Y_3Al_5O_{12}$ crystal) yield a spot size of less than 1 μm half-width in the FOV. Digital and analog modulation inputs of the laser diode are used to adjust laser power and illumination time.

In order to use the laser system to bleach selected patterns (for example point, line, circle) with defined location, intensity, and timing, integration into the microprobe control software is indispensable. The microprobe control software is a plugin to Image-Pro-Plus (Media Cybernetics Inc., USA) [2] and is extended to control the laser system. An automatic calibration routine relates deflection of the laser scanner to the position of the focus in the FOV via subsequent deflection and imaging of the laser focus on a scintillator.

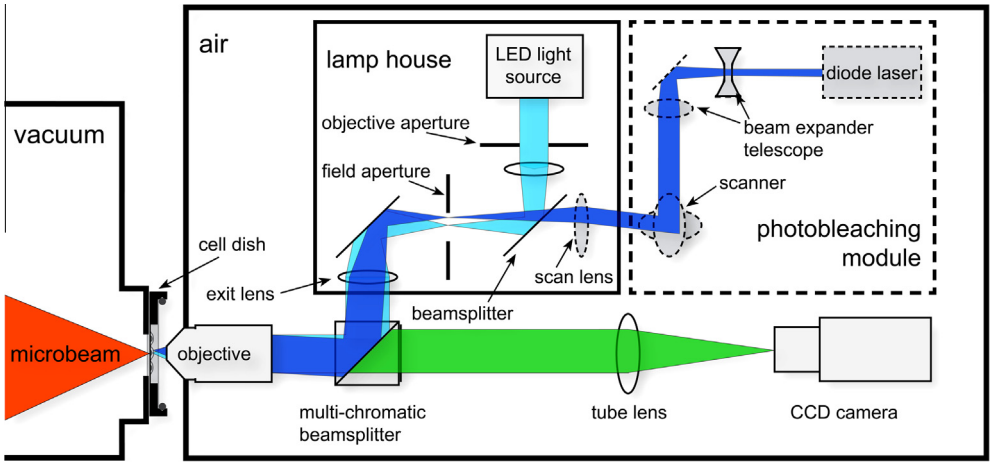

Fig. 1. Schematic view of the biological end-station with extended laser setup (dashed line).