Methylene blue- and thiol-based oxygen depletion
for super-resolution imaging

Philip Schäfer, Sebastian van de Linde, Julian Lehmann, Markus Sauer, Sören Doose*

Department of Biotechnology & Biophysics, Biozentrum, Julius-Maximilians-University
Würzburg, Am Hubland, 97075 Würzburg, Germany

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Corresponding Author

* Email: soeren.doose@uni-wuerzburg.de

Supporting Information

This material contains additional information on material and methods, on results and discussion, as well as a scheme and ten figures S1-S10 with data as described in the figure captions and discussed in more detail within the main text.
MATERIALS & METHODS:

**Chemicals:** Methylene blue (MB), 2-aminoethanethiol (also β-mercaptoethylamine, MEA), potassium hydroxide were purchased from Sigma Aldrich (Germany). MEA was also purchased from AppliChem (Germany). Cy5-NHS was purchased from GE Healthcare (Germany). Disodium salt and tetrasodium salt ethylenediaminetetraacetic acid (EDTA) and hydrogen chloride were purchased from Carl Roth (Germany). Aqueous solutions were prepared with Millipore-purified water (18.2 MΩ-cm) and the pH was adjusted by the addition of sodium hydroxide.

For the enzymatic oxygen scavenging system GluOx a stock solution was prepared, which contained 2,000 U/ml glucose oxidase, 40,000 U/ml catalase, 25 mM potassium chloride, 22 mM tris(hydroxymethyl)aminomethane - hydrogen chloride at pH 7.0, 4 mM tris(2-carboxyethyl)phosphine (TCEP). The solution was kept in 50 % glycerin at -20°C. All chemicals were purchased from Sigma Aldrich (Germany). Throughout this project the enzymatic oxygen scavenging system GluOx was always taken from the stock solution, with a constant ratio glucose oxidase to catalase, and 2.5-5 % (w/v) glucose was added. As the speed of oxygen scavenging is limited by the concentration of glucose oxidase the concentration of the enzymatic oxygen scavenging system GluOx in the sample is always indicated by the concentration of glucose oxidase.

**Antibodies:** Mouse anti-β-tubulin antibodies (T5201) were purchased from Sigma Aldrich (Germany). Goat anti mouse F(ab')2 fragments (A10534, Life Technologies, Germany) were labeled with Cy5-NHS (PA15101, GE Healthcare, Germany) according to standard coupling protocols given by the supplier. Gel filtration columns (Sephadex G-25, GE Healthcare) were used to purify the conjugates. The degree of labeling was determined by absorption spectroscopy.
(V-650 Photometer, Jasco, Germany) to about one. Antibody solutions were stored in 0.2 % sodium azide in phosphate buffered saline (PBS) solution.

**pH monitoring:** The pH was measured with a pH-electrode GPHR 1400A Digital pH/mV-Meter (Greisinger Electronic, Germany) with a working temperature of 0-50 °C. The error of the instrument was specified by the manufacturer to be <0.02 pH.

**Immunocytochemistry:** In order to stain the microtubule network of HeLa cells, indirect immunocytochemistry was applied. HeLa cells were grown in LabTek II chambers (Nunc, Germany) and fixed according to \(^1\). Cells were first fixed for 2 min with 0.3 % glutaraldehyde and 0.25 % Triton X-100 in cytoskeleton buffer (CB; 10 mM MES pH 6.1, 150 mM NaCl, 5 mM EDTA, 5 mM glucose, 5 mM MgCl\(_2\); Sigma Aldrich, Germany) and then fixed for 10 min in 2 % glutaraldehyde in CB. Afterwards, cells were washed three times with PBS and incubated with blocking buffer (BB; PBS containing 5 % (w/v) BSA) for 30 min. Cells were then incubated with mouse monoclonal anti-β-tubulin antibodies dissolved in BB for 60 min and with Cy5-labelled goat anti-mouse F(ab')\(_2\) fragments for 60 min, respectively. Three washing steps with PBS containing 0.1 % (v/v) Tween-20 (Sigma Aldrich) were performed after each staining step.

**Super-resolution imaging microscopy:** The sample was embedded in (i) 100 mM MEA, (ii) 100 mM MEA + 10 U/ml GluOx (with 5 % (w/v) glucose), (iii) 100 mM MEA + 1 μM MB, (iv) 100 mM MEA + 5 μM MB, or (v) 100 mM MEA + 50 μM MB; all buffers were set to pH 7.4. The sample was placed on an inverted microscope (IX-71, Olympus, Germany). The beam of a 641 nm diode laser (Cube 640–100C, Coherent, Germany) was reflected by a dichroic mirror (FF650-Di01, Semrock, USA) and focused onto the back-focal-plane of an oil-immersion objective (60x, NA 1.45, Olympus). Before being imaged on an electron-multiplying CCD camera (Ixon DU897, Andor, UK), fluorescence light was filtered by band- and longpass filters.
(BrightLine 697/75, RazorEdge 647, Semrock). Additional lenses were used to generate an image pixel size of 107 nm. 20,000 frames were recorded with a frame rate of 100 Hz at irradiation intensities of 5 kW/cm². Single-molecule image stacks were analyzed and reconstructed with rapid/STORM V2.21. Only fluorescence spots containing more than 500 photons were analyzed. Double-spot emission was analyzed as described in 4. Reconstructed images were binned with 20 nm/pixel and displayed in false color.

RESULTS & DISCUSSION:

Dimer formation: In aqueous solution the planar aromatic structure of MB promotes stacking interactions due to hydrophobic and pi-stacking interactions. MB is thus known to form dimers with an equilibrium binding constant between 2,000 and 5,000 M⁻¹ (reviewed in reference 5). Such dimers exhibit spectral properties of non-fluorescing H-dimers with a blue-shifted absorption maximum at 605-610 nm and an extinction coefficient of around 9×10⁴ M⁻¹cm⁻¹ at 610 nm (versus 3.9×10⁴ M⁻¹cm⁻¹ for the monomer at this wavelength) 5. Considering that MB monomer and dimer spectra are indistinguishable below 400 nm (Fig. S3) the single MB state indicated by the UV spectra around the isosbestic points consists of both monomers and dimers. When looking at the ratio between absorption at the monomer peak (665 nm) and at the dimer peak (610 nm) at the start of the dark reaction and comparing this value with ratios for pure MB solutions at different concentrations (Fig. S3) it appears that 70 to 90 % of all MB molecules (with a total concentration of 42 µM) represented in Fig. 2 are in a monomeric state. This is consistent with expectations from the dimer equilibrium constant 5-7. A slight increase of this ratio during the dark reaction clearly indicates that MB can be reduced both as monomer and as dimer. With each reduction of a single MB entity within a dimeric complex, the electronic structure is
modified to reconstitute the monomer spectrum of the molecular complex. This results in a relative increase of the contribution from monomeric MB to the observed absorption spectrum even if monomeric and dimeric MB reacted with equal probability.

REFERENCES


Scheme S1: Molecular structure of methylene blue (MB+) and leucoMB (MBH).
Fig. S1: Discoloration of MB in aqueous MEA solution. Absorbance was measured at 665 nm in a stopped flow instrument after mixing MB with aqueous MEA solution. Data were recorded for the dark reaction in mixtures of 42 µM MB (pH 7.8) with 45 mM MEA. Three repeated measurements are overlaid with the light exposure from the spectrometer lamp being shut off at different times. The grey curve was recorded with continuous illumination. The black curve was recorded with intermittent illumination. The red curve was recorded about 13 min after the solvents were mixed. All data curves overlay proving that the observed reaction is independent from the spectrometer irradiation.
**Fig. S2:** Absorption spectra for the dark reaction of MB and MEA as shown in Fig. 2. Two isosbestic points in the near UV spectrum evolve. Considering that dimers and monomers exhibit identical spectra in the UV (Fig. S3) interconversion between two states is indicated, representing a MB monomer/dimer mixture at the start of the reaction and the MB leucobase at the end of the reaction.
**Fig. S3:** Dimer formation of MB. (A) Absorption spectra of MB in aqueous solution at different concentrations. Data were recorded with 1 µM (light blue), 5 µM (dark blue), 50 µM (violet), and 250 µM (black) MB concentration and normalized to the absorbance at 247 nm. (B) Ratio of absorbance at 665 nm to absorbance at 610 nm is plotted for the recorded spectra. The continuous line is added as a guide to the eye.
Fig. S4: Temperature–dependent dark reaction between MB and MEA. (A) Pseudo-first order rate constants for the dark reaction of 42 µM MB and 55 mM MEA at a pH of 7.3 (black square), 7.8 (blue circle), 8.2 (red triangle), 8.6 (green diamond) are displayed as function of inverse temperature $T^{-1}$ and reveal Arrhenius behavior. (B) Activation enthalpies $E_A$ as extracted from the data in (A) are plotted as function of pH.
Fig. S5: Temperature–dependent photoinduced reaction between MB and MEA. (A) Pseudo-first order rate constants $k_{\text{fast}}$ for the photoinduced fast reaction of 42 µM MB and 55 mM MEA at a pH of 7.8 (blue triangle), 8.2 (red circle), 8.6 (black square) are displayed as function of inverse temperature $T^{-1}$ and reveal Arrhenius behavior. (B) Pseudo-first order rate constants $k_{\text{slow}}$ for the slow reaction, resembling the dark reaction, of 42 µM MB and 55 mM MEA at a pH of 7.8 (blue triangle), 8.2 (red circle), 8.6 (black square) are displayed as function of inverse temperature $T^{-1}$ and also reveal Arrhenius behavior. (C) Activation enthalpies $E_A$ as extracted from the data in (A) and (B) are plotted for the fast reaction (open circles) and the slow reaction (black squares) as function of pH.
Fig. S6: Absorbance time traces of MB in aqueous solution during laser irradiation (irradiation power 3.27 mW) with and without oxygen. Data was recorded in mixtures of 42 µM MB and 55 mM MEA (pH 7.8) at various O$_2$ concentrations. O$_2$ concentration was adjusted by mixing an oxygen-depleted MEA solution with an oxygen-saturated buffer solution. The fractions of oxygen-saturated volumes were 1/11 (black), and 10/11 (cyan). On the left axis the absorbance ratio (absorbance at 665 nm versus absorbance at 610 nm) is given indicating a slight increase of the monomer versus dimer population during the reaction. On the right axis the absorbance at 605 nm is indicated.
Fig. S7: Oxygen exchange between the atmosphere and the observation volume (Erlenmeyer flask, volume of 265 ml). (A) Oxygen was monitored over time during constant nitrogen purging of the gas-liquid interface. Exponential oxygen depletion was observed with a rate constant of $(2.3\pm0.1)\times10^{-4}$ s$^{-1}$ due to diffusional transport of oxygen through the gas-liquid interface. Alternatively, the oxygen concentration was monitored after 10 min of purging the aqueous solution with nitrogen and exposing the interface to the standard atmosphere. Exponential oxygen intake from the surface was found to take place with similar rate constants of $(3.6\pm0.1)\times10^{-4}$ s$^{-1}$. (B) The influence of oxygen exchange through the gas-liquid interface was tested for oxygen depletion through oxidation of 50 mM MEA in an aqueous solution (pH 8.6). Oxygen was depleted to lower levels but with similar rate constants when the interface was constantly purged with nitrogen ($k=(8.7\pm0.2)\times10^{-4}$ s$^{-1}$, lower curve) as compared to depletion with an interface exposed to standard atmosphere ($k=(8.6\pm0.5)\times10^{-4}$ s$^{-1}$, upper curve).
**Fig. S8:** Oxygen depletion by the glucose oxidase/glucose/catalase system (GluOx) in aqueous solution. (A) O$_2$ concentration was measured over time using a Clark electrode and exponential decays were observed. The aqueous solution contained 0.01 U/ml GluOx (i.e., 0.01 U/ml glucose oxidase, 0.2 U/ml catalase; with 5% (w/v) glucose). (B) Characteristic rate constants extracted in aqueous solutions with 5% (w/v) glucose and a GluOx concentration as indicated. The time resolution is limited by the finite response time of the electrode which we estimated from this data and further experiments to be (0.086±0.004) s$^{-1}$. Below this rate the pseudo-first order rate constant scales linearly with GluOx concentration with a slope of (0.18±0.01) s$^{-1}$U$^{-1}$ml.
**Fig. S9:** Absorbance time traces of MB in aqueous solution at different $O_2$ concentrations. Data were recorded for the dark reaction in mixtures of 42 µM MB (pH 8.2) with 55 mM MEA at various $O_2$ concentrations. $O_2$ concentration was adjusted by mixing an oxygen-depleted MEA solution with an oxygen-saturated buffer solution. The fractions of oxygen-saturated volumes were 1/11 (black), 2/11 (red), 5/11 (green), 8/11 (blue), 10/11 (cyan).
**Fig. S10:** Super-resolution imaging of Cy5-labeled microtubules in HeLa cells by dSTORM. The data was recorded using an imaging buffer containing 100 mM MEA and 1 µM MB (pH 7.4). (A) Diffraction-limited wide-field fluorescence image showing a subpopulation of individual Cy5 molecules that blink in successive frames. In addition bright spots of constant fluorescence intensity (white circles) appear after 10 to 30 min of irradiation (~1 kW/cm² at 641 nm) that represent MB accumulations; (B) Super-resolved image reconstructed with rapidSTORM from a set of 4000 images (33 Hz frame rate). In areas around accumulated MB (white circles) no Cy5 localizations can be determined.