The study of protein recruitment to UV-induced DNA lesions can be distorted by photoconversion of DNA dyes like Hoechst or DAPI [version 1; referees: 1 approved]

Verena Hurst¹,², Susan M. Gasser¹,²

¹Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland
²University of Basel, Basel, Switzerland

Abstract
A common approach used to assess DNA repair factor binding in mammalian cells is to induce DNA damage with a UV laser and follow the movement of GFP-tagged proteins to the site of damage. Often these measurements are performed in the presence of the blue DNA intercalating dye Hoechst or DAPI, which is used to label nuclear DNA. A UV-induced switch of Hoechst and DAPI from a blue-light to a green-light emitter will give a false positive signal at the site of damage. Thus, photoconversion signals must be subtracted from the overall green-light emission to determine true recruitment. Here we demonstrate the photoconversion effect and suggest control experiments to exclude false-positive results.

Keywords
Photoconversion, Hoechst, DAPI, UV laser, DNA repair
Abbreviations
DAPI: 4', 6-diamidino-2-phenylindole; UV: ultraviolet light; U2OS: human bone osteosarcoma epithelial cells; YOYO-1: tetracationic homodimer of Oxazole Yellow; GFP: Green fluorescent protein; 53BP1: Tumor suppressor p53-binding protein 1; XRCC1: X-ray repair cross-complementing protein 1; FEN-1: Flap endonuclease 1; PARP-1: Poly (ADP-ribose) polymerase 1; Ku70: 5'-deoxyribose-5-phosphate lyase Ku70/X-ray repair cross-complementing protein 6, MDC1: Mediator of DNA damage checkpoint 1; SMARCA5: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5

Introduction
A variety of DNA binding dyes, such as DAPI, Hoechst, Vybrant DyeCycle Violet and YOYO-1, can change their optical properties upon exposure to light. Whereas the induction of YOYO-1 blinking is an intended change that is exploited for super resolution microscopy, the photoconversion of DAPI, Hoechst or Vybrant DyeCycle Violet during multicolor fluorescence microscopy is unexpected and can lead to false-positive signals.

Upon UV exposure or a low pH, the emission spectra of DAPI, Hoechst and Vybrant DyeCycle Violet shift from the blue to the green wavelength with detectable signals in the yellow and orange. This shift makes the signal indistinguishable from the emission of other standardly used fluorescent proteins such as GFP. An experimenter expecting the DNA dyes to emit in the blue can misinterpret the green signal as that arising from another probe in the sample. This risk has been raised previously, yet the artefact is rarely controlled for.

With respect to these findings, a microscopic setup like the one used to study the localization of repair proteins to a UV laser-induced zone of DNA damage can be particularly problematic. Very commonly, cell nuclei are stained with DAPI or Hoechst and a restricted part of the nucleus is exposed to a strong UV laser. The protein of interest is detected in the green channel thanks either to its fusion to GFP or else through an antibody labelled with a green light-emitting fluorophore. Unfortunately, photoconversion of the DNA dye is usually not checked. Here will illustrate the problem and suggest necessary controls.

Results
To study the recruitment of a potential DNA damage related protein, we made use of a previously established setup in which cell nuclei are sensitized with Hoechst, DNA damage is induced with a UV laser, and the recruitment of a protein of interest is measured over time by fluorescence microscopy. Unexpectedly, cells stained with Hoechst that did not express any GFP-tagged protein showed a similar increase in the green channel at the UV damage site, as cells expressing the GFP-tagged protein (Figure 1). The detected increase in signal was not due to protein recruitment to the damage site, since there was no GFP-tagged protein in the cell. Moreover, in cells expressing the GFP-tagged protein that were not stained with Hoechst, there was no increase in signal intensity at the UV damage site. This demonstrates conclusively that the increase in fluorescence in the green channel was a false-positive result. Raw images are available on figshare.

Discussion
We illustrate here that one should avoid exposing DAPI or Hoechst to a strong UV laser if one is imaging green light emitting probes such as GFP or a secondary antibody coupled to fluorescein/Alexa488. This is because photoconverted DAPI and Hoechst emit strongly in the same channel. As an alternative nuclear marker, we suggest employing a fluorescently tagged protein that localizes at the nuclear periphery and does not interfere with the experimental process.

Figure 1. Representative U2OS cell nucleus before and after UV-induced photoconversion of Hoechst.
We note that in addition to particular situations in which one induces damage, the photoconversion of DAPI can occur during standard dual color microscopy. To minimize artefacts one should be careful about the order in which dyes are observed, and visualize the green channel prior to exposing to short-wave light.

Methods
U2OS cells (a gift from Prof. Primo Leo Schaer, Department of Biomedicine, University of Basel) were incubated with 1.5 µg/ml Hoechst 33342 (Thermo Fisher Scientific, H1399) for at least 30 minutes prior to photoconversion. Photoconversion was induced with a VisiFRAP module (Visitron) mounted on the backport of the microscope and equipped with a 405 nm laser (Toptica, illumination power at the objective 12.8 mW). Confocal images were acquired with an Olympus IX81 microscope equipped with a PlanApo 100x/1.45 TIRFM oil objective, a CSU-X1 scan-head (Yokogawa), an Evolve 512 EMCCD camera (Photometrics), a 491nm laser (Cobolt Calypso 100), a 488/568 dichroic (Semrock Di01-T488/568-13x15x0.5), a band-pass 525/40 emission filter (Semrock FF01-525/40-25) and controlled with the VisiView Software (Visitron). Images in Figure 1 show maximum intensity projections of stacks covering 7 µm.

References


Data availability
Raw images of the stacks taken during this study are available on figshare. DOI: https://doi.org/10.6084/m9.figshare.7583960.

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

Media
The three available avi files, C1 green, C2 blue and composite, represent a time series of maximum intensity projections showing the UV-induced emission change of the DNA intercalating dye Hoechst from the blue to the green region of the visible spectrum. Under live conditions, a Hoechst-stained cell nucleus was irradiated with 405 nm UV laser light along a predefined pattern (#). A time series of image stacks was acquired (25 equally spaced time points over 65s, stacks covering 7-µm sample depth) in two channels (C1 “green”: 491/525 nm, C2 “blue”: 405/450 nm). DOI: https://doi.org/10.6084/m9.figshare.7583960.

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This short report by Hurst and Gasser exposes an important experimental detail that is often, but not always, overlooked: Hoechst-sensitized cells irradiated with UV light produce a signal in the same region of the spectrum as GFP. Consequently, without the proper controls, it may lead to false positives. That is, that it may be falsely concluded that GFP-tagged proteins are recruited to laser-induced damage.

In the discussion, they point out potential controls to that would prevent an experimenter to make this mistake. Specifically, they propose leaving out Hoechst or DAPI altogether, using the appropriate control cells that do not express the GFP-tagged protein of interest, using another nuclear marker, such as a protein localizing to the nuclear periphery, or using a different fluorescent protein. Those are all good ways of getting around the reported problem. I would also add the use of a UVC source (Dabin et al., 2018).

References

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** DNA repair, chromatin, expanded trinucleotide repeat disorders

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