



Photoconversion of DAPI and Hoechst dyes to green and red-emitting forms after exposure to UV excitation

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Abstract

The fluorescent dye 4'-6-Diamidino-2-phenylindole (DAPI) is frequently used in fluorescence microscopy as a chromosome and nuclear stain because of its high specificity for DNA. Normally, DAPI bound to DNA is maximally excited by ultraviolet (UV) light at 358 nm, and emits maximally in the blue range, at 461 nm. Hoechst dyes 33258 and 33342 have similar excitation and emission spectra and are also used to stain nuclei and chromosomes. It has been reported that exposure to UV can convert DAPI and Hoechst dyes to forms that are excited by blue light and emit green fluorescence, potentially confusing the interpretation of experiments that use more than one fluorochrome. The work reported here shows that these dyes can also be converted to forms that are excited by green light and emit red fluorescence. This was observed both in whole tissues and in mitotic chromosome spreads, and could be seen with less than 10-s exposure to UV. In most cases, the red form of fluorescence was more intense than the green form. Therefore, appropriate care should be exercised when examining tissues, capturing images, or interpreting images in experiments that use these dyes in combination with other fluorochromes.

Keywords DAPI · H33258 · H33342 · Fluorescence · Photoconversion · Wavelength · Chromosome · *Drosophila*

Introduction

The fluorescent DNA-binding dyes DAPI, Hoechst 33258, and Hoechst 33342 have been widely used to stain nuclei and chromosomes for fluorescence microscopy. They are excitable by light in the UV region and emit broadly in the blue region. They may be used in combination with fluorochromes that are excited by longer wavelengths and that emit in the green or red regions of the spectrum to identify distinct biological molecules and their relative localizations within a cell or tissue. The ability of specific excitation and emission filter combinations to separate signals is critical for the identification and localization of each fluorophore. If fluorescent signals overlap, it can create significant difficulty for determination of the true locations of each associated molecule. For this reason, double or triple labeling experiments will make use of excitation/emission combinations that exhibit substantial

spectral separation, such as UV excitation/blue emission, blue excitation/green emission, and green excitation/red emission combinations. Even with these well-separated colors, crosstalk between channels may still occur and cause problems for interpretation. Spectral imaging can provide a solution to overlapping emission spectra (Malik et al. 1996; Hiraoka et al. 2002), but if fluorophores show unexpected excitation and emission spectra, it becomes considerably more challenging to unmix the signals and assign them to the appropriate molecules.

It has been reported that exposure of DAPI or Hoechst dyes to the UV excitation, that is used for observing nuclear staining, can convert them to forms that are excited by blue light and emit green fluorescence (Piterburg et al. 2012; Jež et al. 2012; Żurek-Biesiada et al. 2013). This is obviously a problem if the protein of interest is also marked by green fluorescence, and may lead to mistaken localization of such a molecule to the nucleus. This report shows that photoconversion of these dyes can also extend to green excitation/red emission, which in most cases was more intense than the form with blue excitation/green emission. The photoconverted forms may lead to misinterpretation of experiments even when using a fluorophore that is thought to be widely separated from the normal excitation/emission spectrum of these dyes.

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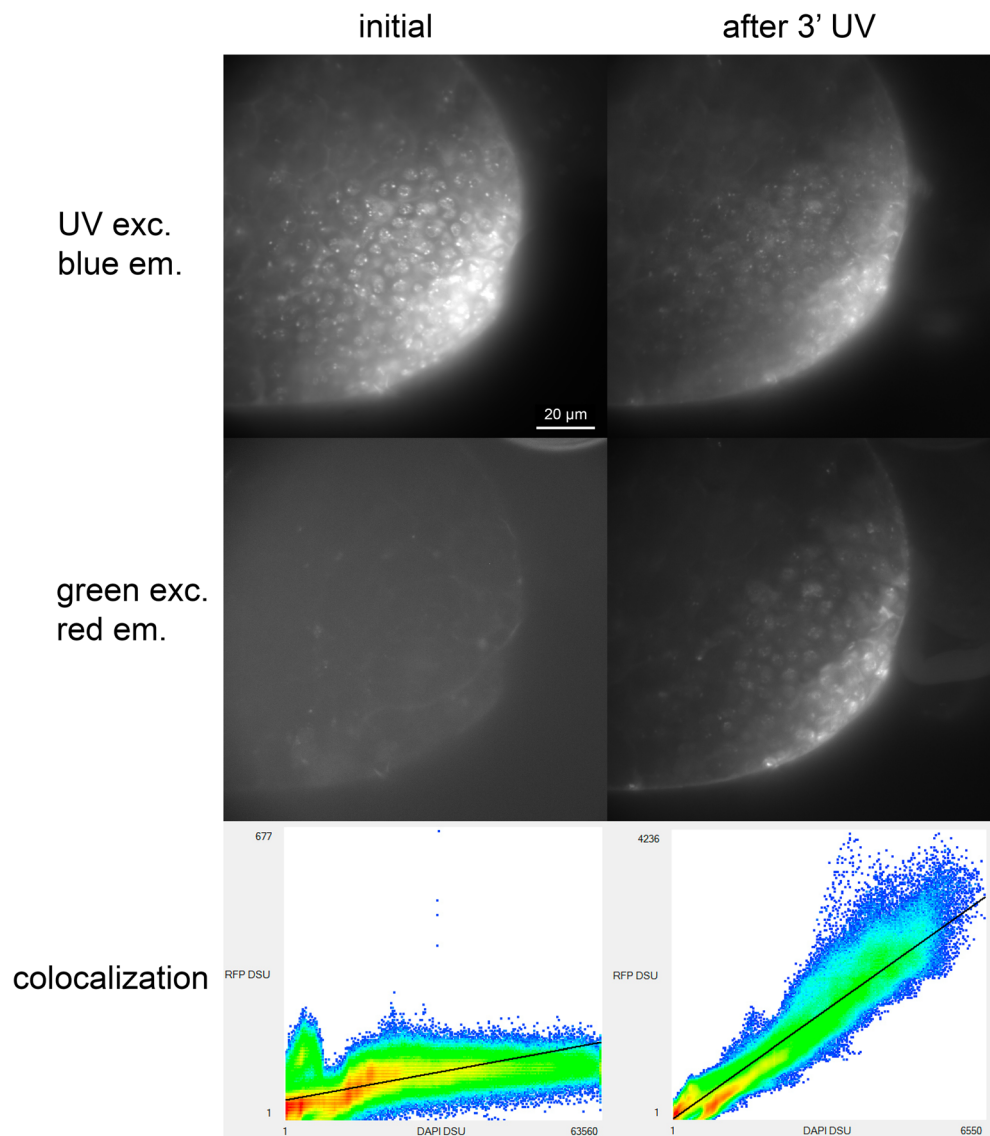
Materials and methods

Testes and accessory glands of *Drosophila melanogaster* were dissected from young males (0–3 days old) in $\times 1$ phosphate-buffered saline (PBS), fixed for 30' in $\times 1$ PBS with 4% paraformaldehyde, then stained in $\times 1$ PBS with DAPI, Hoechst 33258 or Hoechst 33342 for 30 min. After destaining in $\times 1$ PBS for 10' or more, testes were mounted in Vectashield (Vector Laboratories), Slowfade Gold (Invitrogen), or $\times 1$ PBS. Cover slip edges were sealed with nail polish. The testes were examined with an Olympus DSU spinning disc microscope using a PlanApo N $\times 60/1.42$ oil objective, 100 W Hg lamp excitation, and equipped with a Hamamatsu Orca Flash4 v2 camera capturing 16 bit images. Filter sets for UV/blue, blue/green, and green/red fluorescence were Olympus part numbers OSF-005, U-MHQ650, and U-MHQ66, respectively. Images were acquired without a neutral density filter in the

excitation path, unless otherwise specified, for times indicated in the figure legends. For timed UV exposures, the DSU spinning disc was removed from the light path, and a neutral density filter was not used. The system was controlled by Slidebook6 software (Intelligent Imaging Innovations), which was also used for analysis. After export from Slidebook, images were assembled in Photoshop with no further image manipulation.

For metaphase chromosome squashes, *Drosophila* larval brains were dissected in $1\times$ PBS, swelled for 10 min. in 0.5% sodium citrate, fixed briefly in 11:11:1 methanol: acetic acid: water, placed in a drop of 45% acetic acid on a siliconized cover slip which was picked up with a microscope slide, then squashed, and frozen on dry ice. The cover slip was removed with a razor blade while the slide was still frozen. The slide was then air dried and mounted with Vectashield containing DAPI, Hoechst 33258 or Hoechst 33342.

Fig. 1 Photoconversion of DAPI to a form with red fluorescence. A *Drosophila* testis was stained with 2 $\mu\text{g}/\text{ml}$ DAPI and mounted in Vectashield. Initially, a z stack was captured for UV excitation/blue emission at 50-ms exposure; green/red at 500 ms. The testis was then exposed to UV for 3 min, and the z series acquisition was repeated. The images shown here are maximum projections of a series of six 0.2- μm z sections. Each image was individually optimized for display by scaling to the minimum and maximum intensity values of the original 16 bit image with gamma = 1.0. The displayed images were then exported from Slidebook as 8 bit gray scale images. The bottom row shows the colocalization of blue (x axis) and red (y axis) intensities at each time point. The points within the scatterplot are colored blue to red where dark blue represents single pixel events and red represents many pixels with identical intensity values. Axes are indicated as RFP (green excitation/red emission) and DAPI (UV/blue)



Examination was carried out using a U Plan Apochromat $\times 100/1.40$ objective as above, except that the disc scanning unit was not engaged: observations were in normal wide-field fluorescent mode.

At least two, and in most cases three or more samples were examined for each experiment reported in the figures. Results for different samples within an experiment were very similar, and typical examples were chosen for the figures.

Results

DAPI photoconversion Testes of the fruit fly, *Drosophila melanogaster*, were dissected from young adults, fixed with paraformaldehyde, stained with DAPI, and examined with an Olympus DSU disc scanning microscope with filters designed for DAPI, GFP, and RFP. In initial examinations, testes from

males expressing a *Drosophila* telomere protein fused to mCherry were examined for evidence of mCherry fluorescence in nuclei. Careful inspection failed to reveal any visible mCherry fluorescence. However, after repeatedly switching to the DAPI filter set to identify nuclei, and then back to the RFP filter set to look for mCherry fluorescence, red fluorescence could be detected, by eye or with the camera. This red fluorescence was suspect because it gained in intensity after each examination with the DAPI filter set. Furthermore, when both channels were captured and overlaid, the brightest DAPI spots, corresponding to centric heterochromatin and the Y chromosome, appeared to correspond fairly precisely to the brightest spots of red fluorescence. Although the mCherry signal was expected to be nuclear, it was expected at telomeres, not centromeres.

To determine whether this red fluorescence might be an artifact of conversion of DAPI into a red-emitting form, we

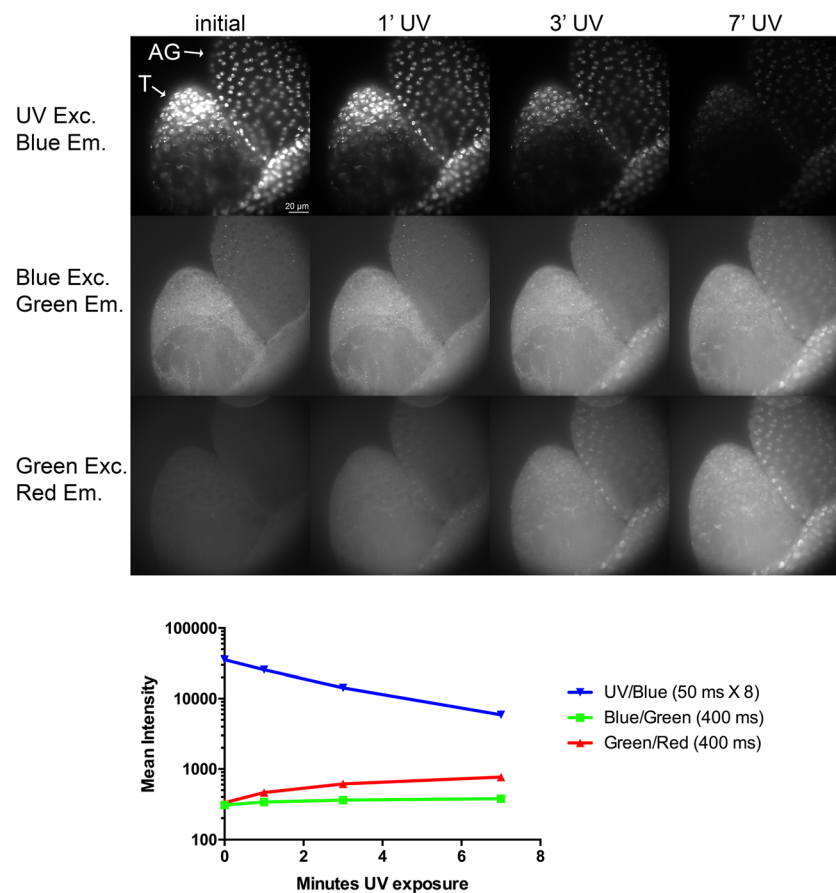
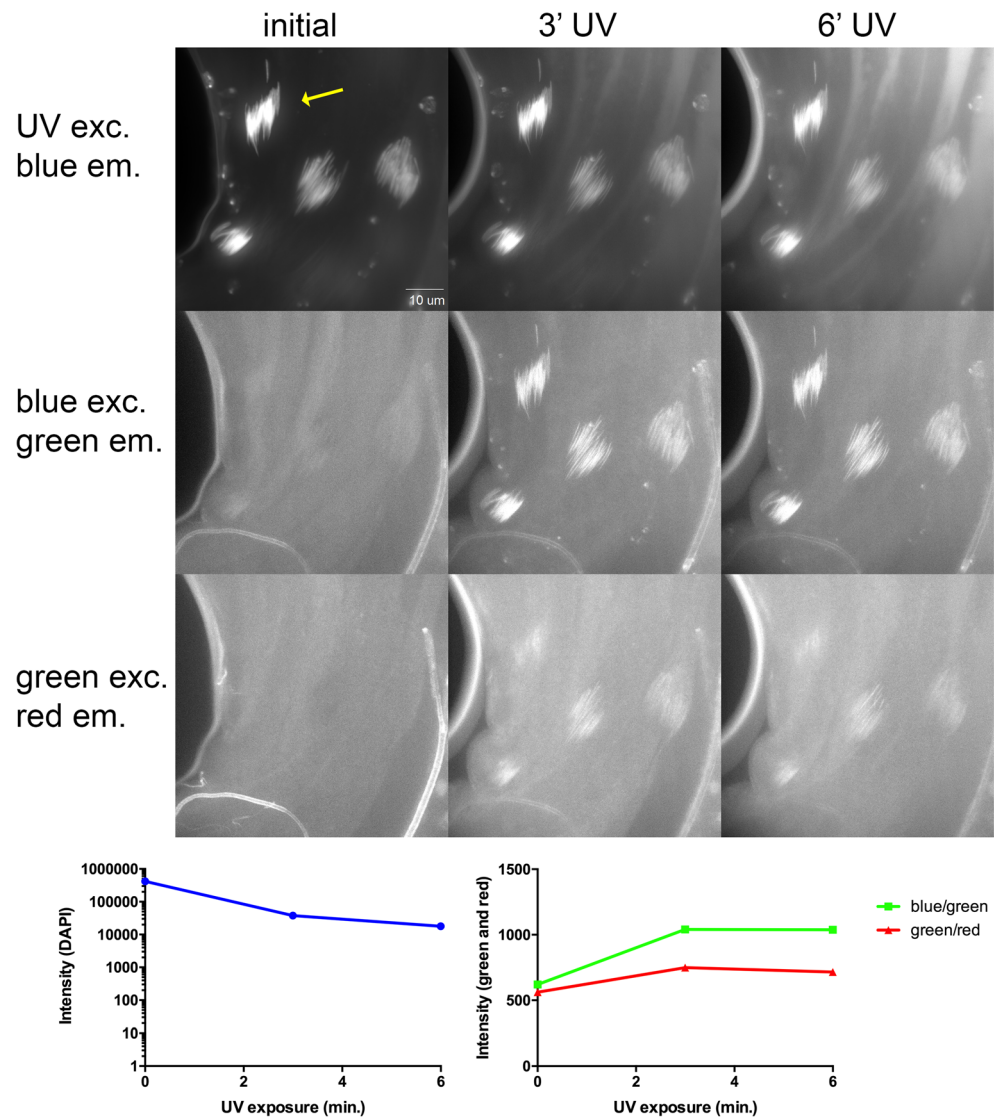


Fig. 2 Photoconversion of DAPI to green and red fluorescent forms. A *Drosophila* testis (T) and accessory gland (AG) were stained with 0.4 $\mu\text{g}/\text{ml}$ DAPI and with an AlexaFluor488 antibody against a *Drosophila* telomere protein and mounted in Vectashield. Single z section images for each channel were captured at the initial time point and after 1-, 3-, and 7-min exposure to UV (cumulative values). Exposure lengths for image capture were 50 ms for the UV/blue channel and 400 ms for blue/green and green/red channels. In this figure, images were scaled to reflect the change in intensity over time. The minimum and

maximum intensity values were determined across the entire time course for each channel. The images from each time point within a channel were adjusted to the same scale, representing 0–99.5% of the full range of intensity values captured over the time course, with gamma = 1.0. Each image was then exported from Slidebook as an 8 bit gray scale image. The change in mean intensity over time across the entire image is plotted in the bottom panel. Values for the UV/blue channel were multiplied by 8 to compensate for their shorter exposure

Fig. 3 Photoconversion of fluorescence in condensed sperm heads. After staining with 2 $\mu\text{g}/\text{ml}$ DAPI and mounting in Slowfade Gold, a *Drosophila* testis was visually scanned briefly in UV/blue with a neutral density filter of 1.5% transmittance in the excitation path to identify a region that had several mature sperm head bundles. A z series was acquired for each channel sequentially, with exposure lengths of 50 ms (UV exc.), 200 ms (blue), and 500 ms (green). The images shown here are maximum projections of those series. Image captures were repeated after 3- and 6-min cumulative exposure to UV. Display of each image was scaled separately to 0–99.5% intensity values of that image, and then exported from Slidebook as an 8 bit gray scale image. To quantitate the change in intensity over time, the UV/blue channel was used to create a mask over the sperm head bundle indicated by an arrow in the initial time point. The graphs below show the change for that sperm head bundle (UV/blue values multiplied by 10; blue/green values multiplied by 2.5, to account for different lengths of exposure). The UV/blue channel is plotted separately because of the extreme difference in scales



examined DAPI-stained testes from *yellow white* genotype males that did not express fluorescent proteins. Initially, nuclei stained with DAPI were readily visible in the UV/blue channel, but red fluorescence was very low. After 3-min exposure to UV excitation, the signal in the UV/blue channel had bleached considerably, although it was still quite visible. At this point, fluorescence with green excitation/red emission was now easily visible and corresponded nearly precisely with the DAPI signal (Fig. 1). As the normal UV-excited blue emission was bleached, there was a corresponding rise in green-excited red emission of DAPI-stained DNA. It is clear that DAPI-stained DNA can be photoconverted into a red fluorescent form.

We carried out two more experiments with DAPI-stained testes to examine the bleaching of DAPI fluorescence and the rise of both green and red fluorescence over time. In the first, we

examined a fixed testis and neighboring accessory gland that were stained with DAPI. In this instance, the testis was also stained with an antibody against a telomeric protein, with the secondary antibody coupled to AlexaFluor488 (Fig. 2). When using the blue excitation/green emission filter combination, the antibody staining was visible as bright puncta within nuclei (not readily seen at this magnification), as expected for a telomeric protein, and a dimmer cytoplasmic background. Although the bright puncta were located within nuclei, they did not in any way resemble the pattern of DAPI staining. Red fluorescence was very low in the initial examination. After 1-min exposure to UV, the UV/blue pattern of nuclear staining was noticeably bleached, and red, and to a lesser extent green, fluorescence began to appear in the same pattern. After longer exposure to UV, blue fluorescence from UV excitation continued to fade, and both green and red fluorescence continued to increase. After 7'

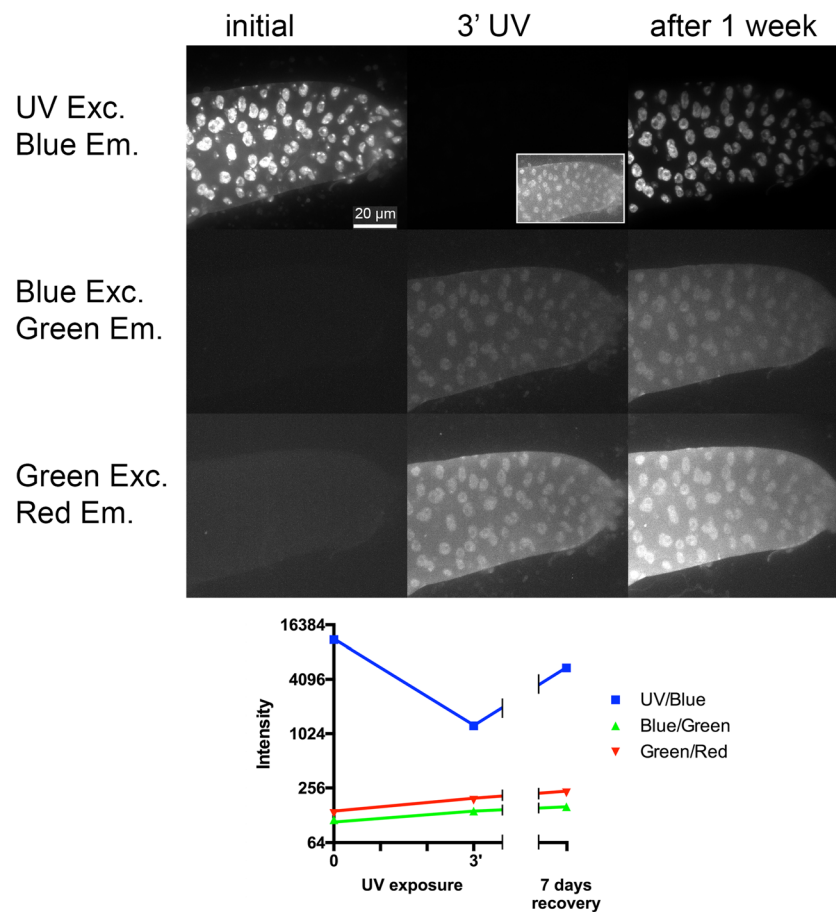


Fig. 4 Bleaching, photoconversion, and recovery after DAPI staining. A *Drosophila* accessory gland was stained with 2 $\mu\text{g}/\text{ml}$ of DAPI, mounted in Vectashield, and visually scanned briefly in UV/blue with a neutral density filter of 1.5% transmittance in the excitation path to identify DAPI-stained nuclei. A z series was acquired for each channel sequentially, with exposure lengths of 500 ms (green or blue excitation), and 50 or 100 ms (UV excitation) using the 1.5% transmittance neutral density filter. After the initial capture, the tissue was exposed to UV for 3 min, and the z series acquisition was repeated. After 7 days of storage in the dark at room temperature, another z series

was acquired. The images shown here are maximum projections of those series. The images from each time point within a channel were adjusted to the same scale, representing 0–99.5% of the full range of intensity values captured in that channel over the time course, with $\gamma = 1.0$. Each image was then exported from Slidebook as an 8 bit gray scale image. The change in mean intensity over the entire tissue is plotted at the bottom. UV/blue values were multiplied by 10 or 5 to compensate for the shorter exposures. Inset figure for UV/blue at 3 min (white box) is an image scaled to the 0–99.5% range of that image showing that, even though it is bleached, stained nuclei are still visible

exposure to UV, both red and green fluorescence strongly resembled the initial pattern of UV/blue fluorescence, and although weaker than the blue fluorescence, even after bleaching, it was easily observed. The green fluorescent form, which has been previously reported as a product of DAPI photoconversion, was weaker than the red fluorescent form in this instance.

Although the intensity graph at the bottom of Fig. 2 shows little rise in the intensity of green fluorescence with time of UV exposure, this is not because photoconversion to the green form did not occur. Instead, this is likely because the AlexaFluor488 was bleaching at the same time that the photoconverted DAPI fluorescence was appearing, and these factors cancelled each other when intensity across the image

as a whole is measured. Direct observation of the tissues clearly showed a change in pattern, with bright puncta decreasing, and a pattern that reproduces the DAPI staining rising.

In a second experiment, we examined maturing sperm heads in the testes of yellow white males that had been fixed and stained with DAPI (Fig. 3). In the *Drosophila melanogaster* testis, 64 haploid spermatids undergo coordinate development, and the needle-like sperm heads are tightly clustered. Initially, the UV/blue staining was very bright, but there was very little fluorescence in the green or red channels. After 3' and 6' exposure to UV, sperm heads were clearly visible in green, and to a lesser degree red, fluorescence. In contrast to the previous experiment, photoconversion to green

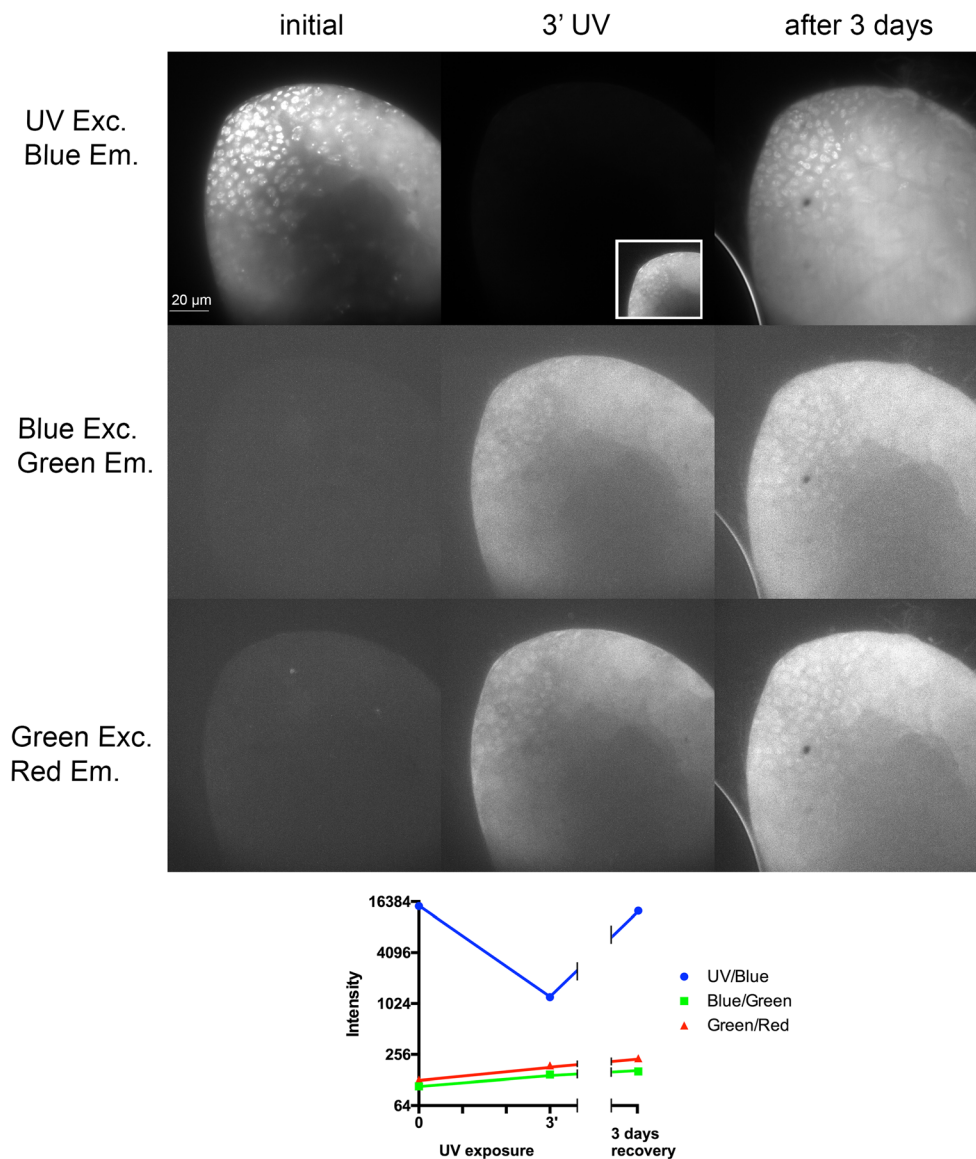


Fig. 5 Bleaching, photoconversion, and recovery after Hoechst 33258 staining. A *Drosophila* testis was stained with 50 $\mu\text{g}/\text{ml}$ of Hoechst 33258. After staining and mounting in Vectashield, a z series was acquired for each channel sequentially, with exposure lengths of 500 ms (green or blue excitation) or 100 ms (UV excitation) using the 1.5% transmittance neutral density filter. After initial capture, the tissue was exposed to UV for 3 min and the z series acquisition was repeated. After 3 days storage of in the dark at room temperature, the z series acquisition was repeated again. The images shown here are maximum projections of those series. The images from each time point within a

channel were adjusted to the same scale, representing 0–99.5% of the full range of intensity values captured in that channel over the time course, with $\gamma = 1.0$. Each image was then exported from Slidebook as an 8 bit gray scale image. Mean intensity was quantified over approximately the distal 1/4 of the portion of the testis imaged here, and is plotted at the bottom. UV/blue values were multiplied by 5 to compensate for the shorter exposures. Inset figure for UV/blue at 3 min (white box) is an image scaled to the 0–99.5% range of that image showing that nuclei are still visible

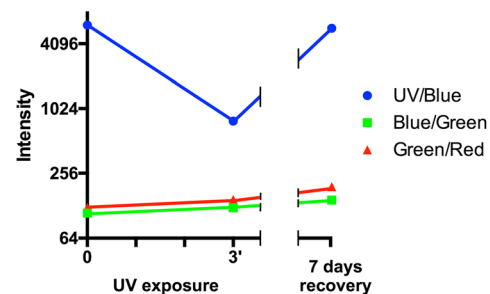
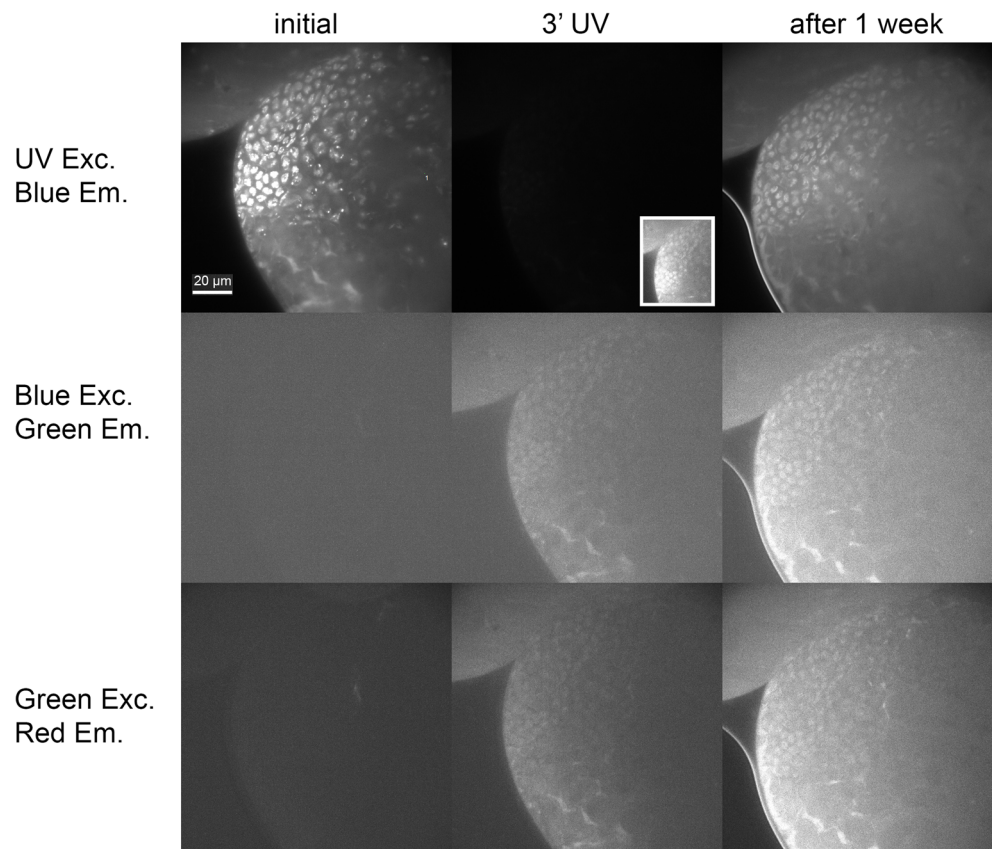
fluorescence was stronger in this case. Similar results were obtained when examining sperm heads within testes mounted in $1\times$ PBS (not shown).

Even at the initial time point, there was evidence of a small degree of fluorescence in the blue/green channel. This is probably a result of the fact that a certain amount of time was spent examining the testis in the UV/blue channel to find an appropriate region for the experiment, and then setting up the

capture of a z series. Accordingly, the testis had been exposed to a small amount of UV before the initial image acquisition.

Bleaching; photoconversion; and recovery of DAPI, Hoechst 33258, and Hoechst 33342 fluorescence Zurek-Biesiada et al. (2013) reported that bleaching and photoconversion of DAPI or H33258 fluorescence were reversed over time. To test whether this would be true in our hands, we examined fixed

Fig. 6 Bleaching, photoconversion, and recovery after Hoechst 33342 staining. A *Drosophila* testis was stained with 50 $\mu\text{g}/\text{ml}$ of Hoechst 33342. After staining and mounting in Vectashield, the testis was imaged as described in Fig. 5, except storage was for 7 days instead of 3 days. Captured images were treated and quantified as described in Fig. 5



and stained testes or accessory glands after 3-min exposure to UV, and again after several days of storage in the dark at room temperature. As seen in Fig. 4, the DAPI-stained nuclei within the accessory gland were strongly bleached in the UV/blue channel after a 3-min exposure to UV. Concomitantly, nuclei became visible in blue/green and green/red channels. After 1 week of storage in the dark at room temperature, we imaged the same section of the accessory gland. We found that the DAPI signal in the UV excitation/blue emission range had returned, though not quite to its original level. However, the photoconverted fluorescence that clearly resembles the initial UV/blue nuclear staining persisted and increased in intensity in both blue/green and green/red channels.

Figures 5 and 6 show equivalent experiments with the Hoechst dyes. We found that these dyes were also photoconverted to forms that are easily visible in both the blue/

green and green/red channels. As was the case with DAPI, the UV/blue fluorescence recovers after several days of storage in the dark, and the photoconverted forms of the Hoechst dyes persisted and increased in intensity during storage.

Examination of metaphase chromosomes Finally, we examined metaphase chromosomes prepared from *Drosophila* larval brains that were stained with these dyes to see how their fluorescence and morphology might be affected by exposure to UV. With DAPI, photoconversion to both the green and red-emitting forms was detected after < 10-s exposure to UV (Fig. 7). Although the DAPI signal in the UV/blue channel was always much brighter than the photoconverted forms, the altered forms were still readily visible, by eye and camera, and their pattern of fluorescence on chromosomes closely matched the UV/blue signal.

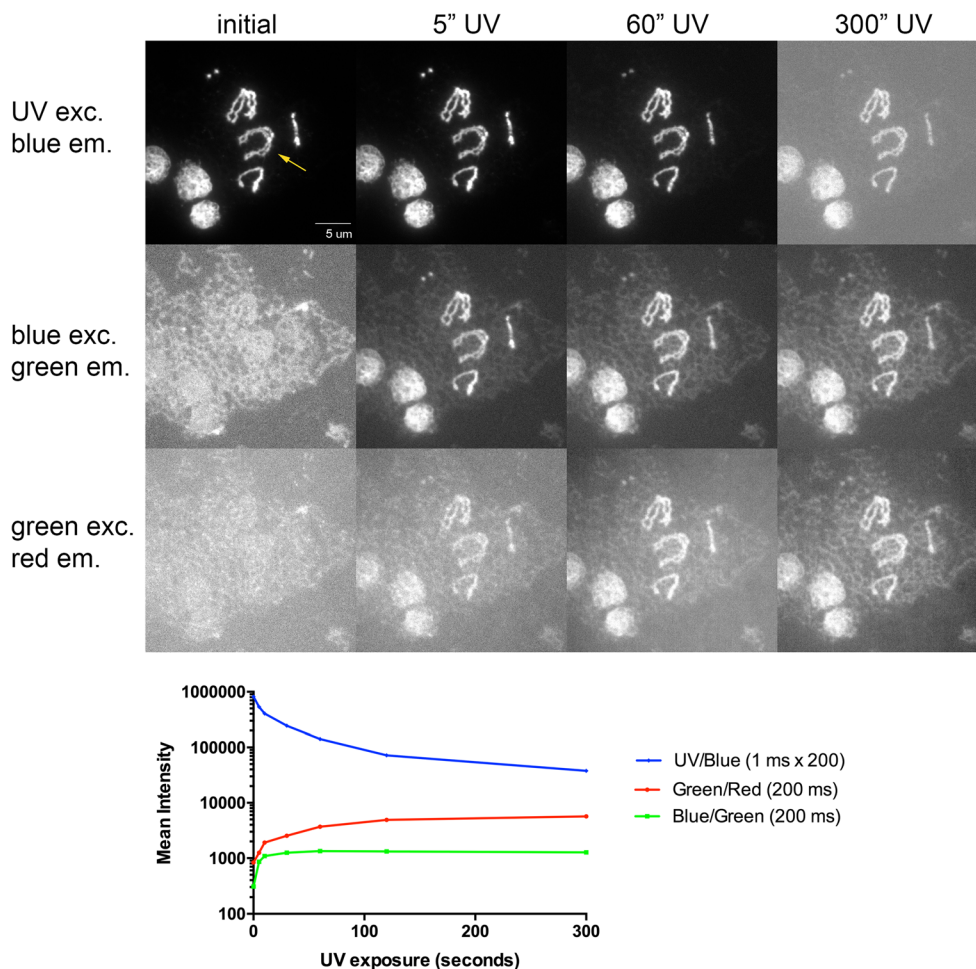


Fig. 7 Photoconversion of fluorescence in mitotic chromosomes stained with DAPI. Chromosomes from *Drosophila* larval brain mitoses were mounted in Vectashield with 10 μ M DAPI. Suitable chromosome spreads were identified with the UV/blue channel and a 1.5% transmittance neutral density filter in the excitation light path. After quickly identifying and focusing (estimated time 2–4 s), the shutter was closed and captures of all three channels were initiated. Exposure length was 1 ms for the UV/blue channel and 200 ms for the blue/green and green/red channels. After the initial capture, chromosomes were exposed

to UV for 5, 10, 30, 60, 120, and 300 s (cumulative exposure lengths), and images reacquired after each interval. A subset of those images is shown here. The display of each image was scaled to the 0–99.5% intensity interval for that image, gamma=1.0, and exported as an 8 bit gray scale image from Slidebook. A mask was generated in the UV/blue channel for the chromosome pair indicated by the arrow in the first image, and mean intensity over that chromosome pair was determined in each channel and for each time point. The change in intensity with UV exposure is graphed at the bottom

The results with the Hoechst dyes were significantly different. Although UV exposure of both forms of Hoechst resulted in photoconversion, only Hoechst 33258 resulted in photoconversion that allowed visualization of chromosomes (Fig. 8). Even then, chromosome morphology was quite poor in both the blue/green and green/red channels, but of the two, was better in the green/red channel. With longer UV exposures, the morphology of chromosomes in the photoconverted channels became worse, and they were not easily recognized. This correlates with a slight bleaching of these channels after their initial rise in intensity.

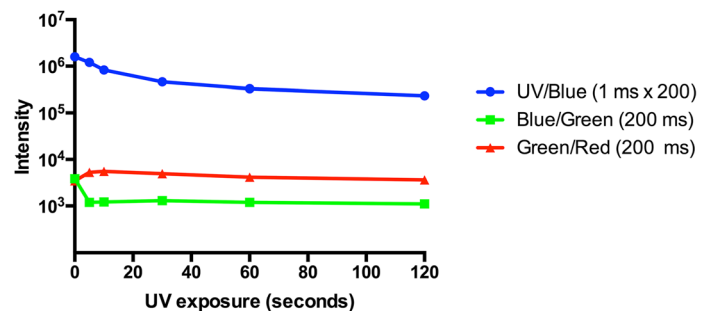
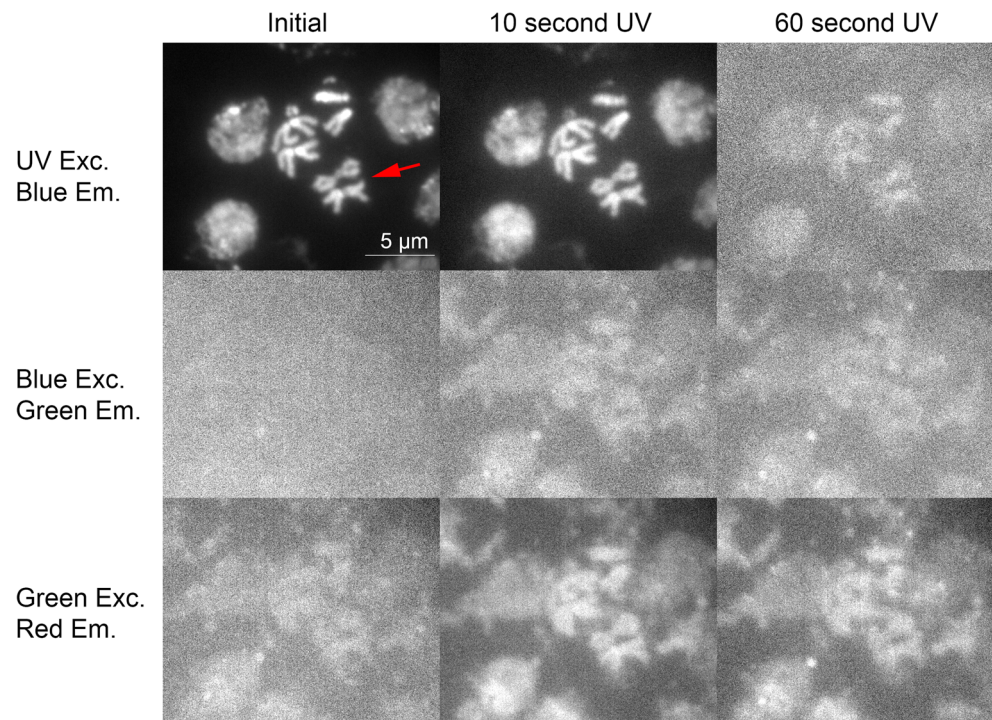
When chromosomes stained with Hoechst 33342 were exposed to UV, they bleached very rapidly. Increased intensity of fluorescence in the blue/green and green/red

channels was detected at the location of the chromosomes after UV exposure, but chromosomes were not recognizable (Fig. 9).

Discussion

Our results show that after exposure to UV, DAPI, Hoechst 33258, and Hoechst 33342 are all converted to forms that are excited by green light and emit red fluorescence. In the case of DAPI, at least, this was true regardless of mounting medium or DAPI concentration. This adds to previous reports which showed these dyes are photoconverted to blue-excited green-emitting forms. In at least some cases, very little exposure to UV is needed to produce the converted

Fig. 8 Photoconversion of fluorescence in mitotic chromosomes stained with Hoechst 33258. Chromosomes from *Drosophila* larval brain mitoses were mounted in Vectashield with 50 $\mu\text{g}/\text{ml}$ of Hoechst 33258. Chromosome spreads were identified and imaged as described in Fig. 7, except that cumulative UV exposure only extended to 120 s. A mask was generated over the chromosome pair indicated by the red arrow in the first image, and mean intensity over that chromosome pair was determined in each channel and for each time point. The change in intensity with UV exposure is graphed at the bottom



forms. Photoconversion of DAPI or H33258-stained mitotic chromosomes was observed after less than 10-s exposure to UV.

In all of our experiments but one, the green/red form was brighter than the blue/green form. The precise degree of photoconversion to the green or red form may partly depend on mounting medium; since in the single case where blue/green fluorescence was greater (Fig. 3), the mounting medium was different than in the other figures. This was not systematically investigated. It has been reported that glycerol in the mounting medium exacerbates the problem of photoconversion, indicating that it can be influenced by composition of the mountant (Jež et al. 2012).

With all dyes, the expected UV/blue fluorescence was always more intense than the photoconverted forms. Nevertheless, the photoconverted forms were easily detected, albeit with much higher relative background. In all cases, DAPI staining produced a cleaner image of nuclei

and chromosomes than the Hoechst dyes and the converted forms of DAPI more precisely duplicated the pattern of DAPI staining in the UV/blue channel.

Although it was reported that Hoechst 33342 had lower photoconversion than DAPI or Hoechst 33258 (Jež et al. 2012; Żurek-Biesiada et al. 2013), and might be preferred in experiments where photoconversion may cause a problem, we did not find that to be the case in our experiments. For instance, when comparing Figs. 4, 5, and 6, the differences in channel intensities after 3-min exposure to UV were very similar between dyes (Table 1). Furthermore, both Hoechst dyes bleached more rapidly than DAPI, which is especially noticeable with metaphase chromosomes (Figs. 7, 8, and 9), with H33342 bleaching most rapidly accompanied by the loss of distinctive chromosome morphology. The rapid bleaching and high background seen with H33342 argue against its use as a substitute for DAPI.

Fig. 9 Photoconversion of fluorescence in mitotic chromosomes stained with Hoechst 33342. Chromosomes from *Drosophila* larval brain mitoses were mounted in Vectashield with 50 $\mu\text{g}/\text{ml}$ of Hoechst 33342. Chromosome spreads were identified and imaged as described in Fig. 7, except that cumulative UV exposure only extended to 120 s. A mask was generated over the chromosome indicated by the red arrow in the first image, and mean intensity over that chromosome was determined in each channel and for each time point. The change in intensity with UV exposure is graphed at the bottom

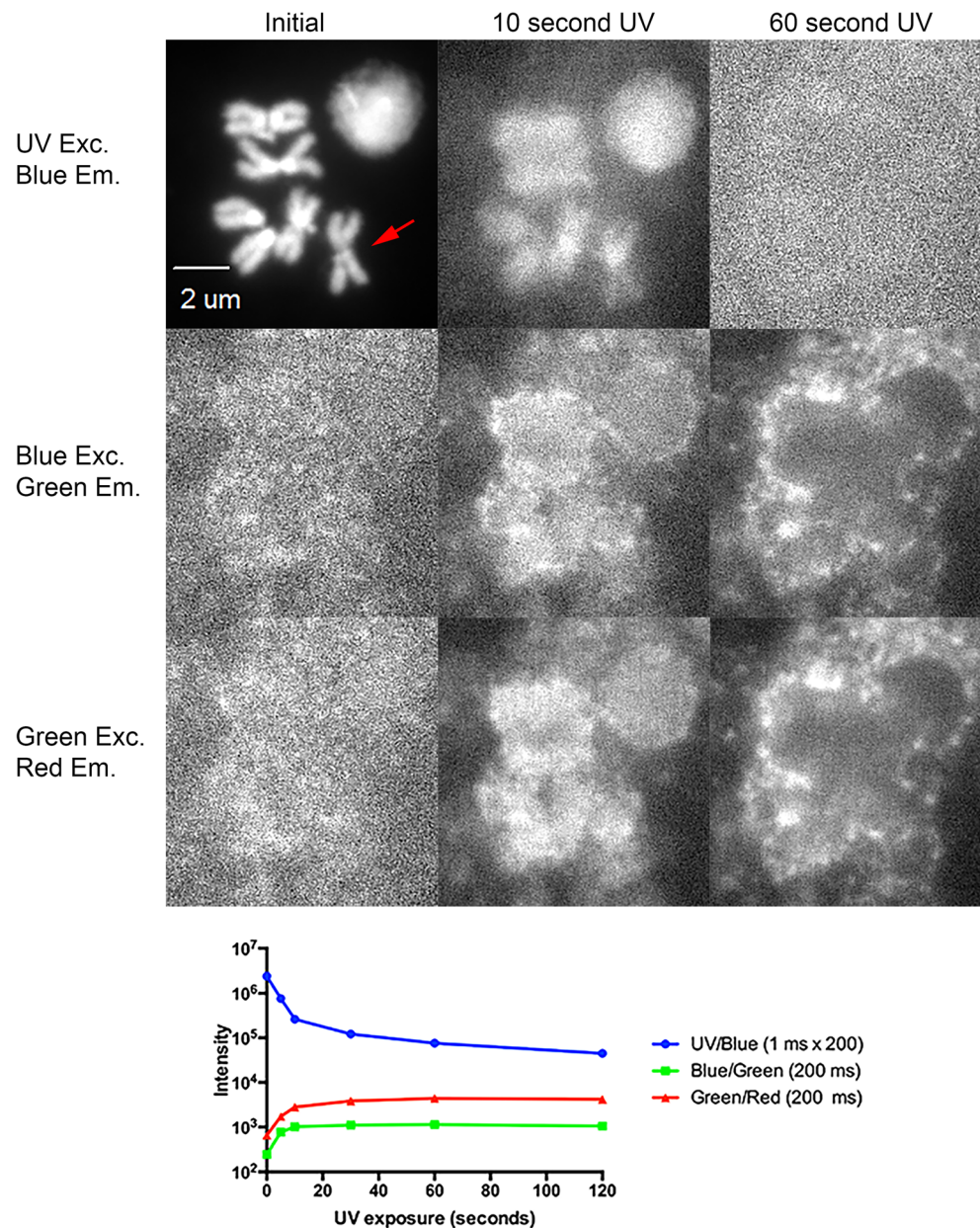


Table 1 Relative fluorescence of each channel after 3-min exposure to UV. The mean intensity over the stained tissue in each channel is shown here. In all cases, the blue/green channel showed the lowest intensity and the remaining channels were compared to this channel. Relative intensities are indicated below the intensity values

	UV/blue	Blue/green	Green/red
DAPI (Fig. 4)	1250 ×8.8	142 ×1	187 ×1.3
H33258 (Fig. 5)	1215 ×8.2	148 ×1	190 ×1.3
H33342 (Fig. 6)	785 ×6.4	122 ×1	146 ×1.2

These results, along with previous findings by others, indicate that appropriate care should be taken when performing experiments with multiple fluorochromes that include DAPI or a Hoechst dye as a nuclear stain. Green or red fluorescence that mimics the pattern of DAPI and Hoechst fluorescence should be considered suspect without appropriate controls, or verification using other DNA dyes, such as propidium iodide or TOPRO3, to identify DNA and nuclei.

Clearly, photoconversion of DAPI is not always a problem and DAPI is often used successfully in combination with other fluorochromes. Previous authors recommended that exposure to UV should be as brief as possible, and sequential image capture should be arranged so that longer wavelengths are

captured first. When following these principles, we readily imaged fluorescence of AlexaFluor488 at telomeres without interference from photoconverted DAPI (e.g., see the initial time point in Fig. 2). Nonetheless, investigators should be aware of the potential for problems that might arise from the photoconverted forms of DAPI and plan image capture accordingly.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or vertebrate animals performed by any of the authors.

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