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Evaluation of Eight Mounting Media and Acrylic Microscope Slides for Hair and Fiber Microscopy

ABSTRACT

At the FBI Laboratory, hair and fibers are often mounted in Permount™ for microscopical examination. In some situations, the fiber color is further analyzed often via ultraviolet-visible (UV-VIS, 200 nm – 800 nm) microspectrophotometry (MSP). A limiting factor during UV-VIS MSP is that current sample preparation method using both Permount™ and glass (i.e., slide and coverslip) is known to exhibit high absorbance in the UV spectral range below 320 nm. As such, a suitable alternative mounting medium and/or material (i.e., slide and coverslip) for hair and fiber slide preparation is desirable. Eight commercially available mounting media and an alternative slide option were identified for further evaluation through microscopist surveys, vendor inquiries, and reviews of scientific literature. The mounting media were evaluated for color, setting time, media autofluorescence, hair and fiber sample autofluorescence suppression or enhancement, and mounting media absorption in the ultraviolet range. Additionally, color discrimination of fibers embedded in these mounting media were characterized via MSP to determine which media offer advantages in the UV region. Multiple candidates performed well in one or more evaluation. In summary, glycerol-based mounting media provided several advantages in terms of use, transparency, and lack of autofluorescence. However, the acrylic microscope slide preparation did not confer a meaningful advantage over glass slide preparation.

Keywords: Fiber Analysis, Light Microscopy, Microspectrophotometry, Mounting Medium

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INTRODUCTION

Currently, Permount™ is being used by several forensic laboratories as a semi-permanent mounting medium for hair and fiber samples, mainly because of its ideal refractive index and of its performance as a matrix for long-term storage [1-4]. When qualitatively assessing hairs and fibers, examiners benefit from the refractive index of Permount™ (RI = 1.51), which allows for the evaluation of the microscopical characteristics in hairs and fibers, and evaluation of Becke lines in fibers. At the FBI Laboratory, hair and fiber samples are mounted in Permount™ on a glass microscope slide and are covered with a glass coverslip for initial examinations, which may include feature comparison, polarized light microscopy, fluorescence microscopy, and microspectrophotometry in the visible range (VIS-MSP). In certain circumstances, when information in the VIS-MSP is limited, fiber samples may be further analyzed in the ultraviolet (UV, 200 nm – 380 nm) region. However, for UV-MSP to be performed, the glass coverslip must be removed, and the fiber samples must be extracted from the mounting medium, Permount™. The samples are then temporarily remounted in glycerol on quartz microscope slides and are covered using quartz coverslips. Note that glycerol and quartz do not absorb in the UV-VIS spectral region and are ideal for UV-VIS MSP. However, since quartz slides and quartz coverslips cost substantially more than glass slides and glass coverslips, and due to glycerol's low refractive index, both are impractical for use during initial microscopic hair or fiber examinations. Additionally, removing fibers from Permount™ is time-consuming and comes with an increased risk of loss or damage to the hair or textile fiber samples in the process. Ideally, forensic hair and fiber examiners would prefer to avoid transferring samples from glass to quartz slides to minimize these risks. The identification of one or more semi-permanent mounting media with a RI suitable for microscopic hair and fiber exams and more cost effective slides/coverslips that do not absorb in the UV spectral region would address this concern.

In an effort to find such an alternative mounting medium to Permount™, we conducted two surveys – a domestic survey and an international survey. This manuscript summarizes the results of these surveys. 39 mounting media were identified and eight were selected for further examination. These media were selected based on popular usage by respondents and literature concerning the analysis of hair, fiber, and other sample types of scientific studies covering different disciplines (forensic and biomedical analyses). Furthermore, the selected candidate mounting media were verified to be in production and commercially available at the time of the study. A mixture of both glycerol-based (water) and organic-based (toluene) mounting media were selected to avoid limiting our selection to only one broad mounting medium class.

We also searched for an alternative to glass and quartz slides and cover slips. Glass exhibits a high absorption below 320 nm, a limiting factor during ultraviolet-visible (UV-

VIS) MSP of fiber examinations. Quartz has low absorption in the UV range but is much more costly. Nunc™ Permanox™ ultraviolet-transparent acrylic slides (UVT-A), a relatively inexpensive ultraviolet transparent (UVT) acrylic slide (Permanox® slides), that exhibits minimal absorption in the UV spectral range, was also evaluated to determine whether a greater UV spectral range could be achieved.

The candidate mounting media were evaluated on their color, setting time, media autofluorescence, fiber autofluorescence suppression or enhancement, and media absorption in the UV range. Additionally, fiber samples were prepared in several different ways: on glass microscope slides covered with glass coverslips, quartz slides covered with quartz coverslips, and Permanox® UVT slides covered with UVT slides. These were characterized via microspectrophotometry (MSP) to determine which sample preparation method offer advantages in the UV spectrum.

The identification of such an alternative mounting medium and/or mounting medium platform could allow forensic hair and fiber examiners to conduct initial examinations (e.g., comparison microscopy, polarized light microscopy, and fluorescence microscopy) and subsequent examinations (e.g., UV-VIS microspectrophotometry) without removing the sample from the prepared slide.

METHODS

Literature review and survey

A thorough literature review was conducted to identify possible candidate mounting media. Mounting media were identified in forensic, anthropological, and biomedical research articles, including mounting media that have been used for several decades. Vendors and technical experts from multiple companies were contacted individually for product information and alternative developments.

A questionnaire developed in conjunction with FBI Laboratory hair and fiber forensic examiners consisted of nine free-response questions, seven of which inquired about specific information on different aspects of slide preparation by the respondent.

The questionnaire was made available to willing participants using an online survey tool between October 2017 and November 2017. Participants were members of the American Society of Trace Evidence Examiners (ASTEE). In total, 159 survey responses were received representing 39 unique mounting media. Entellan New™, Glycerol, Permount™, Water and Xylene, were reported well over 5%. Permount™, 19% of responses, was the most used mounting medium in North America. Of the microscopists' reported mounting media,

Entellan New™ [5]and Permount™ [6] were chosen for further evaluation. The six additional mounting media CC/Mount™ [7], Eukitt UVR® [8], Fluoromount-G® [9], FluorSave™ [10], Omnimount™ [11], and ProLong™ [12]were identified via vendor inquiries and scientific literature.

Additionally, Nunc™ Permanox® Ultra-Violet Transparent Acrylic (UVT-A)[13] cell culture slides were included to test for improved transparency over glass without the expense of spectral grade quartz. Glycerol was included in the final list to serve as a control for autofluorescence and UV-transparency tests.

Macroscopic color

The color of each mounting medium was evaluated by placing large volumes of mounting media (400 µl) in capped glass vials and small volumes of mounting media (100 µl) on glass slides covered with glass coverslips, and storing them at room temperature for up to one year. The prepared glass vials and glass slides were photographed by the Forensic Imaging Unit of the FBI Laboratory in separate groups (i.e., glass vial group and glass slide group). Digital photographs of the vials and slides were taken over a white background. A programming language, Python [5], was used to compare pixel values at various sampling regions for intensities of red, green, and blue values via use of histograms generated from sampling areas of the images for glass vials. Water in glass vials, empty glass vials, water on glass slides, and empty glass slides were used as negative controls, while hematoxylin and eosin (H&E) staining solution was used as a positive control for color.

Image quality

A polyester fiber labeled "orange"[14] was used as a reference fiber for image quality tests. The microscopic characteristics of single strands of fibers in mounting media were examined visually under brightfield illumination using a Leica FS4000 comparison

[5] Millipore-Sigma; Billerica, MA
[6] Fisher Scientific; Pittsburgh, PA
[7] Sigma-Aldrich; Allentown, PA
[8] Electron Microscopy Sciences; Hatfield, PA
[9] SouthernBiotech; Birmingham, AL
[10] Millipore-Sigma; Billerica, MA
[11] National Diagnostics; Atlanta, GA
[12] Thermo Fisher Scientific; Waltham, MA
[13] Nalge Nunc International; Rochester, NY
[14] Monsanto Company; St. Louis, MO

microscope (Leica DM 4000B microscope with the Leica FS 4000 optical bridge)[15] and photos were taken with a Leica MC 190 HB camera. The following microscope settings were applied: 400 times magnification, field diaphragm 10, aperture diaphragm 16.

Setting time and ease of handling

Setting time was evaluated by placing 100 µl of mounting medium in the center of large (75x50x1.0mm), pre-cleaned glass microscope slides. The prepared slides were placed in a fume hood for various intervals of time – five-minute intervals for the first two hours and 30-minute intervals for the next six hours. Shortly before the end of each interval, an outline of the mounting media coverage area was traced on the dry side of the microscope slide. After eight hours, another large slide was placed on top of the mounting media as a cover. The preparation was then immediately pressed by a weight (821 g) for one minute. After pressing, the weight was lifted, and the outline of mounting media coverage was traced on the topmost slide of the preparation. The slides were allowed to sit overnight before being scanned. The coverage areas of the mounting media were measured in pixels and scaled to millimeters using ImageJ [6,7]. Outlines were traced using a Wacom Cintiq 21UX tablet[16]. Comparisons between initial coverages and post-pressed coverages were documented through the change in area over the aforementioned 36 time intervals. The mounting medium was considered to be set once there was no change in coverage area. Set times were verified by checking the coverage of the mounting media at the time interval in which the respective mounting medium was observed to set. In this manner, continuous force of the topmost slide alone displacing liquid mounting media was taken into account beyond the observed setting time.

Additionally, three sets of slides with a single human head hair segment mounted in each mounting medium were allowed to undergo prolonged setting and were stored in transparent slide mailers exposed to air and light, exposed to light, and protected from light for several months. Images of noteworthy slides were taken under the stereomicroscope at 400 times magnification after 233 days.

Ease of removal

Hair segments were mounted on glass slides using approximately 200 µl of mounting medium; care was taken to allow a portion of the hair to stay above the mounting medium for solidity testing. For each mounting medium, one set of samples was protected with coverslips while another identical set was left unprotected. Hair segments in the unprotected set were allowed to dry from exposure to continuously flowing air in a fume

[15] Leica; Wetzlar, Germany

[16] Kabushiki-gaisha Wacom/Wacom Co., Ltd.; Saitama, Japan

hood for 48 hours. Unprotected preparations were then probed for solidity by pulling the portion of the hair sample above the mounting medium and observing whether or not the hair was removed or if the entire slide preparation was lifted.

Mounting media preparations were tested for ease of clearing agent dissolution to simulate scenarios in which entire slide preparations had to be disassembled. Preparations were completely immersed in the manufacturer's recommended clearing agent and the time it took for the clearing agent to loosen the coverslip from the glass slide was monitored. For the purpose of testing ease of clearing, xylene (xylene substitute) was tested on organic-based mounting media regardless of the existence of complementary, specialized clearing agents. NeoClear™ and HistoClear-II™ were also explored for Entellan™ New and Omnimount™, respectively. Water (Millipore water)[17] was used for aqueous-based mounting media. All clearing agents were applied dropwise to each sample. The samples were then checked for ease of hair segment removal and mounting media clearing. For preparations that included coverslips, diamond blades were used to cut a small square around a portion of the embedded hair segment. Additionally, slides with coverslips were immersed in appropriate clearing agents for 30 minutes, 60 minutes, and overnight to see if the entire coverslip could be lifted from slide.

Human toxicity

Information on toxicity was obtained from MSDS and manufacturer specifications for each mounting medium.

Autofluorescence

The impacts of autofluorescence were examined by measuring the capacity to see a proteinaceous fiber, such as a human hair shaft, versus a cellulosic cotton fiber specimen. The proteinaceous fiber is known to exhibit autofluorescence. A highly pigmented black human scalp hair shaft served as the proteinaceous fiber, while an undyed cotton fiber from a reference fiber collection served as the cellulosic fiber. Slide preparations were imaged at 200 times magnification using brightfield and fluorescent microscopy. Fluorescence was achieved by using Olympus™ BX53F microscopes[18] as part of the Leeds™ LTC comparative microscope system[19]. Fluorescence was observed through three filter sets: WBV[20] (Wide bandpass Blue/Violet), WB[21] (Wide bandpass Blue), and WG[22] (Wide

[17] Millipore-Sigma; Billerica, MA

[18] Olympus Corporation; Shinjuku, Tokyo, Japan

[19] Leeds Forensic Systems, Inc.; Minneapolis, MN

[20] LCT-19000 WBV (UV/DAPI) Longpass – EX – 375/28, BS – 415. EM – 435lp, peak = 375 nm, bandwidth = 28 nm

bandpass Green). Control background was achieved using a brightfield filter with no brightfield light source and the shutter closed. Images were obtained via live camera streaming and SPOT imaging software[23]. Mounting media on glass slides were imaged at 200 times magnification at a focal plane where a test fiber was in focus. Exposure times and other camera settings were kept constant across preparations (i.e., Exposure: 700ms, Brightness: 0.75, Gamma = 1, Gain = 1, Contrast = 0.69, Color Temperature = 5000, Full chip area, and No binning). An empty glass slide and a glass coverslip preparation was used as the baseline to determine autofluorescence in the absence of media. Pixel intensity values of images were measured using ImageJ software [6,7]. Sample and background areas were outlined using a Wacom Cintiq tablet for images where fiber samples were present.

The ratio of the fluorescence intensity of the sample (i.e., cotton fibers, hair segments) to the intensity of background noise were measured by quantifying mean grey values at equally distributed regions along the sample area. The ratio of the entire sample intensity to the entire background noise was also quantified and included in the ratio. Sample-to-background autofluorescence was quantified using three filters: WBV, WB, and WG. Sample-to-noise ratios for intensities within the same group (cotton fibers vs. hair segments) that are further away from one (in both the $x>1$ and $x<0$ direction) indicated a greater contrast between sample intensity and background intensity with enhanced visualization.

Fiber deterioration

Slide preparations with commercially available fibers mounted in candidate mounting media were kept in the dark for over a year. Visual examination of the fibers was conducted using a Leeds™ LTC comparative microscope system to check for signs of fiber deterioration of mounted samples compared to reference fibers of the same type that were not stored in any mounting medium.

Transparency to ultraviolet light

In a first step, the absorbance of slides and respective coverslips was tested. No mounting media or fibers were used during this step. Quartz served as a transparency standard throughout the MSP trials since its UV absorbance is virtually zero. Glass was first compared to quartz. To quantify absorbance, the intensity of transmitted light passing

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[21] LCT-19002 WB (GFP/FITC) Longpass – EX – 480/30, BS – 505. EM- 545lp, peak = 480 nm, bandwidth = 30 nm

[22] WG (TRIT/Cy3) Longpass – EX – 540-25, BS – 565. EM – 575lp, peak = 565 nm

[23] SPOT Imaging; Sterling Heights, MI

through glass alone was compared to the intensity of light passing through quartz. Positive readings above zero were considered absorbance readings. Oscillations in instrument readings beyond the 500 nm range were considered as zero absorbance. All tests were conducted using quartz as a reference to establish ground truth MSP spectral data.

In a second step, the absorbance of mounting media themselves was tested. Absorbance was measured for each mounting medium in glass slide preparations with glass coverslips to reflect casework conditions. The total absorbance was determined by calculating the integral of the spectrum line that represented each mounting medium's absorbance across the entire UV-VIS range collected by a CRAIC microspectrophotometer (202.49 – 799.32 nm). Additionally, first derivative analysis was conducted to reveal similarities between spectra of different mounting media. Again, all tests were conducted using quartz as a reference to establish ground truth MSP spectral data.

In a third step, tests for how mounting media absorbance affects fiber sample readings were conducted solely on glass slides and glass coverslips. Fibers used in microspectrophotometry were gathered as reference fibers from reference collections. Fibers with homogenous textures known to absorb in the ultraviolet region and visible spectrum were chosen – acrylic, polyester, rayon, and nylon. Additional fibers were purchased [24], namely nylon fibers from the master nylon sample pack (including 15 homogenously dyed fabrics) and acrylic fibers from the acrylic belt webbing sample pack (including 9 samples with two colors each). The perceived macroscopic color described by the manufacturers of each were orange for acrylic; maroon or grey for polyester; purple for rayon; and red, gold, or blue for nylon. Refer to a full list of fibers used in this study in Supplementary Table 1. Only select fiber results are discussed in this study to illustrate major differences in mounting media.

In a fourth step, Permanox® slides were also tested to see if it was possible to acquire additional information deep into the ultraviolet region using a quartz alternative and using an aqueous-based mounting medium. Blue, white, and red nylon fiber measurements were documented using glass, quartz, and Permanox® slides. Since Permanox® slides were intended for cell culture usage, another Permanox® slide was used in place of a coverslip.

A CRAIC Microspectra 121 Microspectrophotometer was used to acquire MSP data using CRAIC Microspectra Imaging UV-Visible-NIR Imaging Package software and GRAMS. Absorbance was measured by subtracting baseline noise of the log of sample/reference measurements from the pixel values; the reference was a spot unoccupied by sample (blank spot) in a spectral grade quartz slide preparation. For fiber measurements,

[24] Seattle Fabrics, Inc.; Seattle, WA

references were comprised of measurements in blank spots of either quartz slide or glass slide preparations with mounting media in-between the coverslip and the slide.

$$A = -\log \frac{I}{I_0}$$

$$\text{ABSORBANCE} = -\log \frac{\text{SAMPLE} - \text{DARK}}{\text{REFERENCE} - \text{DARK}}$$

RESULTS AND DISCUSSION

Literature review and survey

Various mounting media were considered as possible candidates based on refractive indices, known color characteristics, price, human toxicity, and recommendations in previous forensic [8,9], anthropological, and biomedical studies. A total of 68 mounting media were selected from the literature. Other mounting media not mentioned in previous literature were chosen from communications with vendors and technical experts/representatives from local and multinational companies.

In addition to conducting a literature review and contacting vendors for new product developments, an electronic survey addressing mounting media usage was made available through the American Society of Trace Evidence Examiners (ASTEE). A total of 76 responses were obtained: 56 (74%) from the United States, 1 (1%) from Canada, 18 (24%) from Europe, and 1 (1%) from Malaysia. The type of facilities represented by respondents were mostly forensic laboratories, but respondents from other types of facilities participated. Data from the respondents revealed a clear prevalence of Permount™ (32 responses) and xylene or xylene substitute usage across all respondents (Figure 1). Most facilities (61%) reported using a combination of temporary (glycerine, xylene, immersion oil, etc.), semi-permanent (Meltmount™, Entellan New™, Permount™, etc.), and permanent (Aroclor™, Norland Optical Adhesive™, DPX™, etc.) mounting media, while the remaining facilities (39%) reported using a single mounting medium for multiple types of analyses. Note that the results of the survey referred to media used as a mounting medium in slide preparations for specified sample types (fiber, hair, or both). Each type of mounting medium was differentially used by respondents for solely synthetic fiber, solely hair, or both fiber and hair analyses. The results of a separate question addressing the type of solvent used to dissolve mounting media or to seal slide preparations did not contribute to mounting media responses. Thus, a facility using xylene as a solvent had no impact on the overall usage count of xylene as a temporary mounting medium. Numerous types of challenges experienced by examiners were reported and involved a range of phenomena that included yellowing of mounting media, fiber degradation, crystallization, and other types of immediate and long-term observations. After responses were lumped into broad mounting medium types, mounting media were compared to see how many challenges

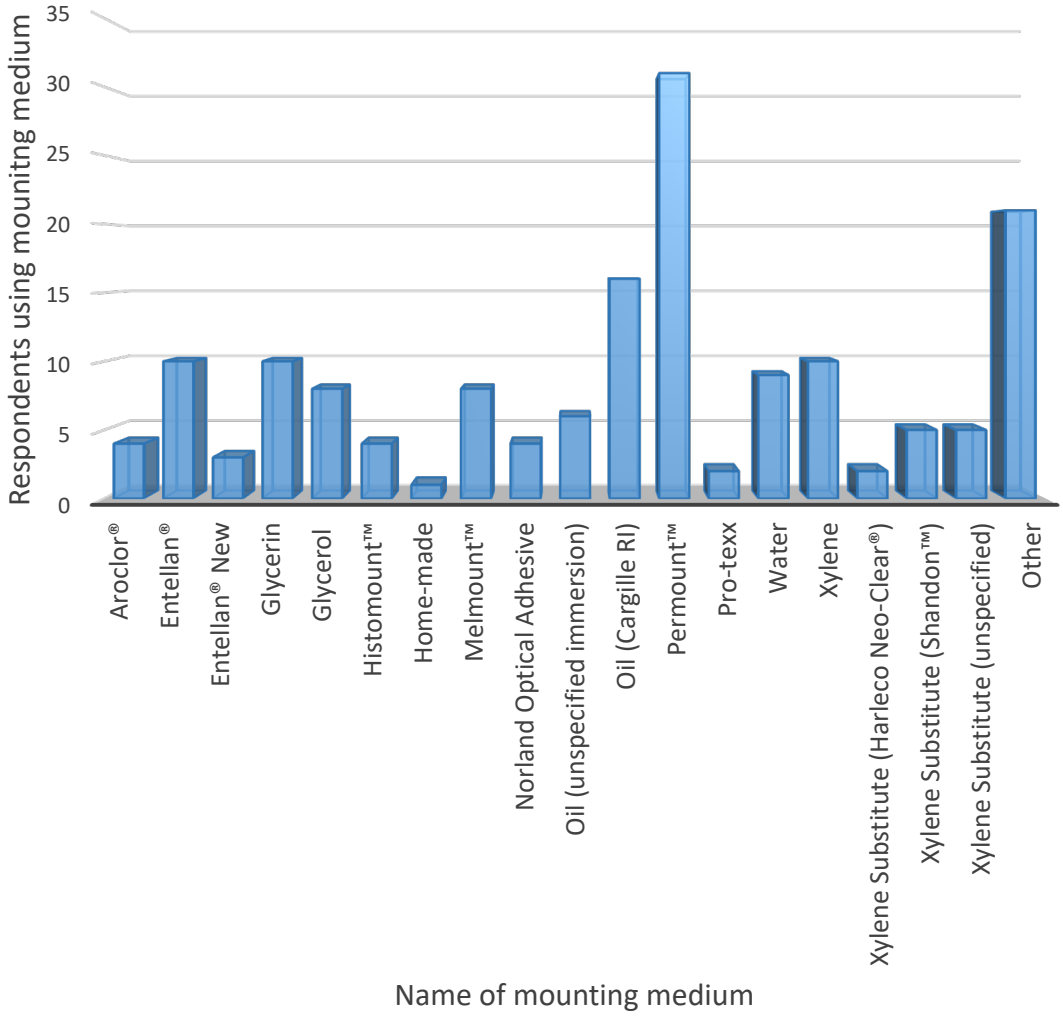

Figure 1: Diversity in mounting media usage from survey respondents. Overall, 39 unique mounting media were used by respondents, representing temporary, semi-permanent, and permanent mounting media. The most popular mounting media was Permount™, with 31 instances of use. "Other" indicates mounting media that was represented by only one respondent.

were faced by examiners using a particular mounting medium. Note that the number of reported challenges is heavily influenced by the frequency of use for a given mounting medium, as mounting media that have yet to see widespread adoption in laboratories either by rarity or by years available on the market may have examiners reporting more problems with increased usage.

Final candidates

Both organic and aqueous-based mounting media were included in the final list of candidates (Table 1). Entellan New™ was included as a candidate due to a strong

recommendation in previous literature [8], survey results, and laboratory personnel; Eukitt™ was included for its ability to be easily cured under ultraviolet light which could provide an advantage with instant solidification. CC/Mount was the first aqueous mounting medium chosen as it had not been found in previous literature. Additionally, the possibility of using CC/Mount with slide materials that were incompatible with organic solvents (acrylic) was considered. Despite its known yellow appearance, ProLong Glass Antifade™ mounting medium (hereafter referred to as ProLong™) was chosen due to the manufacturer's claim that the mounting medium prevents photobleaching of samples under various wavelengths for extended periods of time. Although a refractive index close to that of glass (1.51) was the first major screening criteria in selecting the final group of candidate mounting media, mounting media such as Fluoromount-G® and FluorSave™ were included due to other desirable characteristics (i.e., clarity, aqueous nature, and minimal toxicity).

Table 1: Final list of candidate mounting media. Nine of 70 commercially available mounting media were considered for further evaluation based on how closely refractive indices were similar to that of glass (RI = 1.51). Note that glycerol/glycerin was added in this study to serve as a positive control mounting medium used to generate ground truth data. Mounting media are sorted in alphabetical order and the RI values were obtained from the vendor's websites. Note that xylene refers to xylene and xylene substitute, and water to molecular biology-grade water that has been purified via a Millipore filtration systems and ultrafiltration systems.

Name	Manufacturer	Base	Macroscopic Color	Clearing Agent	RI (20°C)
CC/Mount™	EMD Millipore, Sigma-Aldrich	aqueous	clear	Water	"very high"
Entellan New™	Thermo Fisher Scientific	organic	clear	Xylene, Neoclear®	1.490-1.500
Eukitt® UVR	O. Kindler	organic	clear	Xylene	1.48
Fluoromount-G®	Southern Biotech	aqueous	clear	Water	1.393
FluorSave™	EMD Millipore, Sigma-Aldrich	aqueous	clear	Water	1.33
Glycerol	Sigma-Aldrich	aqueous	clear	Ethanol	1.4722
Omnimount™	National Diagnostics	organic	clear	Xylene, HistoClear® II	1.5118
Permount™	Thermo Fisher Scientific	organic	yellow	Xylene	1.518-1.521
ProLong ™ Glass Antifade	Thermo Fisher Scientific	aqueous	yellow	Unknown	1.52

Additionally, an alternative slide preparation was considered in order to test for improved transparency over glass. Permanox® slides were used in tandem with aqueous mounting media identified after performance evaluations. Since Permanox® slides are known to react poorly with organic reagents, they were not used with organic reagents. Other types of slides that claimed to have improved transparency over glass slides were either too expensive or not readily available for practical laboratory use, and therefore were not considered for this study.

Macroscopic color

Glass vials containing 400 µl of each mounting medium, as well as glass slides containing 100 µl of each mounting medium were analyzed for macroscopic color. Hematoxylin and eosin[25] staining reagent served as a positive control for color and opacity, while water served as a negative control for no color and for clarity.

With respect to the vials containing 400 µl of mounting media, only Permount™ and ProLong™ exhibited a tint of color (Figure 2A). All other mounting media were completely transparent before and after curing. However, the yellow tint was difficult to see when mounting media were spread into a slide preparation (Figure 2B). The same effect on tint visibility holds true for hematoxylin and eosin on slides. Quantitative color assessment using Python [5] revealed Permount™ and ProLong™ retained a noticeable level of RGB intensities in slide preparations. All other mounting media displayed histograms nearly identical to that of water (Figure 3).

With respect to the glass slides containing 100 µl of mounting media, macroscopic color was almost indistinguishable from water (Figure 2B). This was also evident when focusing in areas of the mounting media not occupied by the polyester fiber segment (Figure 4).

[25] Sigma-Aldrich; Allentown, PA

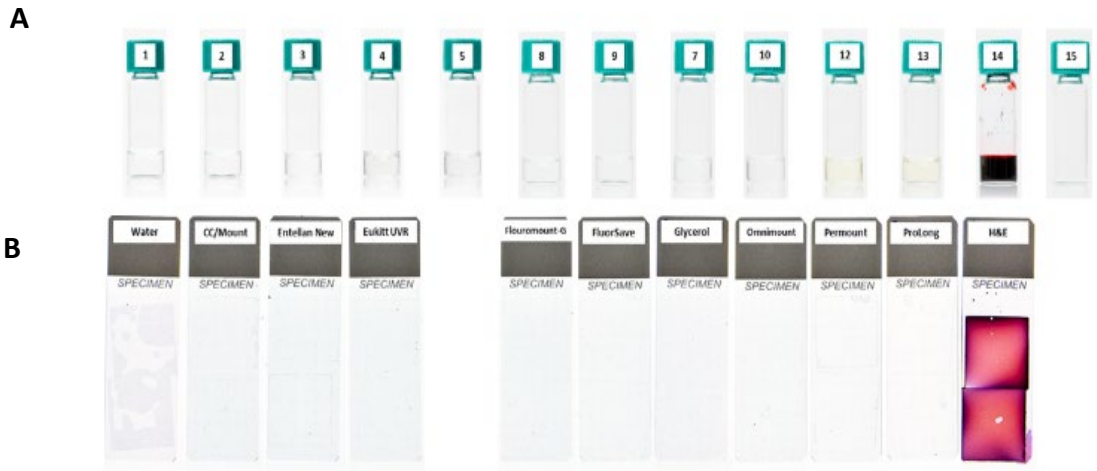

Figure 2: Macroscopic color of selected mounting media. (A) Large (400 µl) volumes. 1 = first water blank, 2 = CC/Mount™, 3 = Entellan New™, 4 = Eukitt® UVR cured by ultraviolet light, 5 = Eukitt® UVR uncured, 6 = second water blank, 7 = glycerol, 8 = Fluoromount-G®, 9 = FluorSave™, 10 = Omnimount™, 11 = third water blank, 12 = Permount™, 13 = ProLong™, 14 = Harris modified hematoxylin eosin (H&E) staining reagent, 15 = empty vial, true blank. (B) Small (100 µl) volumes. Images courtesy of the FBI Laboratory Forensic Imaging Unit (FIU).

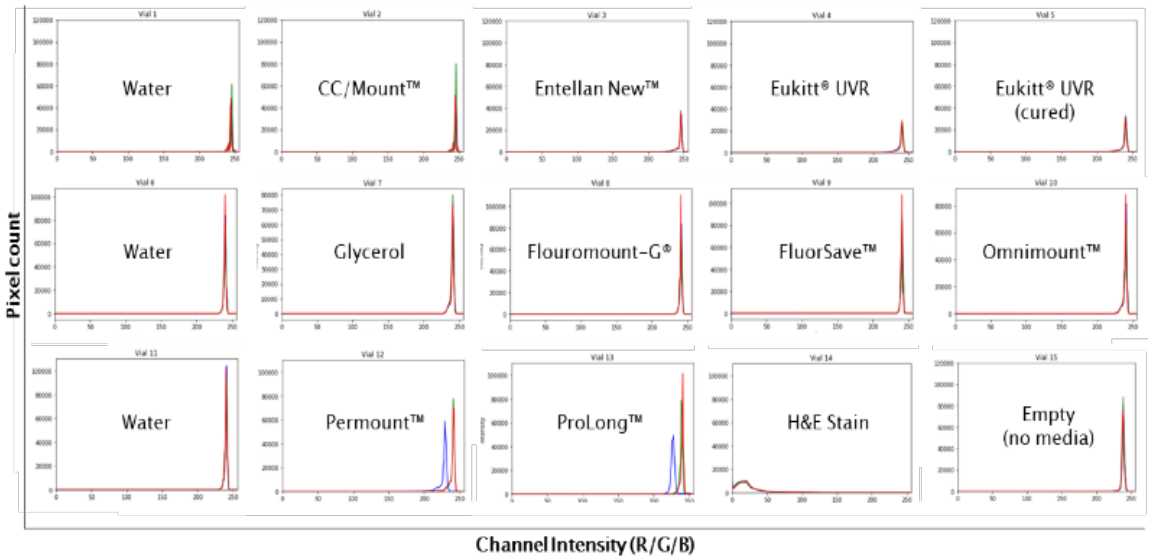

Figure 3: Macroscopic color of mounting media. Histogram of 8-bit integer values for R (red intensity, red lines), G (green intensity, green lines), and B (blue intensity, blue lines) components for images of colorless water, Permount™, and ProLong™. All other mounting media displayed histograms nearly identical to that of water in the same image.

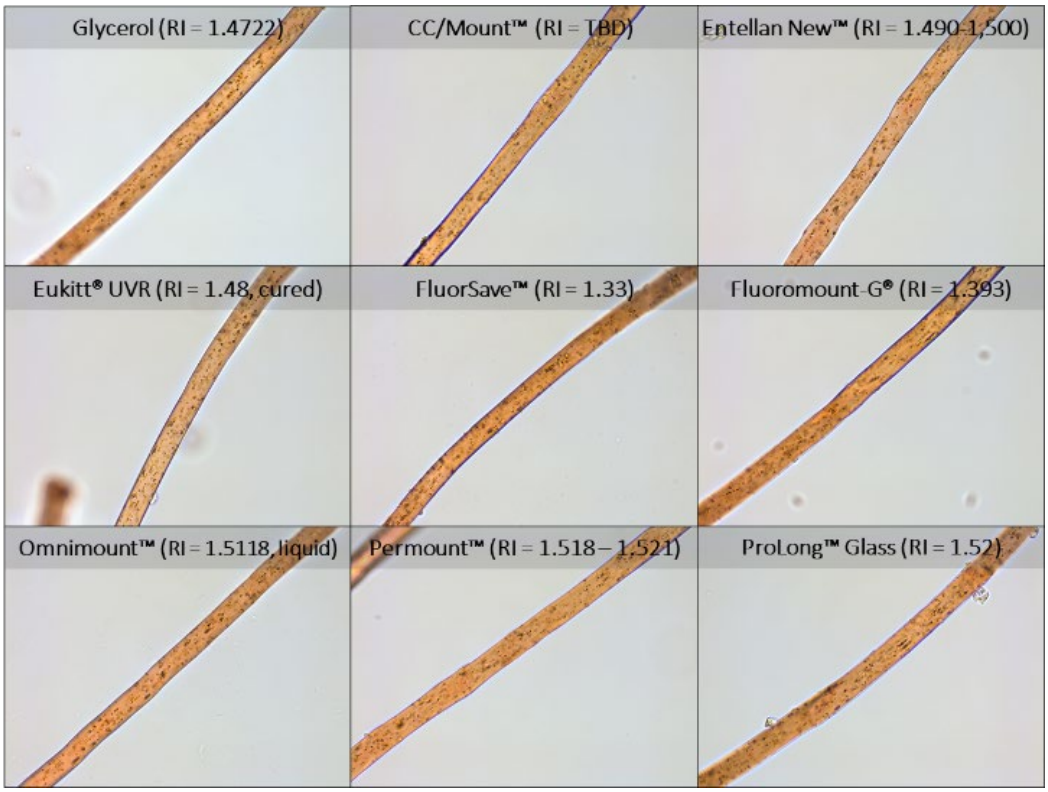

Figure 4: Image quality of fiber in different mounting media. A polyester fiber was split into segments and mounted in each candidate mounting medium on glass slides. Images were taken at 400 times magnification through a Leica FS4000 comparison microscope, Eukitt® UVR has been cured.

Image quality

The microscopic characteristics of single strands of orange polyester fibers in mounting media were evaluated and revealed extremely low reliefs (Figure 4). Relief, a combination of a Becke line and a dark shadow, can be described as an observable contrast between the fiber and the mounting media. When light passes between transparent materials with very different refractive indexes, a high relief is produced at the interface between the material's edge [10] (Schaefer T., 2020). When light passes between two transparent materials with similar refractive indexes, relief is low [10] (Schaefer T., 2020). Observed relief is also a function of the microscope used for fiber sample examination, including the numerical aperture of the condenser and objective used. The polyester fibers prepared in Permount™ (RI of approximately 1.518 – 1.521), Omnimount™ (RI of approximately 1.5118), ProLong™ (RI of approximately 1.52), and Entellan New™ (RI of approximately 1.490 – 1.500) exhibited the lowest reliefs. Polyester fibers prepared in Eukitt UVR® (RI of approximately 1.48), CC/Mount™ (RI not available from manufacturer), FluorSave™ (RI of approximately

1.358), and Fluoromount-G® (RI of approximately 1.393) exhibited moderate relief in some areas along the fiber sample.

Setting time and ease of handling

Desirable setting time was defined as complete solidification of the mounting media in slide preparations within one hour. With the exception of Eukitt® UVR, which was not tested for setting, only two mounting media (Omnimount™ and Permount™) were unable to set within the duration of the setting trials as reflected in the change in area coverage beyond the 24hr time point (Figure 5).

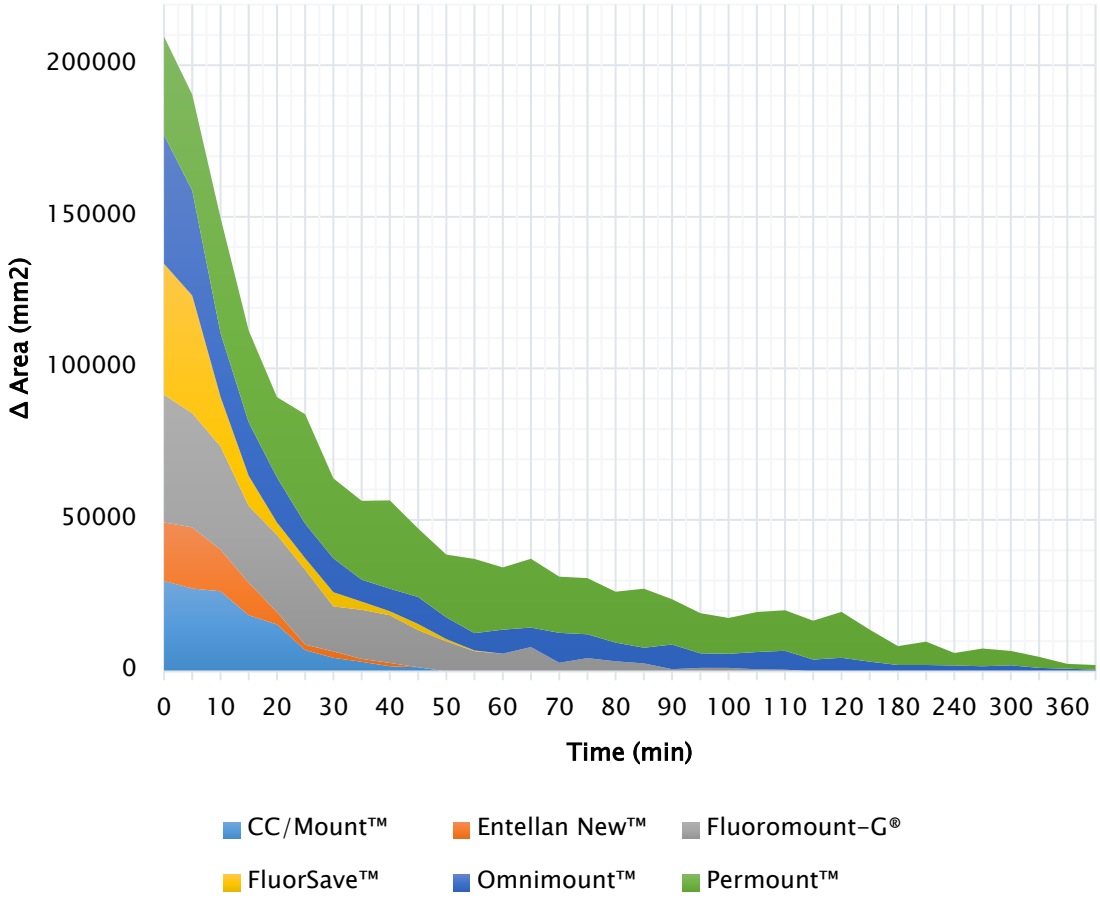

Figure 5: Changes in coverage area of mounting media over time. Change in area represents overall spread in coverage for mounting media sandwiched between two microscope glass slides when constant force applied to coverslip for 1min (n=36 replicates). Eukitt UVR not pictured due to instantaneous curing with UV-light. ProLong™ not pictured due to limited volume in original container. Glycerol not tested.

Entellan New™ and CC/Mount™ were the fastest to set, solidifying in under one hour (45 minutes and 50 minutes, respectively). FluorSave™ set by the one-hour mark, with Fluoromount-G® setting within two hours. As previously mentioned, Omnimount™ and Permount™ were unable to set within 390 minutes (6.5 hours), while Omnimount™ dried at a faster rate (about 32mm^2/min past 100 minutes) than Permount™ (about 9mm^2/min past 100 minutes). After three repeats, two of three Permount™ slide preparations which were allowed to dry overnight were still able to be moved without significant effort. Eukitt® UVR required exposure to UV light for only 30 seconds for complete curing ProLong™ was not tested during this phase of the study because the same volume of mounting medium was required for fair evaluation of setting time. Although manufacturers state less volume is required of ProLong™ when compared to other mounting media, the available volume of ProLong™ was not sufficient to match the volume of other mounting media. Furthermore, use of excessive volumes of ProLong™ may not represent proper usage recommended by manufacturers.

While the selected mounting media varied in viscosity, this quality did not determine the extent to which the mounting media set. In other words, once mounting media were solidified, no additional changes in coverage area were seen, such as shrinkage or involution. Although Permount™ had the greatest change in coverage area for all time intervals when compared to other mounting media, it is important to note that the drop of Permount™ allowed to dry on the slide was surrounded by a thin film as early as ten minutes. Unlike other organic-based mounting media which spread considerably upon application, Permount™ was able to maintain its initial shape on the glass preparation before weights were placed on the coverslip, similar to the aqueous-based mounting media.

The state of each mounting medium after drying time was also observed. Fluoromount-G® and Permount™ remained in a soft, semi-solid state that was easily moved when pressure was applied to their preparations. When cured, Eukitt® UVR remained somewhat soft when no coverslip was applied to the mounting medium droplet but was completely solidified otherwise. All other mounting media became completely solidified as droplets or as proper preparations under coverslips; this may be a factor to consider with respect to the timeframe one has to place specimens in mounting media and may or may not affect the ease of preparing a slide.

For ease of handling, mounting media like Permount™ and FluorSave™ can remain semi-solid for a period of time (in the case of Permount™, well beyond 24 hours). CC/Mount™ and Entellan New™ were the fastest setting mounting media, completely hardening within an hour. Both were easy to dispense and maintained shape after application on glass slides, allowing for quicker storage and more flexible options in terms of slide orientation while avoiding the possibility of dripping. Eukitt UVR only required a 30-second exposure

to ultraviolet light in order to cure. Among the slowest setting mounting media were Omnimount™ and Permount™; coverslips on top of Permount ™remained easily moveable after two weeks in proper storage conditions. This may or may not be considered an advantage, depending on the use of appropriate clearing agents.

Ease of removal

Appropriate clearing agents for each mounting medium were tested on preparations that were cured for 48 hours to test the ease of sample removal. These preparations consisted of hair segments mounted with and without coverslips on glass microscope slides (Table 2).

Table 2: Ease of removal and clearing agent compatibility. Entellan New™ and Omnimount™ were tested with clearing agents specifically developed for them. Resistance implies the hair segment is only movable with significant input from user.

Mounting Medium	**Drop (dried 48hr)**	**Clearing Agent**	**Retrieval**		**Immersion**		
			Dropwise application	***Excision***	***30min***	***60min***	***24hr***
CC/Mount™	solid	Water	instant	instant	X	X	X
Entellan New™	solid	Xylene Substitute	instant	instant	X	X	X
Entellan New™	solid	NeoClear™	instant	instant	X	X	X
Eukitt® UVR (cured)	soft but solid	Xylene Substitute	some resistance, but easily removed	instant	X	X	✔ softened
Eukitt® UVR	liquid	None	none required	instant	N/A		
Fluoromount-G®	soft, film-like	Water	instant	instant	✔		
FluorSave™	solid	Water	instant	instant	✔		
Omnimount™	solid	Xylene Substitute	not removable	instant	✔		
Omnimount™	solid	Histo-Clear™	instant	instant	✔		
Permount™	soft but solid	Xylene Substitute	resistance after many drops	some resistance	✔		
ProLong™	solid	Xylene Substitute	instant	instant	X	X	✔

Fluoromount-G®, Permount™, and cured Eukitt® UVR were solidified but soft in drop-form exposed to air. However, when under coverslips, only Permount™ remained moveable while Fluormountt-G® and cured Eukitt® UVR were not. All other mounting media were completely hardened after the drying period, both as drops and when prepared between glass slides and glass coverslips, with the exception of uncured Eukitt UVR which remained liquid.

Direct dropwise application of xylene substitute on organic mounting media allowed to dry by exposure to continuous airflow resulted in instant release of the embedded hair segment for all mounting media except Permount™ and Omnimount™. Instant release is defined as the loosening of the hair segment when the hair segment was gently moved by forceps. In the case of Permount™, the hair segment was loosened but provided further resistance when moved gently by forceps. Further application of xylene substitute decreased resistance of the hair segment until complete release. Hair segments embedded in Omnimount™ were not released by the application of xylene substitute, while the rest of the preparation remained completely unaffected by xylene substitute. However, hair segments in Omnimount™ were easily released by the application of Histo-Clear II™. As for aqueous mounting media, direct dropwise application of water on media allowed to dry via continuous airflow resulted in the instant release of hair segments in all aqueous-based mounting media.

To simulate scenarios in which portions of a sample must be retrieved from mounting media, preparations were subject to cutting by a diamond blade. When portions of coverslips were compromised by a diamond blade, dropwise application of the appropriate clearing agent resulted in instant release of both the coverslip segment and the hair segment in all mounting media. Unlike in exposed mounting media drop preparations, hair segments under coverslips were embedded in a thinner and more uniformly distributed mounting media, leaving less mounting media for the solvent to potentially dissolve.

Mounting media preparations were tested for ease of clearing agent infiltration to simulate scenarios in which entire slide mounts had to be disassembled. When completely immersed in an appropriate clearing agent, preparations varied in the time needed to loosen the coverslip from the glass slide. Within 30 minutes, coverslips in Omnimount™, Permount™, Fluoromount-G®, and FluorSave™ detached from the slides and mounting media with ease. This was true of Omnimount™ in both xylene substitute and Histo-Clear II™. After 60 minutes, CC/Mount™, Entellan New™, Eukitt® UVR, and ProLong™ were not infiltrated by the clearing agent and did not release their coverslips, remaining completely solidified with no bubbles or visible indications of interaction between the mounting media and the clearing agent around the perimeter of the coverslip. Overnight immersion of these remaining preparations resulted in softening of Eukitt® UVR so that the coverslip

could be removed. CC/Mount™ was unable to be infiltrated. In xylene substitute and NeoClear™, Entellan New™ was unable to be softened or dissolved.

When mounting media containing a single hair per slide were stored for long periods of time, all semi-permanent mounting media except Eukitt® UVR did not display noticeable features (Figure 6). However, Eukitt® UVR preparations showed significant rippling of texture after 233 days. The mounting media lifted the coverslip away from the glass slide. The texture of the mounting medium was gelatinous and did not re-solidify. Interestingly, drops of cured Eukitt® UVR that were not protected with coverslips but were stored in slide mailers protected from light did not become gelatinous and remained solidified throughout the 233 days, similar to other mounting media.

Human toxicity

Toxicity of mounting media was also taken into account (Table 3). Aqueous-based mounting media do not warrant as many Globally Harmonized System (GHS) of Classification and Labelling of Chemicals warnings as organic-based mounting media. Although there is not sufficient data available for further toxicity classifications of glycerol-based mounting media (e.g., ProLong™, CC/Mount™, Fluoromount-G™, and ™FluorSave™), the potential for hazardous effects on the users of these reagents may be less than for mounting media with xylene or toluene as their base. Aqueous mounting media (i.e., CC/Mount™, Fluoromount-G®, and FluorSave™) can be cleared by using water as a solvent, whereas organic-based mounting media (i.e., Entellan New™, Eukitt® UVR, Omnimount™, and Permount™) must use xylene substitute or a specialized clearing agent to loosen samples from preparations (Table 2). Interestingly, ProLong™ is compatible with xylene substitute and instantly releases samples. Use of mounting media with less hazardous components leads to use of clearing agents with less hazardous components which ultimately leads to increased safety for laboratory personnel.

Autofluorescence

The autofluorescence of the empty glass slide preparation was determined to be a mean grey value of 3,609 ± 678, setting the threshold of what was considered noise when observing the autofluorescence of other mounting media (shaded region, Figure 7). Ideally, regions in which materials are not autofluorescent will appear black. All mounting media except Permount™ exhibited autofluorescence macroscopically comparable to the empty glass slide preparation. For Permount™, autofluorescence was high as the areas examined through the WBVI filter were lit in an intense, light-blue light (Figure 8). The autofluorescent intensity of Permount™ through the WBV filter (mean grey value = 39,570) was quantified to be 30% greater than that of its noise levels (mean grey value = 1,323), while other mounting media exhibited autofluorescence of less than 3% (Figure 9). The

lowest autofluorescent intensity through the WBV filter was exhibited by Omnimount™ (mean grey value = 3,283, 2.4% greater than noise).

Grey cotton reference fibers were imaged alongside segments of human hair shafts originating from the same donor and same strand of hair. Visibility of these natural fibers was assessed through the WB (480 nm ± 30), and WG (≥565 nm) filters (last two not pictured). For all mounting media except Permount™, cotton fibers appeared blue through the WBV, i.e., color values existed in the red channel (Figure 10). Grey cotton fibers in CC/Mount™ and FluorSave™ appeared to have less autofluorescence than when mounted in glycerol. ™Grey cotton fibers were visible in Permount™ despite being against a highly fluorescent, blue background. Similarly, for all mounting media except Permount™, human hair shafts appeared blue, but with varying autofluorescent intensities. Entellan New™, Omnimount™, and Eukitt® UVR exhibited highly autofluorescent hair shafts in comparison to other mounting media. In Eukitt® UVR, the high autofluorescence of the hair shaft was reflected, contributing to background autofluorescence that was not present when specimens of less intense autofluorescence (cotton fibers) were examined. Autofluorescence of hair shafts in Permount™ were still visible, appearing as a dark shade of blue against a highly fluorescent, light blue background.

Note that the fluorescence intensities of cotton fibers were calculated with a smaller sample area and larger background area when compared to the larger hair shaft samples, measured in pixels (px). The average cotton fiber area was 37,205 px (Standard deviation, SD = 13,961) against an average cotton fiber background area of 1,011,363 px (SD = 13,972), whereas the average hair fiber area was 127,300 px (SD = 21,404) against an average hair background area of 921,284 px (SD = 21,402).

For the WBV filter, cotton fibers experienced sample-to-background intensity ratios furthest from 1.0 when imaged in Entellan New™ mounting medium (ratio of 2.6, n=26 measurements). In contrast, the sample to background intensity ratio for ProLong™ was much lower (ratio of 1.37, n=26 measurements), though the cotton fibers were still easily discernible. Interestingly, the ratio for Permount™ was 1.0 for cotton fibers, indicating equal autofluorescence of the sample and the background on average. Both the cotton fiber and empty space occupied only by Permount™ appeared similar in intensity. Qualitatively, this can be seen in the difficulty of distinguishing cotton fiber samples in WBV images (Figure 10).

As for the other natural fiber set, hair shafts in Omnimount™ had the highest sample-to-background intensity (ratio of 12.57, n=26 measurements), with the hair shaft region-of-interest contrasting with a dark background. Since the ratio is so high, this amount of autofluorescence in the sample that is absent in the background of Omnimount™ empty space pointed to the ability of Omnimount™ to image hair shafts well in the WBV filter with

less intensity than is required by the other mounting media. Hair shafts in mounting media whose ratios were closest to one include glycerol (ratio 1.1, n=26 measurements) and Fluoromount-G® (ratio 1.1, n=26 measurements).

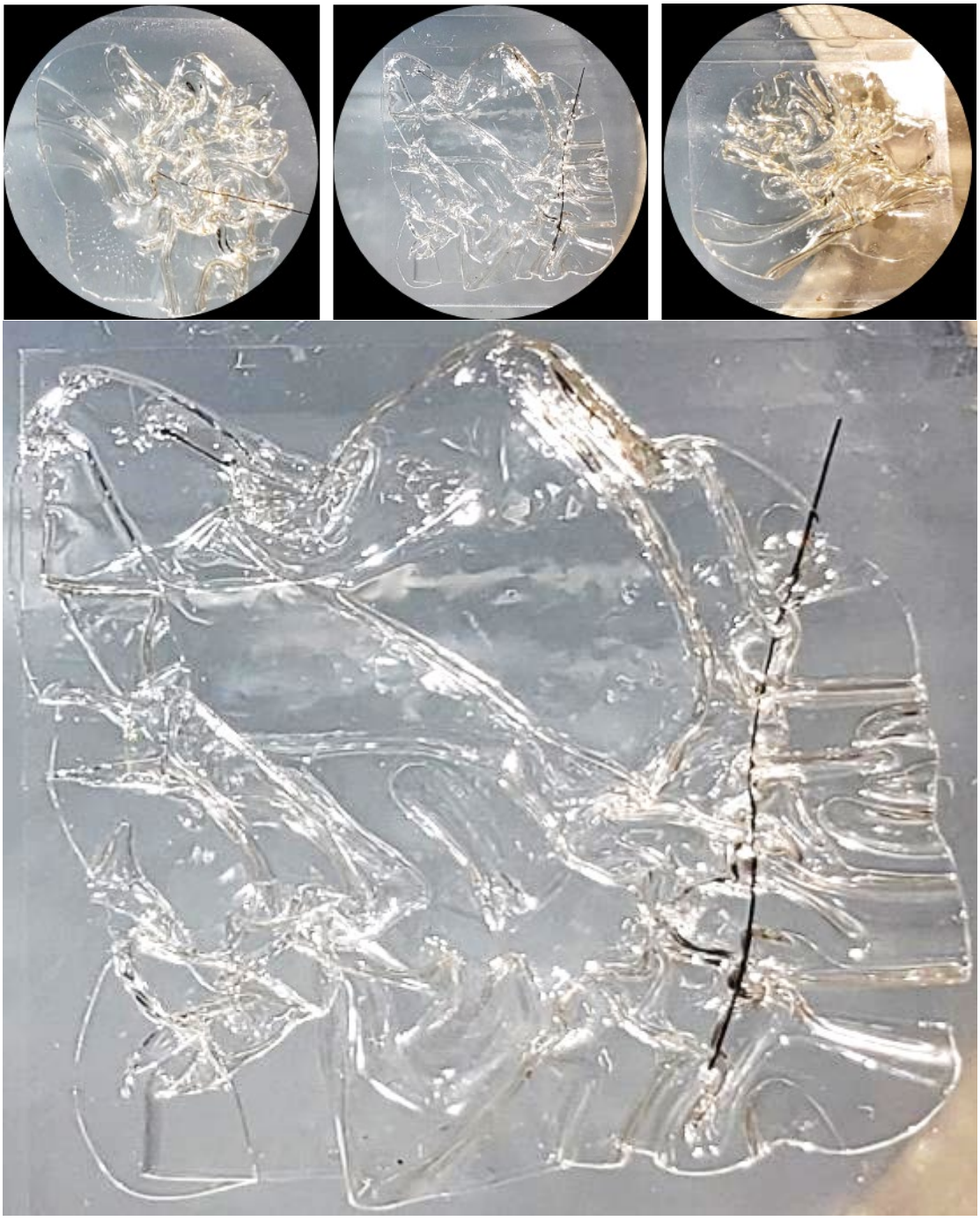

Figure 6: Unusual drying result for hairs mounted in cured Eukitt® UVR. Slides were stored for 233 days exposed to air (A), within a slide mailer exposed to light (B), and within a slide mailer protected from light (C). (D) Detail of rippling texture for dried mounting media as pictured in (B).

Table 3: Mounting media toxicity notes. Globally Harmonized System hazard labels indicate different hazards. Glycerol-based mounting media had minimal hazards. Due to the novelty (relative to Permount™) of some the mounting media, insufficient data exists to determine toxicity components.

ID	Hazards	Hazardous Compound	Inhalation	Skin contact	Reproductive hazard	Target organ toxicity	Other
Entellan New™		Xylene	Respiratory oedema formation	Chapped skin, dermatitis (long-term)	None	None	None
Permount™		Toluene	Headache, dizziness, nausea, vomiting	Irritant	Birth defects to unborn child	Central nervous system (single exposure)	None
Eukitt® UVR		Xylene	Nausea, shortness of breath, vomiting, anorexia	Dermatitis	No data available	Central nervous system (depression)	Anorexia
ProLong™		Glycerol	No data available	No data available	No data available	No data available	None
CC/Mount™		Glycerol	No data available	No data available	No data available	No data available	None
Fluoromount-G™		Sodium azide, glycerol	Insufficient data for classification	Insufficient data for classification	Insufficient data for classification	Insufficient data for classification	None
FluorSave™		Unknown	No data available	No data available	No data available	No data available	Aggravation of medical condition
Omnimount™		Naptha (Petroleum)	No data available	Harmful irritant	No data available	No data available	None

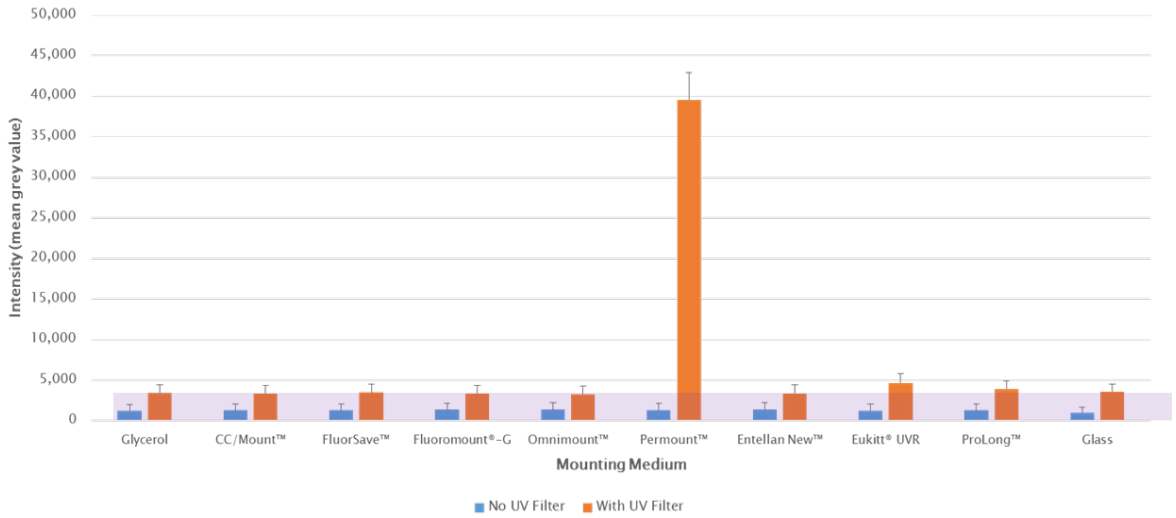

Figure 7: Autofluorescence intensity for candidate mounting media. Mean grey value measurements of candidate mounting media viewed through no UV filter (blue) and UV filter (orange, WBV, 375 nm, ± 28 nm, 200 times magnification, 700ms exposure). A mean grey value further away from a value of 1 for filter group indicates high autofluorescence by mounting medium. The shaded region denotes the threshold for glass autofluorescence.

Figure 8: Appearance of autofluorescence for candidate mounting media through WBV filter. Mounting media viewed through excitation filter with a peak of 375 nm, ± 28 nm (WBV Longpass). Images taken at 200 times magnification, using the same exposure time for all mounting media (700ms).

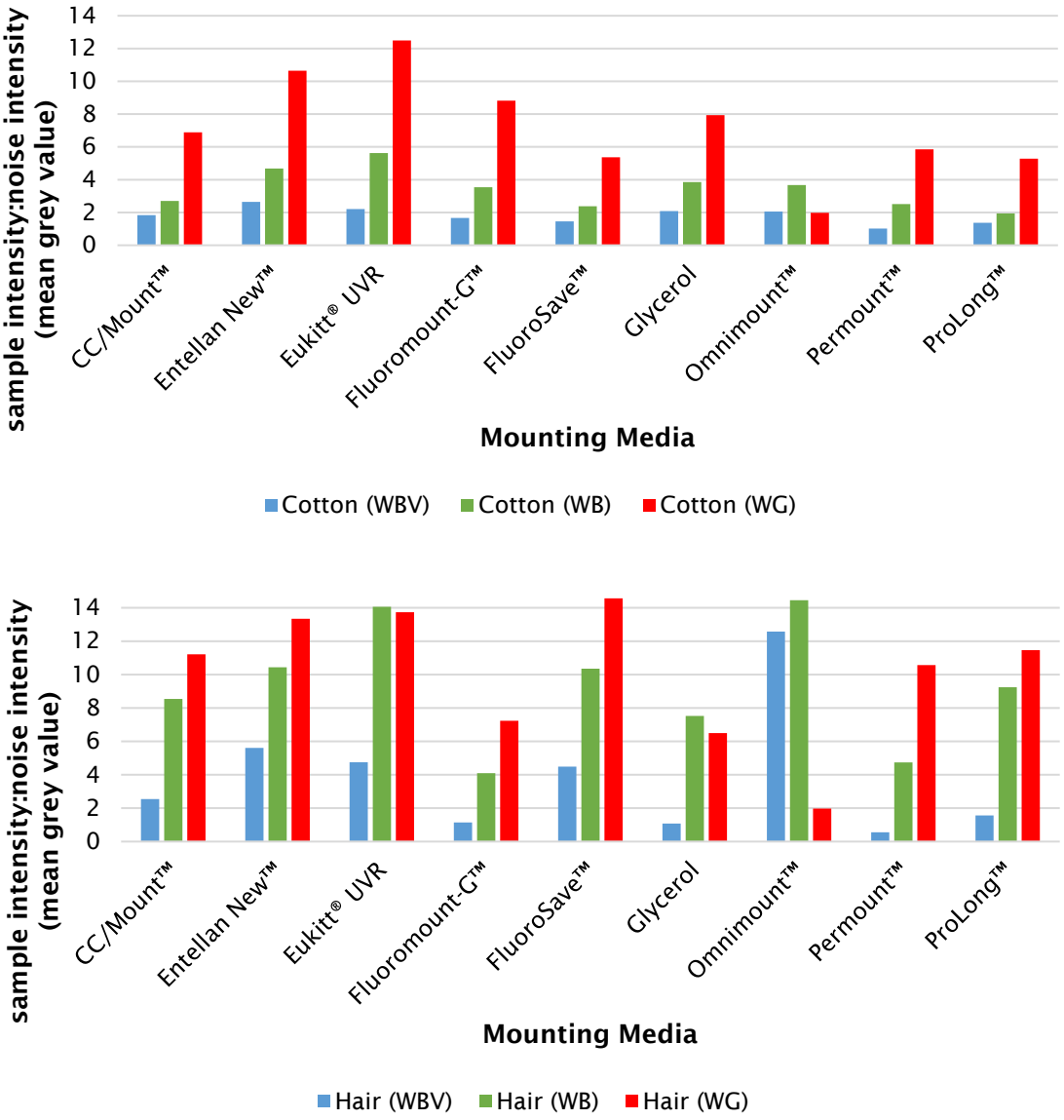

Figure 9: Autofluorescence of human hair and cotton fiber specimens in mounting media through three excitation filters. Mean grey values of representative areas and whole samples versus background mean grey values were quantified through WBV, WB, and WG filters for grey cotton fibers (Top) and hair shaft segments from the same donor (Bottom).

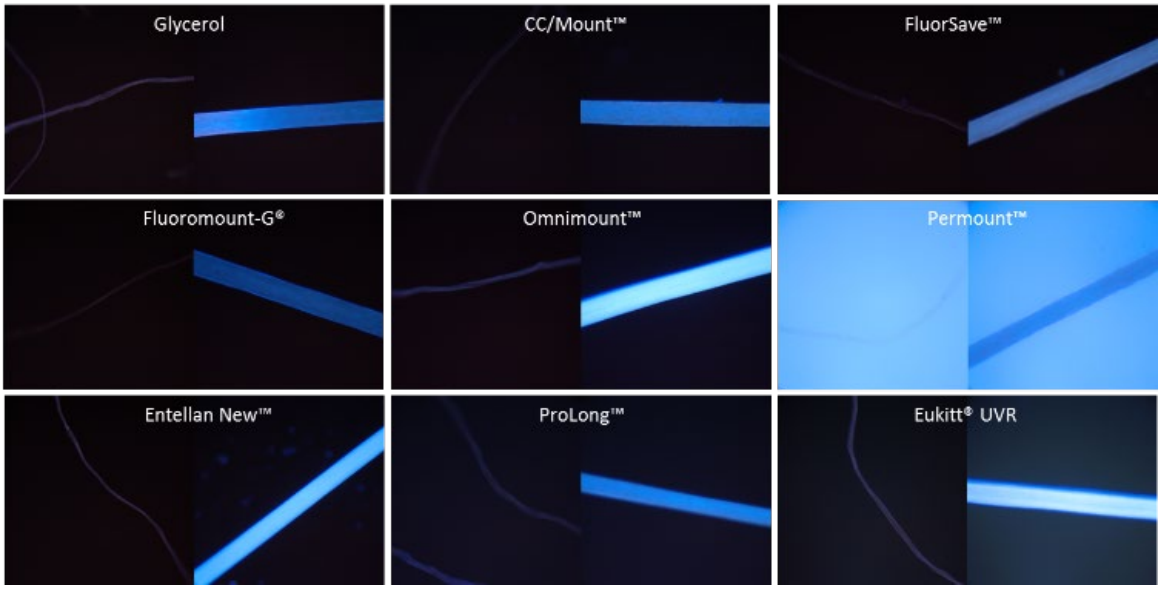

Figure 10: Autofluorescence of human hair and grey cotton fiber specimens in candidate mounting media mounted on glass microscope slides and glass coverslips. Cotton fibers and hair shafts imaged with UV light (WBV, 753 nm, 200 times magnification, 700ms exposure excitation filter with a peak of 375 nm, ± 28 nm). Each panel represents respective mounting media with cotton fibers (left side) displaying less noticeable autofluorescence than hair shafts (right side).

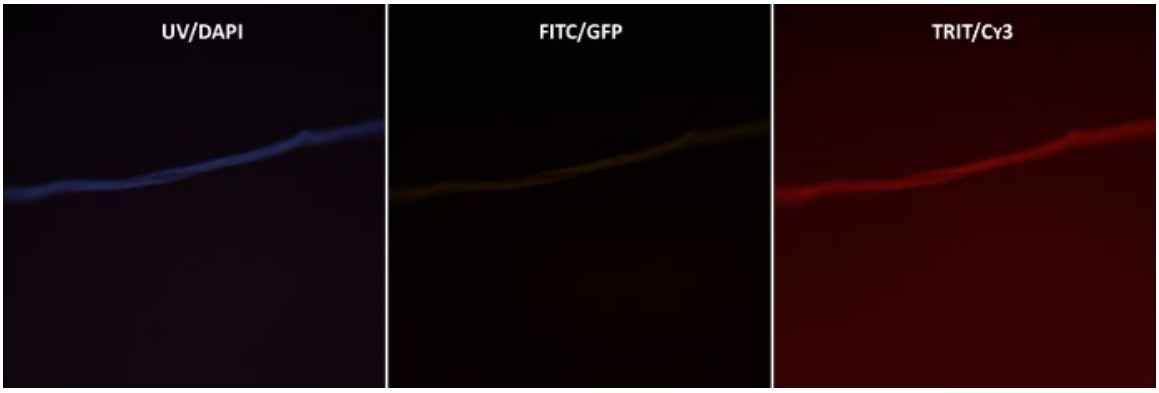

Figure 11: Autofluorescence of cotton fiber in Omnimount™ through three filter sets

Preparations with ratios closer to one indicated the weak autofluorescence of the hair shaft in these mounting media. Of particular interest is the sample-to-background intensity ratio of hair shafts in Permount™, which was the only value <1 (ratio 0.56, n=26 measurements) (Figure 9). A ratio of less than one indicates the mounting media itself displayed greater autofluorescence than the sample embedded in it; this was observed qualitatively when viewing the hair shaft of a darker shade of blue against a light blue background (Figure 10).

Not surprisingly, the highest ratios for both types of samples for the majority of mounting media were in the WG channel, except for Omnimount™ (cotton and hair), Glycerol (hair), and Eukitt® UVR (hair). Among the sample-to-background ratios through the WG filter, hair shafts in FluorSave™ had the most contrast with their environment, while Omnimount had the least contrast in this filter. Qualitatively, it was similar to the high amount of autofluorescence of fibers in Permount™ through the WBV filter, although in the WG filter the difference was not as drastic (Figure 11).

Fiber deterioration

There was no evidence of fiber deterioration or fiber alteration when compared to the same reference after one year in any of the mounting media, for any of the observed fiber types (i.e., hair, cotton, acrylic, polyester, rayon) (Data not shown).

Transparency to ultraviolet light

With respect to the absorbance measurements of slides and respective coverslips, glass absorbance was detected between 200-340 nm, with a maximum absorbance of 1.3AU occurring at approximately 273 nm (Figure 12).

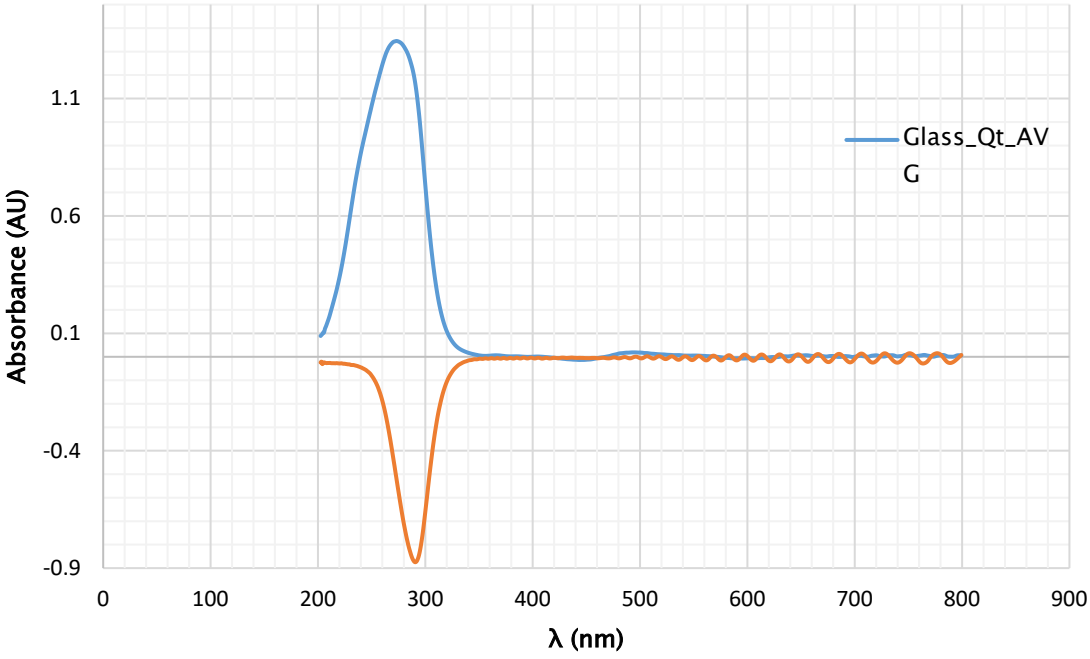

Figure 12: UV absorbance of glass compared to quartz. Blue refers to MSP readings using quartz as reference sample and glass as the test sample. Orange refers to MSP readings using glass as reference sample and quartz as a test sample.

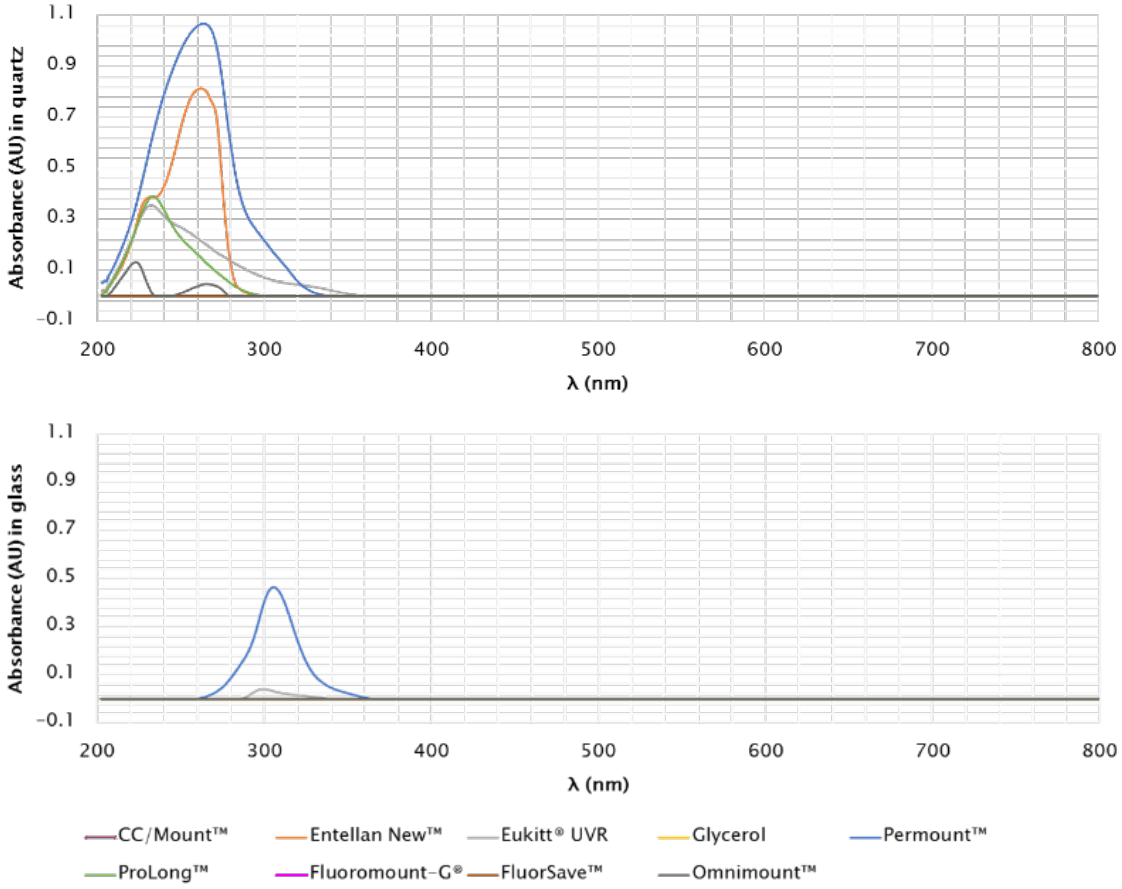

Figure 13: Comparison of UV absorbances for mounting media without fiber samples. (Top) Absorbance was quantified using quartz slides and quartz coverslips. Blue bars represent the range of non-zero absorbance readings for mounting media in quartz. (Bottom) Absorbance was quantified using glass slides and glass coverslips. Negative absorbance values were corrected to be zero in all data. Orange bars represent the range of non-zero absorbance readings for mounting media in glass. Negative absorbance readings were converted to zero. Absorbance values were recorded from 200 nm to 800 nm wavelengths (only 200-400 nm displayed for clarity).

For absorbance measurements of mounting media in quartz preparations, Omnimount™ (two peaks), ProLong™, Permount™, Eukitt® UVR, and Entellan New™ absorbed in the 200 nm – 360 nm range, whereas glycerol, FluorSave™, Fluoromount-G®, and CC/Mount™ did not absorb across the entire spectrum (Figure 13, top panel). The absorbance of aqueous-based mounting media was significantly less than instrument noise (± 0.3 absorbance). All organic-based mounting media observed absorbed to some extent in the transparent slide preparation. In glass preparations, only Eukitt® UVR and Permount™ exhibited absorbance in addition to the baseline absorbance of glass (Figure 13, bottom panel, n=6 readings per mounting medium), although the absorbance of Eukitt® UVR' was minimal (maximum absorbance less than 0.1AU).

Distinct absorbance ranges were seen for mounting media that exhibited absorbance in quartz, (Figure 13) and the extent of absorbance (Figure 14) across 200 nm to 800 nm. In quartz, Omnimount™ absorbed at two ranges: 206 nm-233 nm, and 245 nm-278 nm, leaving a window of transparency under 300 nm (233 nm-245 nm); combined, the two absorbance peaks for Omnimount™ accounted for a low total absorbance of 24AU relative to other mounting media (Figure 15). Other mounting media absorbed in the ultraviolet region more broadly. Entellan New™ had the smallest continuous absorbance range (202 nm-295 nm) with the integral of the absorbance curve of 37AU for a single peak, the second-highest amount. Permount™ had an absorbance range of (202 nm-337 nm) with a total of absorbance of 61AU, the highest total absorbance of the group. Eukitt® UVR exhibited absorbance across the longest range of wavelengths (202 nm-350 nm) but had one of the lowest total absorbance of 21AU. Although ProLong™ had an absorbance range comparable to that of Entellan New™ (202 nm-299 nm), it had the lowest total absorbance among mounting media with a single absorbance peak (20AU total). Aqueous-based mounting media (i.e., CC/Mount™, Fluoromount-G®, and FluorSave™) exhibited virtually zero absorbance in the ultraviolet region when in quartz slide preparations, comparable to glycerol.

Only two mounting media absorbed in the ultraviolet region when prepared on glass slides. Permount™ absorbed broadly (260 nm-364 nm) with a total absorbance surpassing that of glass by 16AU, suggesting an additive light-blocking effect of Permount in the UV region. Eukitt® UVR also absorbed in the ultraviolet region, but at a smaller range (286 nm-340 nm), surpassing the absorbance of glass by a total of 1AU across a narrower spectrum of 286-460 nm. Considering the maximum absorbance of Eukitt® UVR in glass for its entire absorbance range as 0.4AU at 299 nm (Figure 14), Eukitt® UVR might be considered UV-transparent on glass but not on quartz, similar to other organic-based mounting media that displayed low absorbance on glass higher absorbance on quartz (Entellan New™, Omnimount™, and ProLong™). Note the shift in wavelength for each respective absorbance peak which may be a result of an additive absorbance effect when the mounting media is

absorbing energy in the glass preparation. No absorbance was detected in the range of 200 nm to 260 nm for any mounting media in glass when glass was used as the reference.

With regards to manufactured fiber absorbance, a maximum can be expected at around 300 nm. This is due to the glass itself preventing any readings below this wavelength, based on the absorbance of glass and mounting media themselves that was determined during transparency tests (Figures 12 and 13). Therefore, if the manufactured fiber exhibits absorbance at or below 300 nm, a deflection and artificial maximum may occur at around 300 nm, where signals below this wavelength cannot overcome the glass absorbance to be detected. Nonetheless, peak detection in the range bordering the absorbance of glass for different fiber types was the main focus of this portion of the study (Figure 15).

For experiments regarding fiber color discrimination that are discussed below, average spectra for different fiber types can be found in Figure 16. Specific values for absorbance, wavelength, their standard deviations, and number of replicates can be found in Table 4.

Orange acrylic fibers in glycerol exhibited a peak at 302.33 nm ± 0.35 with an absorbance of 0.16AU ± 0.01, as well as a maximum peak at 470.75 nm ± 0.35 with a maximum absorbance of 0.51AU ± 0.02 (n=5). The acrylic fiber produced similar readings when mounted in Entellan New™, Omnimount™, and FluorSave™, the latter of which had the highest absorbance readings for both peaks. However, the acrylic fiber mounted in Permount™ exhibited a reduced absorbance of 0.01AU ± 0.01 (n =5) at 315.21 nm ± 0.00 (n=5), a minimum not found in spectra taken with other mounting media. Similarly, ProLong™ exhibited a diminished signal at approximately 300 nm, but did not result in a minimum. The highest inter-variability of measurements of the same acrylic fiber occurred in FluorSave™ mounting medium (n=6), while the lowest occurred in Fluoromount-G® (n=6). Orange acrylic fibers in Fluoromount-G® had greatest peak distinction as evidenced by steeper slope values in the first peak range as defined by glycerol. First derivative analysis of the first peak for all mounting media further illustrate the fact that the spectra of fibers in Permount™ and ProLong™ have a different shape with different peaks and regions of concavity when compared to the other spectra of acrylic fibers in other mounting media and glycerol. Acrylic fibers in Permount™ display the opposite concavity when compared to measurements taken in all other mounting media.

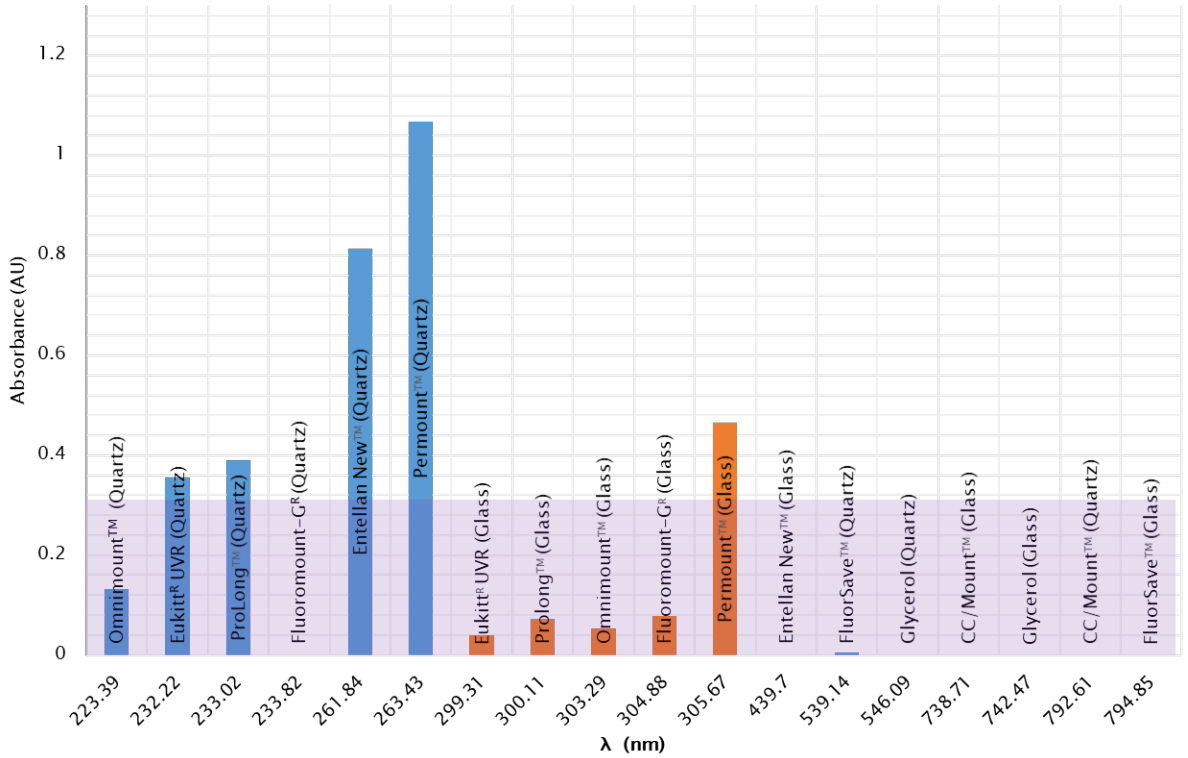

Figure 14: Summary of maximum absorbance at specific wavelengths for each mounting medium. Absorbance at each maximum for individual mounting medium spectrum in quartz (blue) and glass (orange). Instrumental noise is considered anything ± 0.30 AU (purple shaded region).

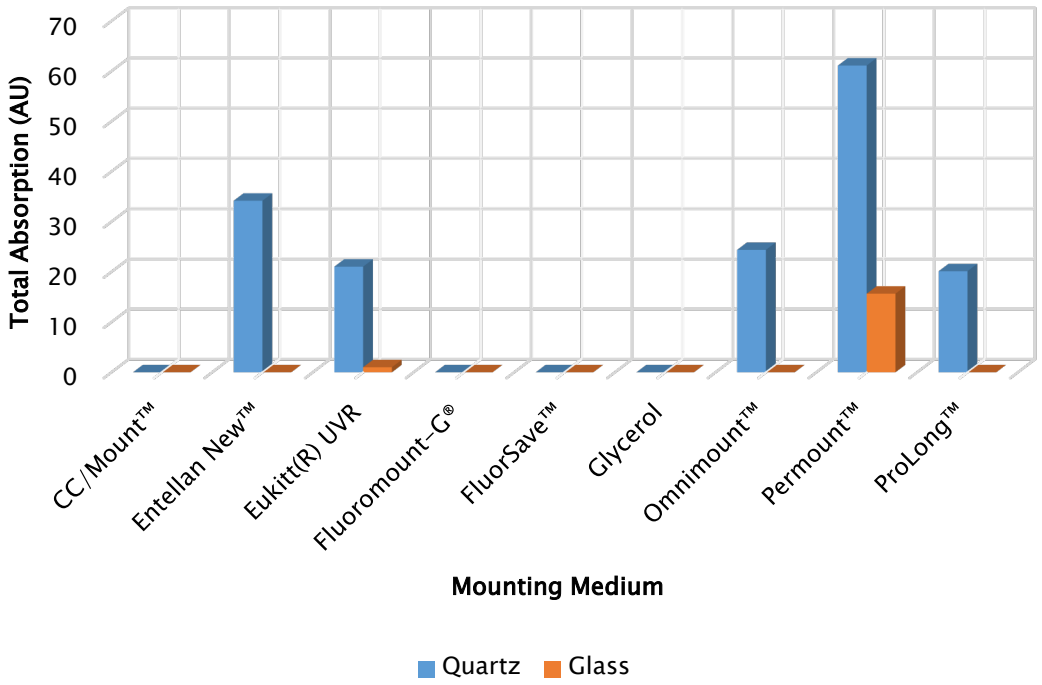

Figure 15: Absorbance integrals. Total amount of absorbance for each mounting medium from 200 nm to 800 nm, taking into account the extent of absorbance for broad and narrow peaks of similar intensities as well as baseline values. Note that quartz absorbance values (blue) reflect the true amount of absorbance in a maximally transparent preparation. In contrast, glass absorbance values (orange) reflect the amount of absorbance from the mounting media that are greater than absorbance coming from glass slide and glass coverslip. The sum of absorbance for all mounting media except Omnimount™ are calculated from a single peak of varying shape that occurs in the ultraviolet region, while the Omnimount™ absorbance results from two distinct peaks.

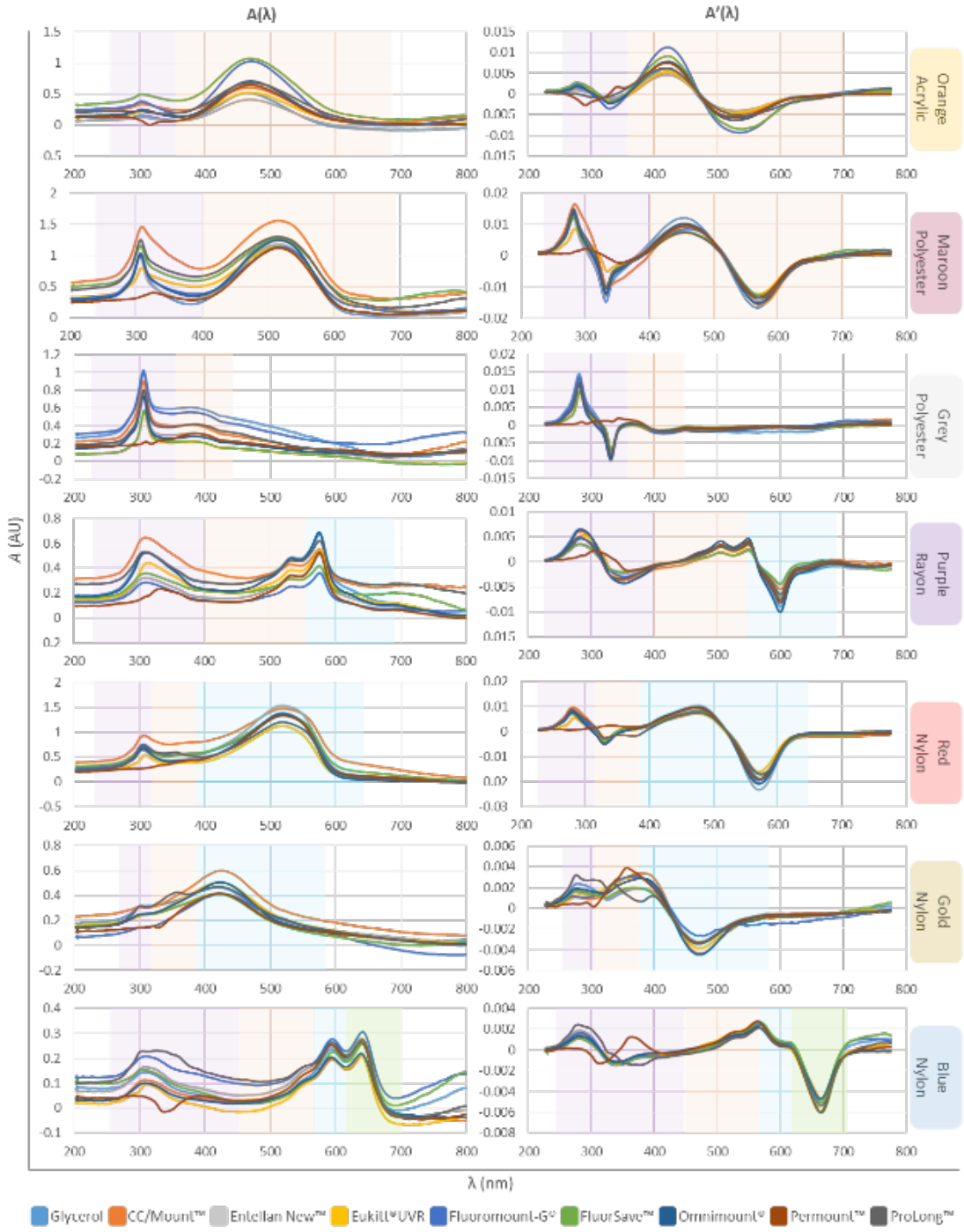

Figure 16: Comparison of MSP spectra of various fibers in mounting media. Average MSP spectra of fibers in different mounting media (Left) and corresponding first derivative curves (Right) for nine mounting media. Regions of interest are highlighted in different colors.

Table 4: Summary of peaks and features of interest. Wavelengths (λ, nm) of all maxima and corresponding absorbance readings (A, AU) with standard deviations (±) were used in side-by-side comparisons for all mounting media. Average values highlighted in blue. Taking into account baseline values when compared to glycerol, mounting media that exhibited high absorbance in the UV range are highlighted in green, while mounting media that exhibited low absorbance in the UV range are highlighted in orange.

		Glycerol	±	CC/Mount™	±	Entellan New™	±	Eukitt® UVR	±	Fluoromount-G®	±	FluorSave™	±	Omnimount™	±	Permount™	±	ProLong™	±	Average (All)	±
Orange acrylic	λ (nm)	302.33	0.35	303.93	0.87	*301.54*	*0.36*	308.28	0.76	302.97	0.44	295.58	20.50	303.29	0.56	258.64	6.21	291.99	10.76	294.96	17.99
	A (AU)	0.16	0.01	0.34	0.02	*0.13*	*0.01*	0.24	0.05	0.38	0.01	0.50	0.12	0.25	0.02	0.14	0.00	0.23	0.02	0.26	0.12
	λ (nm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	315.21	0.00	-	-	-	-
	A (AU)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.01	-	-	-	-
	λ (nm)	470.75	0.35	467.18	1.02	467.80	0.78	467.91	0.29	471.22	0.43	468.32	2.68	469.35	1.10	468.96	0.82	470.13	2.14	468.83	2.46
	A (AU)	0.16	0.01	0.60	0.03	0.41	0.01	0.52	0.04	1.02	0.01	1.07	0.13	0.71	0.04	0.65	0.02	0.68	0.06	0.68	0.22
			n=5		*n=5*		*n=5*		*n=5*		*n=7*		*n=5*		*n=5*		*n=5*		*n=5*		*n=9*
Maroon polyester	λ (nm)	306.47	0.00	309.25	1.09	306.31	0.36	309.08	1.00	306.79	0.43	306.63	0.35	307.10	0.35	331.31	11.98	306.94	0.43	309.38	6.42
	A (AU)	1.02	0.02	1.46	0.05	0.99	0.03	0.79	0.12	1.03	0.08	1.15	0.07	1.02	0.04	0.48	0.07	1.25	0.06	1.01	0.30
	λ (nm)	514.27	2.04	514.92	1.87	516.57	1.27	515.40	2.75	518.12	1.15	516.88	0.65	516.72	0.00	515.95	3.00	514.09	0.69	515.95	1.50
	A (AU)	1.29	0.08	1.56	0.07	1.17	0.04	1.14	0.08	1.14	0.07	1.26	0.06	1.24	0.03	1.12	0.06	1.30	0.04	1.25	0.14
			n=5		*n=6*		*n=5*		*n=7*		*n=5*		*n=5*		*n=5*		*n=13*		*n=5*		*n=9*
Grey polyester	λ (nm)	305.67	0.00	305.99	0.44	305.78	0.30	306.81	0.42	306.36	0.30	305.67	0.00	305.67	0.00	344.00	8.31	306.47	0.00	310.96	14.38
	A (AU)	1.02	0.04	0.92	0.07	0.80	0.10	0.56	0.05	1.01	0.07	1.11	0.04	0.73	0.02	0.25	0.04	0.80	0.06	0.74	0.25
	λ (nm)	377.02	2.51	370.43	13.66	381.29	2.13	372.73	15.86	376.90	4.71	378.01	1.98	382.73	2.98	385.57	2.17	384.78	0.96	381.02	2.61
	A (AU)	0.61	0.06	0.43	0.07	0.32	0.10	0.22	0.03	0.55	0.08	0.77	0.03	0.28	0.04	0.31	0.04	0.41	0.05	0.37	0.14
			n=5		*n=5*		*n=7*		*n=7*		*n=7*		*n=5*		*n=5*		*n=6*		*n=5*		*n=9*
Purple rayon	λ (nm)	309.76	0.55	309.81	2.27	310.18	3.47	313.46	0.36	309.65	1.23	309.65	2.56	310.91	0.43	332.06	1.50	308.53	0.43	312.91	7.24
	A (AU)	0.53	0.04	0.65	0.06	0.32	0.03	0.44	0.04	0.28	0.01	0.36	0.04	0.53	0.04	0.24	0.02	0.53	0.02	0.45	0.13
	λ (nm)	536.38	5.99	531.57	0.64	535.92	6.88	532.96	0.55	533.35	0.43	536.82	9.09	532.65	0.42	533.16	0.39	532.96	0.78	532.88	0.81
	A (AU)	0.49	0.03	0.43	0.04	0.35	0.02	0.39	0.02	0.25	0.02	0.32	0.05	0.48	0.03	0.34	0.02	0.46	0.04	0.41	0.06
	λ (nm)	576.00	0.29	575.80	0.42	576.11	0.00	575.96	0.34	576.11	0.00	575.34	0.63	575.34	0.00	575.92	0.38	575.34	0.00	575.77	0.41
	A (AU)	0.67	0.04	0.55	0.03	0.51	0.02	0.54	0.03	0.36	0.03	0.42	0.06	0.69	0.05	0.53	0.03	0.62	0.05	0.57	0.08
			n=7		*n=5*		*n=6*		*n=5*		*n=6*		*n=7*		*n=5*		*n=4*		*n=5*		*n=9*
Red nylon	λ (nm)	304.72	0.36	299.31	0.00	305.67	0.00	308.53	0.43	305.35	0.43	304.72	0.36	304.88	0.00	349.28	0.00	305.04	0.35	310.61	14.56
	A (AU)	0.64	0.04	0.85	0.15	0.76	0.01	0.54	0.03	0.75	0.02	0.71	0.01	0.67	0.02	0.37	0.04	0.72	0.04	0.68	0.16
	λ (nm)	320.76	0.00	320.76	0.00	320.76	0.00	320.76	0.00	320.76	0.00	375.75	30.74	320.76	0.00	389.50	0.00	320.76	0.00	334.51	3.42
	A (AU)	0.52	0.05	0.82	0.19	0.62	0.02	0.45	0.03	0.63	0.03	0.59	0.02	0.52	0.02	0.44	0.03	0.61	0.05	0.58	0.05
	λ (nm)	519.20	0.65	499.67	0.00	518.27	0.00	517.96	0.42	519.20	0.85	519.97	0.34	517.81	0.42	518.89	0.35	518.27	0.55	518.96	0.72
	A (AU)	1.38	0.07	1.40	0.13	1.53	0.05	1.12	0.04	1.35	0.02	1.35	0.05	1.37	0.02	1.33	0.05	1.20	0.04	1.35	0.12
			n=5		*n=6*		*n=5*		*n=5*		*n=5*		*n=5*		*n=5*		*n=5*		*n=5*		*n=9*
Gold nylon	λ (nm)	315.34	7.22	300.76	12.11	316.63	7.46	313.49	5.13	306.61	20.92	312.82	11.09	319.97	0.00	315.84	7.97	307.13	9.95	319.71	0.79
	A (AU)	0.35	0.09	0.31	0.06	0.25	0.02	0.24	0.06	0.15	0.03	0.25	0.08	0.26	0.03	0.16	0.01	0.32	0.07	0.25	0.06
	λ (nm)	379.27	0.00	379.27	0.00	379.27	0.00	379.27	0.00	379.27	0.00	379.27	0.00	379.27	0.00	379.27	0.00	357.97	0.71	349.28	0.00
	A (AU)	0.44	0.11	0.46	0.11	0.35	0.02	0.40	0.08	0.32	0.08	0.35	0.06	0.40	0.02	0.33	0.03	0.42	0.08	0.31	0.06
	λ (nm)	418.69	1.25	425.75	3.72	421.70	2.28	422.35	3.72	423.89	8.73	418.30	5.71	423.73	1.53	421.54	2.44	421.56	3.26	421.00	1.82
	A (AU)	0.51	0.12	0.59	0.13	0.42	0.03	0.49	0.09	0.41	0.09	0.41	0.04	0.51	0.01	0.42	0.03	0.46	0.08	0.47	0.06
			n=6		*n=5*		*n=5*		*n=6*		*n=5*		*n=6*		*n=6*		*n=5*		*n=6*		*n=9*
Blue nylon	λ (nm)	299.31	0.00	299.31	0.00	299.31	0.00	299.31	0.00	299.31	0.00	299.31	0.00	308.06	0.80	291.03	4.20	322.87	9.60	308.94	9.06
	A (AU)	0.14	0.02	0.16	0.11	0.16	0.01	0.07	0.01	0.20	0.06	0.15	0.03	0.10	0.01	0.05	0.01	0.23	0.04	0.14	0.06
	λ (nm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	337.88	0.71	-	-	-	-
	A (AU)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.02	0.03	-	-	-	-
	λ (nm)	593.60	0.34	593.85	0.64	593.14	0.64	592.22	0.00	601.56	15.76	595.90	0.34	592.99	0.00	593.29	0.42	594.65	0.76	594.09	1.16
	A (AU)	0.24	0.00	0.25	0.13	0.27	0.01	0.21	0.01	0.28	0.04	0.23	0.01	0.20	0.03	0.25	0.01	0.26	0.02	0.24	0.03
	λ (nm)	639.30	0.42	639.31	1.77	638.99	0.34	637.46	0.34	641.42	0.87	641.73	0.34	638.84	0.00	638.84	0.00	640.74	0.42	639.85	1.32
	A (AU)	0.26	0.00	0.25	0.11	0.28	0.01	0.21	0.01	0.31	0.04	0.26	0.01	0.22	0.03	0.26	0.01	0.27	0.01	0.25	0.03
			n=5		*n=8*		*n=5*		*n=5*		*n=5*		*n=5*		*n=5*		*n=5*		*n=6*		*n=9*

When in glycerol, maroon polyester fibers exhibited a first peak at 306.47 nm ± 0.00 with an absorbance of 1.02AU ± 0.02, as well as a maximum peak at 514.27 nm ± 2.04 with a maximum absorbance of 1.29AU ± 0.08 (n=5). In contrast, Permount™ had a diminished peak at 331.31 nm, 53% less than the average absorbance for this particular peak. Unlike in glycerol, first peak absorbance values for CC/Mount™ and ProLong™ were comparable to peak absorbance for their second peaks. Eukitt® UVR-mounted maroon polyester fibers exhibited variability for the first peak near the UV-threshold. As a consequence of the variability exhibited at the first peak, the highest overall inter-variability of measurements for the same fiber occurred in Eukitt® UVR mounting medium (n=6), while the lowest occurred in Entellan New™ (n=6). Highest overall inter-variability of measurements for the same fiber occurred in Eukitt UVR (n=6), while the lowest occurred in Entellan New™ (n=6). Additionally, baseline values were higher than that of glycerol for CC/Mount™, FluorSave™, and ProLong™. First derivative analysis of this region points to the wavelength of peak detection as the major contributor of variability, and not the extent of absorbance or the shape of the peak. With the exception of Permount™, tight clustering of first derivative spectra shows that the average spectra for maroon polyester fibers in all other mounting media were of similar shape.

Grey polyester fibers in glycerol had a single peak that was cut-off in the ultraviolet region at 305.67 nm ± 0.0 (1.02AU ± 0.04), shouldering off into a smaller peak of at 377.02 nm ±2.51 (0.61AU ± 0.06, n=5). All mounting media with grey polyester fibers except Permount™ displayed similar spectra. Lacking the characteristic peak in the ultraviolet region, Permount™ instead displayed a minimum absorbance of 0.18AU ± 0.03 near this region, at 311.9 nm ± 9.13 (n=6). For grey polyester fibers, the highest inter-variability of measurements occurred in Entellan New™ and Fluoromount-G®. Entellan New™ had variation in absorbance values throughout the visible spectrum while maintaining general peak shape, indicative of a baseline contribution. In contrast, Fluoromount-G® exhibited variation in regions between peaks. It is important to note that unlike maroon polyester fibers, grey polyester fibers possessed unevenly distributed dye. First derivative analyses show spectra for all mounting media except Permount™ clustered tightly.

Fibers with a thicker diameter were also tested. Purple rayon fibers in glycerol had a strong peak near the ultraviolet region at 309.76 nm ± 0.55, with an absorbance of 0.53AU ± 0.04 (n=7). Two other peaks registered in the red end of the visible spectrum. Purple rayon fibers in all mounting media displayed a similar pattern across the spectrum. Permount™ exhibited a slightly diminished first peak that appeared at a wavelength 19.15 nm greater than the average. In contrast, purple rayon fibers in CC/Mount™ had a first peak which, on average, greatly exceeded the absorbance of peaks in the visible region by 51%. First derivative curves for the average spectra of rayon fibers in all mounting media

except Permount™ shared the same critical and inflection points, with varying slopes in both the ultraviolet and visible region. Despite major differences in peak intensities and baseline absorbance, purple rayon fibers in all mounting media except Permount™ exhibited a similar spectral shape.

Nylon fibers known to have strong absorbance below 300 nm were also tested (red, gold, and blue nylon fibers). Red nylon fibers in glycerol displayed a peak at 304 nm ± 0.36 (absorbance of 0.64 AU ± 0.04, n=5), as well as a strong peak in the visible region at 519.2 nm ± 0.64 (absorbance of 1.38 AU ± 0.07, n=5). The subtle peak detected between the strong ultraviolet region peak and the strong visible region peak was most pronounced in ProLong™, occurring at 354.81 nm ± 0.35 with an absorbance of 0.59 AU ± .05 (n=5). However, fibers in Permount™ did not display the first peak near the ultraviolet region, instead displaying a shifted peak of similar shape at 349.28 nm ± 0.0 with an absorbance of 0.37 AU ± 0.35 (n=5). This signal shifted at a wavelength 38.7 nm greater than the average wavelength of the first detectable peak for all mounting media. Red nylon fibers in all other mounting media displayed spectra patterns reflecting those in glycerol, but ProLong™ displayed an additional signature between the ultraviolet peak and the visible peak (0.59AU ± 0.05 at 354.81nm ± 0.35, n=5). First derivative analysis shows that this extra peak can be considered a shoulder as A' reaches 0 before the appearance of this peak. In ProLong™, a change in concavity occurs before the appearance of this peak. As with the case of maroon and grey polyester fibers, red nylon fibers displayed strong similarities in shape in the visible region across all mounting media except Permount™, based on first derivative curves.

Gold nylon fiber peaks were varied among measurements in different mounting media. When in glycerol, gold nylon fibers displayed a peak of 0.38AU ± 0.10 at 349.28 nm and 0.51AU ± 0.12 at 418.69 nm ± 1.25 (n=6). Similar patterns were measured observed for fibers in Entellan New™, Eukitt® UVR, Fluoromount-G®, FluorSave™, and Omnimount™. A more distinct second peak in the visible region was detected in CC/Mount™. Fibers in Permount™ displayed a similar peak pattern, but the first peak at 315.84 nm ± 7.97 did not bleed into the adjacent, stronger peak of 0.42AU ± 0.03 at 421.54 nm ± 2.44 (n=5). Three overlapping peaks were observed in the range of 300-420 nm for gold nylon fibers mounted in ProLong™ at 307.13 nm ± 9.95 (0.32AU ± 0.07, n=6), 357.97 nm ± 0.07 (0.42AU ±0.08, n=6), and 421.57 nm ± 3.26 (0.46AU ± 0.08, n=6). The detection of an extra component contributing to the overall spectra of gold nylon fibers in ProLong™ between 360 nm and 420 nm was not found in other mounting media. The widest variation of spectra readings occurred in FluorSave™ (within the range of 300 to 500 nm) and Fluoromount-G® (beyond 420 nm). First derivative analysis of gold nylon fibers demonstrated the variability in the ultraviolet region between measurements of the same fiber in different mounting media. While similarity is highlighted in first derivative curves,

dissimilarity is equally accentuated as the average spectra did not appear very different from one mounting medium to another. The shapes of the spectra for all mounting media were similar only in the visible region (last detected peak). Overall, based on both the average spectra and the first derivative curves, Entellan New™ and FluorSave™ closely matched glycerol. ProLong™ revealed the presence of more peaks when compared to other mounting media. The least informative in the UV region for this particular fiber set was Permount™.

Blue nylon fibers exhibited a pattern of three peaks in all mounting media except Permount™. In glycerol, the fibers exhibited a peak of 0.13AU ± 0.02 at the 299.31 nm region (n=5), another peak of 0.24AU at 593.60 ± 0.34 nm, and a maximum peak of 0.26AU at 639.30 ± 0.42 nm (n=5). Instead of a peak, fibers mounted in Permount™ displayed a trough in absorbance of -0.02AU ± 0.03 at 337.88 nm ± 0.71 (n=5). This signature was not found in the spectra of blue nylon fibers in other mounting media. However, Permount™ spectra in the visible region were similar to those obtained in other mounting media. Despite the variation in readings for Fluoromount-G® and ProLong™, spectral patterns remained similar to the control spectra. First derivative analysis demonstrates blue nylon fiber spectra measured in all mounting media except Permount™ have similar shape throughout the ultraviolet and visible spectra.

A small 0.8 nm – 2.39 nm gain in the ultraviolet region at which information was obtained was observed when changing the slide type from glass to the less-absorbent UVT acrylic slide (Figure 17); however, a gain in intensity in the ultraviolet region was observed. For blue nylon fibers, the ultraviolet peak occurs on quartz at 247.84 nm at an absorbance of 0.59AU. The same peak occurs in Permanox® at 305.67 nm and 0.27AU, while glass measurements occur at 308.06 nm at 0.14AU (Figure 17). Visible spectrum peaks occurred for quartz, Permanox®, and glass at 630.55 nm, 638.08 nm, and 639.6 nm, respectively, with absorbance generally the same (0.21AU – 0.30AU). For white nylon fibers, measurements of the first peak were measurable in quartz and Permanox® but not in glass. Comparing quartz and Permanox®, there was a shift in peak detection of 22.29 nm (maximum at 283.38 nm and 305.67 nm for quartz and Permanox®, respectively). For red nylon fibers, a similar shift in peak of 42.24 nm was detected (maximum at 261.84 nm and 304.08 nm for quartz and Permanox®, respectively). In all cases, spectra from quartz and glass, but not Permanox®, were comparable in the visible region with similar peak maxima, intensity, and overall shape. The currently available UVT acrylic slides provided only limited increased sensitivity in the UV region compared to the less expensive glass slide alternative. For the three fibers studied, the benefits of the slightly improved sensitivity did not appear to outweigh the increased cost of the slides. In addition, peak maxima in the ultraviolet region are shifted significantly or even diminished when taking measurements in

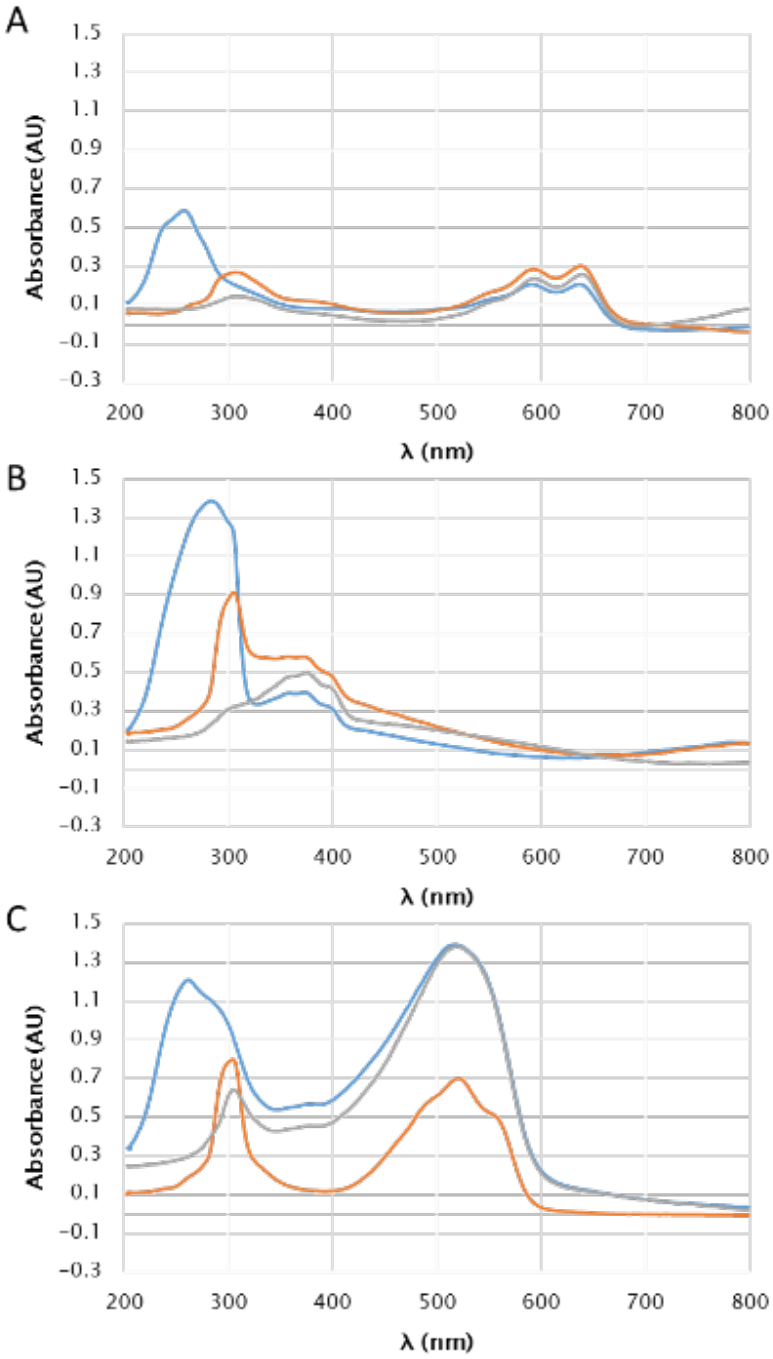

Figure 17: Average shifts in fiber spectra through quartz alternative. (A) Blue, (B) white, and (C) red nylon fiber spectra were compared with readings when mounted in glycerol on full-sized quartz slides and coverslips (blue line), Permanox® slides and cover-slide (orange line), and standard glass slides and coverslips (grey line). Slight information gain in the ultraviolet region below 300 nm was gained by using Permanox® slides when compared to glass slides.

slides and coverslips that are more absorbent than quartz.

Finally, mounting media that were more UV-transparent than glycerol displayed the same spectra shape for multiple fiber types. Some mounting media, such as CC/Mount™ and Fluoromount-G® achieved higher signal values when compared to glycerol on glass in terms of peak distinction in both the ultraviolet and visible regions, while others like ProLong™ revealed peaks that were not detected in any other mounting medium for certain fibers. In all cases, Permount™ displayed diminished peak heights or no detection of peaks at all in the ultraviolet region.

CONCLUSION

Overall, mounting media displayed different strengths and weaknesses. Color, toxicity, ease of handling, and ease of retrieval were characteristics that were immediately evident upon first use. This study shows the evaluated mounting media exhibited different advantages and disadvantages, particularly in terms of clarity, setting time, autofluorescence, UV transparency, and color discrimination of samples. Resolution of peaks in the visible spectrum may also be interesting. Aqueous mounting media overall performed well in the UV spectral range, but glass was the major limiting factor. Further work needs to be conducted in finding more cost-effective alternatives to quartz slides that can be used in place of glass slides. Before any firm recommendations can made, further comprehensive validations must be done to evaluate the long-term conditions and stability of water-based mounting media after several months of storage at room temperature and under highly non-temperature controlled conditions.

A couple of preliminary recommendations can be made. By far, the easiest to use mounting medium is CC/Mount™. It is the least hazardous, easy to use, and transparent for the purposes of MSP. Other glycerol-based mounting media like Fluoromount-G™ provide similar advantages. If a sample must be extracted, transferred, remounted, and placed into multiple preparations, mounting media like CC/Mount™ will be the most beneficial to use due to its ease of extraction. Unlike other solvent-based mounting media, the risk of losing parts or all of the fiber sample are significantly reduced if using CC/Mount™. This is especially true when dealing with hair samples, the examination of which are often done in conjunction with DNA analysis. CC/Mount™ may also be a suitable alternative to glycerol because it is less prone to smearing or dripping, and can be used to temporarily store samples in a solidified mounting medium. While Permount™ retains practicality in casework usage, other mounting media offer several advantages over it.

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Supplementary Table 1: Fiber inventory. Collection of natural and synthetic fibers used in microspectrophotometry aspects of the project. Note that color descriptions are macroscopic observations and do not necessarily represent the true color of the fiber set. Casework reference fiber refers to a collection of commercially available fibers samples purchased and used by hair and fiber examiners in our laboratory for training and validation purposes.

Type	ID	Color Description	Source
Acrylic	B-39	Orange	Casework reference fiber
Acrylic	B-37	Gold	Casework reference fiber
Cotton	-	Grey	Casework reference fiber
Cotton	JOMA C-wt	White	Terry by Wells Lamong CE 0072
Cotton	JOMA C-o	Orange	Terry by Wells Lamong CE 0072
Rayon	Coloray	Violet	Colovay courtallids #8
Polyester	1 - Blue	Blue	Casework reference fiber
Polyester	2 - Yellow	Yellow	Casework reference fiber
Polyester	3 - Orange	Orange	Casework reference fiber
Polyester	4 - Grey	Grey	Casework reference fiber
Polyester	5 - Green	Green	Casework reference fiber
Polyester	6 - Green	Green	Casework reference fiber
Polyester	7 - Yellow	Yellow	Casework reference fiber
Polyester	8 - Green	Green	Casework reference fiber
Polyester	9- Green	Green	Casework reference fiber
Polyester	10 - Green	Green	Casework reference fiber
Polyester	11 - Green	Green	Casework reference fiber
Polyester	12 - Maroon	Maroon	Casework reference fiber
Polyester	13 - Yellow	Yellow	Casework reference fiber
Polyester	14 - Light Green	Light Green	Casework reference fiber
Nylon	Nylon 1	royal blue	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 2	brick red	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 3	purple	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 4	orange	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 5	white	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 6	forest green	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 7	gold	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 8	wine berry	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 9	kelly	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 10	red	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 11	black	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 12	maroon	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 13	silver	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 14	navy	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 15	black	Seattle Fabrics, Inc. Batch #518

Supplementary Table 1: Fiber inventory (Continued)

Type	ID	Color Description	Source
Nylon	Nylon 16	forest green	Seattle Fabrics, Inc. Batch #518
Nylon	Nylon 17	black	Seattle Fabrics, Inc. Batch #518