Raman spectroscopy as a tool for the collection management of microscope slides

Thomas Schmid^{a*}, Robert Jungnickel^a, Birger Neuhaus^b, Jens Riedel^a, Janina Kneipp^{a, c},
Carsten Lüter^b

Abstract

Throughout the history of preparation of biological samples for microscopy the choice of the embedding medium was sometimes dictated merely by availability of the used media. Thus, a plethora of resins and other organic polymers as well as complex mixtures are found to serve as mounting agents in microscope slide collections of museums of natural history, impeding the work for both curators and conservators. Dramatically, in some cases the used embedding media can already be observed to have undergone crystallization and other decomposition processes within few years of mounting demanding immediate action in restoring as well as an imminent precaution in conservation. Therefore, an unambiguous chemical identification of the used agent as well as its current aging stage is of great interest for the biologist community. The technical demands on the analytical approach to obtain this information can be straightforwardly identified. Any used technique has to be non-destructive, yield in molecular information allowing for a chemical identification of the used embedding agents and allow for a spatially well-defined interrogation in a thin sample slice, typically through a transparent cover slip. In this contribution we present a thorough study of the applicability of Raman spectroscopy for the described task. The obtained results clearly demonstrate the successful feasibility of the chosen method for a) a clear distinction between different media, b) the elucidation of the chemical composition of a multicomponent medium and c) an unambiguous identification of real unknown samples by a distinct assignment to a previously recorded spectral library. This library database was built up by recording pure embedding agents and will be provided to the general public. In combination with a Raman spectrometer, it can be an invaluable tool for future curation and conservation endeavors devoted to microscope slide collections at natural history museums.

Keywords

Taxonomy, embedding medium, coverslip seal

Abbreviations

DPX Distrene Plasticizer Xylene

N.A. numerical aperture

USNM followed by catalogue number of United States National Museum of Natural
History, Washington, D.C., Smithsonian Institution

ZMB collection “Vermes” of Museum für Naturkunde Berlin (formerly Zoological
Museum Berlin)

1. Introduction

Small biological specimens or their organs were historically first studied as dry mounts, macerated or sliced material, and in liquids (Bracegirdle, 1978). Proper fixation of biological specimens did not arise before 1833 (Jacobson: chromic acid), 1846 (Blanchard: mercuric chloride, more widely established after 1878 by Lang), 1851 (Clarke: acetic acid plus ethanol), 1882 (Flemming: osmium tetroxide), and 1893 (Blum: formaldehyde) and represented more an alchemistic approach than a scientific one (Bracegirdle, 1978: pp. 60–62). Also, staining of specimens was not developed systematically but by trial and error, so Bracegirdle (1978: p. 65) states “[...] in the 1870s and 1880s especially, virtually every coloured substance was tried out for its microscopical effects and reported in the literature”. It was first Ehrlich to establish “the basic theoretical framework of staining” in 1880 (Bracegirdle, 1978: p. 66). The first resin mounts go back to 1795 using venetian turpentine, and mounts in Canada balsam were introduced around 1832 (Bracegirdle, 1978: p. 88). Another resin still used today is Euparal described by Gilson (1906). This author did not provide a formula of the ingredients but requested commercial companies to produce the medium. For this reason, Euparal seems to have been discredited among scientists for a long time (Bracegirdle, 1978: p. 91). Among the water-based embedding media, gum with glycerin was first used by Klebs in 1869 and by Stevenson in 1876 (Bracegirdle, 1978: p. 79).

Warnings about problems with embedding media were uttered by various authors (Ewing, 1939; Higgins, 1960, 1988; Amrine & Manson, 1996; Brown, 1997; Neuhaus, 2013; Neuhaus & Kegel, 2015), summarized for gum-chloral media by Upton (1993) and more generally by Neuhaus and Schmid (2016). Reasons mentioned for the deterioration of slides were partly contradictory and contained numerous assumptions, e. g., “incursions of air, too much chloral hydrate, too little chloral hydrate, crystallization of chloral hydrate, poor quality gum arabic,

1 presence of phenols, absorption of atmospheric moisture, too much glucose, contamination of
2 medium by ringing compound, breakdown of ringing compound, incorrect heat treatment of
3 slide after preparation, etc., have been cited as causes.“ (Upton 1993, p. 273). Reviewing the
4 microscope slide collection of the Natural History Museum of London, Brown (1997)
5 reported that Canada balsam seemed to last for at least 150 years contrary to almost all other
6 embedding media used. Few experiments on the properties of the components of slides have
7 been conducted (Allington & Sherlock, 2007a, b). Modern non-destructive methods from
8 materials science for the identification of ingredients of embedding media such as X-ray
9 fluorescence analysis and infrared spectroscopy were used in a single event and merely
10 mentioned in a footnote (Garner & Horie, 1984). Protocols for salvaging slides were
11 published occasionally and came to different solutions for different taxa and different types of
12 slides (Moore, 1979; Fain, 1980; Garner & Horie, 1984; Brunner & Blueford, 1986; Gunter &
13 Brown, 2005; Brown & Boise, 2005, 2006; Allington-Jones, 2008; Jacinavicius et al., 2013).
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34 In this study, we apply a non-destructive method, *viz.*, Raman spectroscopy, to microscope
35 slides in order to a) create a spectral library database of various embedding media and their
36 ingredients, b) identify chemical ingredients of selected embedding media, and c) verify
37 unknown media of real microscope slides from natural history collections. We expect this
38 technique to become an invaluable tool in the collection management of microscope slides.
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51 **2. Material and methods**

52 **2.1 Microscope slide samples**

53 All microscope slide samples used in this study originate from the natural history museums in
54 Berlin, Copenhagen, and Washington, D.C. The most important data are summarized in Table
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1, some slides are shown in Figure 1a–g. Often, it was unknown at which time exactly the specimens were embedded.

2.2 Mounting media and further chemicals

Mounting media were obtained from several sources via the Museum für Naturkunde (Berlin, Germany): Berlese's mounting medium, polyvinyl lactophenol (subsample taken by B. Neuhaus at the Zoological Institute at Göttingen); Hoyer's mounting medium (mixed during this study, see section 2.4); Canada balsam (charge: ZC893291 250), Canada balsam (charge: 6092544), Entellan neu, Kaiser's glycerol gelatin (Merck KGaA); Canadabalsam nat. (charge: 35524371), Roti-Histokitt II (charge: 35573927) (Carl Roth GmbH + Co. KG); Eukitt (ORSAtec GmbH formerly O. Kindler GmbH); DPX mountant for Histology (Fluka); Euparal grün (Waldeck GmbH & Co. KG); Araldite resin, hardener DDSA, accelerator BDMA (Agar Scientific); Fluoromount G (SouthernBiotech); Vectashield mounting medium (Vector Laboratories); Technovit 7100 (Heraeus Kulzer GmbH); Permout (Fisher Scientific); Pertex (origin unknown). Gum arabic (Ph. Eur., spray dried) and glycerol were purchased from Roth, while chloral hydrate was received from the old stock of the Museum für Naturkunde (Berlin, Germany). The potential composition of the mounting media is listed in Table 2 based on the literature and on Materials Safety Data Sheets. Probably, not all ingredients are mentioned, because they are proprietary of the manufacturer. The composition of natural resins like Canada balsam, gum arabic, and sandarac is based on the analysis of the original ingredients or of the raw resins; the final products may contain fewer or different chemicals (Lillie et al., 1953: p. 58).

2.3 Raman microscope

All spectra presented here were collected using a LabRam HR 800 instrument (Horiba Jobin Yvon, Bensheim, Germany) coupled to an upright BX41 microscope (Olympus, Hamburg, Germany). For excitation, two different lasers were employed: a HeNe laser having a wavelength of $\lambda = 632.8$ nm (red) and a diode laser with 785 nm wavelength (close to the near-infrared (NIR) spectral range). Approximately 10 mW of laser power at 632.8 nm or approximately 30 mW at 785 nm were focused onto the samples by using a 50x/ $N.A. = 0.75$ objective or a 50x/ $N.A. = 0.55$ long working distance objective in the case of liquid and powder samples, respectively (see also section 2.4). As described below (see Eq. (1)), with a numerical aperture ($N.A.$) of 0.75, the lateral resolution of the microscope is approx. 520 nm at $\lambda = 632.8$ nm and approx. 640 nm at $\lambda = 785$ nm. The diameter of the focal spot is approximately twice this value. The same objective lens was used for both, excitation and collection. The collected light was dispersed by a grating with 300 grooves/mm and detected by a liquid-nitrogen cooled charge-coupled device (CCD) detector (operating temperature: -126°C) with 1024 x 256 pixels leading to spectral resolutions of approx. $2\text{ cm}^{-1}/\text{pixel}$ at 632.8 nm and approx. $1.5\text{ cm}^{-1}/\text{pixel}$ at 785 nm.

2.4 Sample preparation

The microscope slide samples from museum collections were placed on the microscope stage and measured without any further preparation using the 50x/ $N.A. = 0.75$ objective lens. For the collection of database spectra, some drops of each mounting medium were placed on conventional microscope glass slides without a cover slip on top and allowed to harden in normal laboratory atmosphere within at least five days prior to the first measurement (50x/ $N.A. = 0.75$ objective). Powder samples of pure chemicals were placed on microscope glass slides and directly measured. Liquid samples, such as glycerol or gum arabic in water

1 were filled into small glass vessels and placed on the microscope stage using a microscope
2 slide as support. In order to avoid the risk of contamination of the objective lens, liquid and
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4 powder samples were measured through the long working distance objective lens
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7 (50x/ $N.A.$ = 0.55).
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11 For assigning Raman bands to individual constituents, we measured spectra of Hoyer's
12 mounting medium produced by mixing its components, Hoyer's mounting medium without
13 chloral hydrate as well as aqueous solutions of chloral hydrate and gum arabic. The chloral
14 hydrate solution was prepared by mixing 0.51 g chloral hydrate with 0.24 g water, yielding
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16 the relative amount of chloral hydrate that is also contained in Hoyer's mounting medium. In
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18 the case of the gum arabic solution, 30 g of the spray dried resin were mixed with 50 g water
19 and dissolved by stirring for 2 days until a clear solution was obtained. For preparing Hoyer's
20 mounting medium, 1.66 g chloral hydrate was mixed with 0.67 g gum arabic solution and
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22 0.17 g glycerol. The variant of Hoyer's mounting medium lacking chloral hydrate was
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24 prepared by mixing 0.67 g gum arabic solution with 0.17 g glycerol.
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39 **2.5 Data processing**

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41 For better comparability, most spectra shown here were stacked by adding arbitrary offset
42 values. In these cases no Raman intensity (y axis) scale is given in the figures. When
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44 necessary, spectra were additionally scaled by multiplication with a factor, which is
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46 mentioned next to the according traces. No further spectra treatment methods such as
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48 smoothing or baseline correction were applied.
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3. Theoretical background of Raman spectroscopy and microscopy

In 1928, C.V. Raman and K.S. Krishnan provided the first experimental proof for the inelastic scattering of light by studying the interaction of sunlight with organic liquids (Raman and Krishnan, 1928). In today's Raman spectroscopy typically visible lasers are employed for excitation. Upon interaction with a sample, several competitive spectral effects can arise. One of them is inelastic light scattering, in which a portion of the light's energy is transferred to the sample molecules, leaving photons with slightly shifted frequencies (*i.e.*, slightly shifted colors). The frequency shift between the laser source and a Raman scattered photon equals the frequency of a molecular vibration of the sample. This Raman shift is usually expressed in wavenumbers (unit: cm^{-1}). The set of all Raman-active resonance frequencies (*i.e.*, the Raman spectrum) of a molecule depends on the masses of its atoms, their distances (bond lengths and strengths) and spatial arrangement, and is therefore a very characteristic “fingerprint” of a molecular structure. Like with real fingerprints, comparison of the whole Raman spectrum of an unknown sample with reference spectra enables the unambiguous identification of sample constituents. Indeed, the “suspects” and their spectra have to be known in advance. Therefore, an important part of this work is the build-up of a spectroscopic database including typical mounting media and some of their constituents. A thorough explanation of the theoretical background of Raman spectroscopy was given by McCreery (2000).

Due to the compatibility of the used Raman microscope with microscope slide samples, the measurements are relatively straight forward. A Raman microscope is a combination of a conventional (confocal) microscope and a Raman spectrometer allowing to observe a sample in transmitted light brightfield microscopy mode and subsequently – after switching the instrument to Raman mode – collecting Raman spectra of selected, microscopic parts of the sample. The lateral resolution, which is defined as the minimum distance of two objects

allowing to identify them as two barely separated objects, depends on the wavelength of light (λ) and the numerical aperture ($N.A.$) of the objective lens. The $N.A.$ of an objective is a measure for the focusing angle and *vice versa* for the size of the solid angle of the cone of collected light. It is a dimensionless fixed number determined by the manufacturer and can typically be found written on the objective's housing. By definition, $N.A. = 1$ – corresponding to 180° focusing and collection angle – is the highest value that can be achieved in air. As the $N.A.$ linearly scales with the refractive index of the medium between the objective front lens and the specimen, higher numerical apertures are available with water or oil immersion objectives, reaching values of up to 1.6. According to the Rayleigh criterion (Rayleigh, 1879), the lateral resolution (R) of a microscope linearly depends on the wavelength of light and is indirectly proportional to the numerical aperture of the focusing lens:

$$R = 0.61 \frac{\lambda}{N.A.} . \quad (1)$$

As a rule of thumb, the lateral resolution is approx. λ when working with air objectives (assuming $N.A. \approx 0.6$) and approx. $\lambda/2$ with immersion objectives (assuming $N.A. \approx 1.2$) (Álvarez-García et al., 2016). Thus, in the visible spectral range from 400 nm to approx. 800 nm wavelength, lateral resolutions of 200 nm to approx. 1 μm can be achieved. The diameter of the focus spot is approximately $2R$. The depth resolution of such instrument is typically on the order of several 10s of micrometers (also depending on λ and $N.A.$) and can be improved down to a few micrometers by spatial filtering: By reducing the size of a pinhole aperture placed in front of the detector, out-of-focus contributions to the signal can be rejected (Everall, 2009). However, the overall signal intensity is reduced by this procedure. A review of modern Raman imaging techniques, including confocal Raman microscopy, was given by Opilik et al. (2013).

1 As more and more user-friendly Raman microscopy instruments become commercially
2 available, this technique becomes accessible to non-expert users. Thus, one has to keep in
3 mind the intrinsic limitations and pitfalls of the method. First of all, the concept of shining a
4 tightly focused laser beam onto microscope slide samples is commonly associated with
5 sample damage. In modern Raman microscopes, however, neutral density filters are
6 integrated, which allow to very precisely control the laser power reaching the sample. In this
7 study we used laser powers on the order of 10 mW, which did not cause any sample damage.
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9 As thermal damage by laser irradiation mainly depends on the actual light absorption
10 coefficient and heat dissipation properties of a sample, the laser power should be chosen with
11 care for each individual type of samples.
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26 Another typical issue is fluorescence. As mentioned above, the exciting laser beam can induce
27 several spectroscopic effects in the sample at the same time. Raman scattering usually is a
28 very weak effect and can easily be overwhelmed by fluorescence, if the sample contains
29 fluorescing contaminants. In contrast to Raman scattering, fluorescence only can take place if
30 the sample absorbs the laser light. Thus, coloring of a sample is an indication for potential
31 fluorescence emission in the visible range of the electromagnetic spectrum. Furthermore,
32 fluorescence emission of a certain fluorophore always occurs at a specific emission
33 wavelength, whereas – as described above – Raman scattering occurs at a frequency or
34 wavelength, respectively, which is slightly shifted from the actual excitation laser. Therefore,
35 switching to another laser wavelength offers the chance to measure the same Raman shifts in
36 a spectral range, which does not interfere with fluorescence emission. For this reason we
37 collected spectra of each sample with two different laser wavelengths. Furthermore we locally
38 (and temporarily) reduced fluorescence emission at each measurement spot by prior
39 illumination with the laser for several minutes (see below).
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2 In the context of this study we also have to mention fluorescence emission bands from glass
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4 slides and cover slips. Especially if the specimen between glass slide and cover slip is only
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6 some 10s to 100 micrometers thin, interference of the measurement volume with glass is
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8 difficult and sometimes even impossible to avoid. As mentioned above, the resolution (and
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10 thus, the measurement volume) depends on laser wavelength and numerical aperture.
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12 Furthermore, depth resolution can be improved by spatial filtering. Indeed, also in this case
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14 switching to another laser might reduce interference from fluorescence. Below, we will
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16 describe our experiences in minimizing spectral glass band contribution (see section 4.3.2).
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26 **4. Results and discussion**

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28 The results and discussion section will be organized as follows. Firstly, a brief description of
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30 the experimental acquisition procedure, which was developed during this study, is given in
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32 section 4.1, followed by the introduction of Raman spectra of pure mounting media (section
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34 4.2). There, the concept of an unambiguous determination of the chemical composition out of
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36 a Raman spectrum will be demonstrated on the example of Hoyer's mounting medium
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38 (section 4.2.2). As a final result, the successful identification of unknown samples by
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40 assignment to a library database of spectra will be shown (section 4.3).
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48 **4.1 Measurement procedure**

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50 Simplified schematics of the Raman microscope setup and the major steps of the
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52 measurement procedure developed within this study are shown in Figure 2. As a Raman
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54 microscope is a combination of a normal (confocal) microscope and a Raman spectrometer,
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56 microscope slide samples are perfectly suited for Raman microcopy investigation without the
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1 need for any sample preparation. Both, pure mounting media on glass slides and microscope
2 slide samples from museum collections were directly placed on the microscope stage. In
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4 transmitted light brightfield microscopy mode, the samples were observed and the optics
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6 (typically with a 50x/ $N.A. = 0.75$ objective lens) were focused. The pure mounting media
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8 were prepared without a cover slip on top. In this case the optics were focused onto the
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10 interface between sample and air. In the case of microscope slide samples with cover slips,
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12 the optics were focused onto the biological specimen and subsequently the microscope stage
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14 was laterally moved by several 10s of micrometers (Fig. 2a). This way the measurement
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16 volume was placed within the layer of the mounting medium (in depth) and away from the
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18 specimen (laterally) in order to collect spectra from the medium without risking to damage the
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20 specimen if too high laser powers would accidentally have been chosen. With this, the sample
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22 was ready for Raman measurements and the instrument was switched to Raman mode with
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24 one of the two laser wavelengths, e.g., 632.8 nm.
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34 For checking the Raman signal intensity and possible fluorescence emission of the sample, a
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36 first spectrum with a short acquisition time of 1 s was collected. Most pure media and all
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38 microscope slide samples from museum collections exhibited fluorescence emission to some
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40 extent. Nevertheless, Raman spectra from all pure media could be collected, only in some of
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42 the microscope slide samples from museum collections strong fluorescence hampered Raman
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44 measurements even after applying the fluorescence reduction procedure described here.
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48 Continuous measurements with 1 s acquisition time each were performed and the spectra
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50 were observed on the screen. Under this online supervision the continuous illumination of the
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52 same spot led to local fluorescence reduction following a pseudo-exponential decay and
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54 reaching a plateau after typically several minutes (Fig. 2b). When a plateau (minimum) was
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56 reached, the Raman spectrum of the sample was measured with 60 s acquisition time (Fig.
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2c). If saturation of the detector occurred (more than 65535 counts on a 16 bit digital detector), the acquisition time was reduced accordingly and the number of accumulations was increased in order to have a consistent exposure time of 60 s for all spectra presented here. For example, instead of collecting a spectrum within 60 s, 2 spectra with 30 s accumulation time each or 5 spectra with 12 s accumulation time each were averaged. In the next step, the instrument was switched to the other laser wavelength, e.g. 785 nm, and the procedure was repeated.

Even some pure media, which were solely prepared from colorless, non-fluorescent compounds exhibited fluorescence emission. We explain this with aging effects as most pure media were measured after being stored and used in museum laboratories for several years to decades. As known, aging is often accompanied by a color change from transparent/colorless to yellow/brownish, which also gives rise to fluorescence emission. Here the underlying physics of scattering (Raman) and absorption followed by emission (fluorescence) has to be recalled. By shining the laser onto a spot for several minutes, the fluorescing compounds absorb the radiation (while leaving the purely Raman-scattering and non-absorbing compounds untouched) and run through several excitation-emission cycles until losing their fluorescence properties. Thus, the fluorophores are locally bleached, whereas the remaining transparent material experiences no chemical transformation. We would like to point out that this bleaching effect is locally very restricted to the measurement volume of approx. $1\text{ }\mu\text{m}^2$ laterally and some 10s of μm in depth. Furthermore, we observed that if the bleached sample is kept in dark on the microscope stage and the exactly same spot is measured again after a few hours, the fluorescence is (at least partly) restored. We explain this “self-healing” effect by diffusion of molecules back into the previously illuminated volume. Thus, even if we very

1 locally change the composition of the sample for the time of the measurement, over a longer
2 time scale the whole procedure is non-destructive to the sample.
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7 As the stability of the wavenumber scale over the whole measurement series is essential for
8 this work, we not only calibrated the spectrometer to the main peak of a silicon standard
9 sample at 520.7 cm^{-1} at the start of each measurement day, but checked for stability of the
10 calibration by several repeated silicon measurements distributed over each measurement day.
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12 The silicon measurements were saved for documentation and the spectrometer was
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4.2 Raman spectra of mounting media

Figure 3 shows the Raman spectra of 12 mounting media collected at the laser wavelengths
 $\lambda = 632.8\text{ nm}$ and $\lambda = 785\text{ nm}$. Similarities between spectra are due to similar chemical
compositions, which allow to sort some of the media into groups. Most importantly, we group
mounting media into water-soluble and water-insoluble. The latter are soluble in organic
solvents, such as toluene.

4.2.1 Water-soluble media

The water-soluble mounting media are: Vectashield, Fluoromount G, Kaiser's glycerol
gelatin, polyvinyl lactophenol, Berlese's mounting medium, and Hoyer's mounting medium.
According to the literature, all of them contain water and glycerol. Most of the knowledge on
the compositions of mounting media discussed in this and the following sections were taken
from a literature research performed by Neuhaus and Schmid (2016). Data in Table 2 is taken
from that study. In addition to water and glycerol some media contain gum arabic (Berlese's
mounting medium, Hoyer's mounting medium), gelatin (Kaiser's glycerol gelatin), polyvinyl

1 alcohol (Fluoromount G and polyvinyl lactophenol), lactic acid (polyvinyl lactophenol), citric
2 acid and sodium phosphate (Vectashield), and chloral hydrate (Berlese's mounting medium
3 and Hoyer's mounting medium) (Upton, 1993; Neuhaus and Schmid, 2016; see Tab. 2). Due
4 to their similar compositions, we term these water-soluble media 'glycerol media'. The sub-
5 group containing gum arabic and chloral hydrate (*i.e.*, Berlese's mounting medium and
6 Hoyer's mounting medium) was previously termed 'gum-chloral media' (Upton, 1993).
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10 We found strong similarities between all spectra of the glycerol media. Band positions and
11 shapes between 750 cm^{-1} and 1500 cm^{-1} almost perfectly match each other for all glycerol
12 media (Fig. 3a, b). Only polyvinyl lactophenol shows some slight deviations in this range.
13 Except for Hoyer's mounting medium (and partly polyvinyl lactophenol and Berlese's
14 mounting medium) this holds true for the whole spectral range. We would like to point out
15 that the broad band at approx. 1370 cm^{-1} , visible in some of the 785-nm spectra (Fig. 3a), is
16 due to fluorescence emission of the glass slide and thus, not part of the spectra of mounting
17 media. Major differences are relatively strong bands at 273 cm^{-1} and 417 cm^{-1} and some other
18 signals, which are specific for the gum-chloral media and polyvinyl lactophenol, which were
19 investigated in this study. At this point, we can conclude that all water-soluble mounting
20 media in this study show very similar spectra, which we explain with their similar chemical
21 compositions, as all of them contain glycerol (Upton, 1993; Neuhaus and Schmid, 2016).
22 Furthermore, the gum-chloral group can be spectroscopically distinguished from other water-
23 soluble media because of at least two strong bands at 273 cm^{-1} and 417 cm^{-1} . Here, an
24 exception is polyvinyl lactophenol, which was previously not ascribed to the gum-chloral
25 media, but also shows these bands in our spectra. Another interesting observation is the
26 presence of these bands in the spectra measured at 785 nm and their absence in the 632.8-nm
27 spectra, which is explained below.
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4.2.2 Interpretation of the Raman spectrum of Hoyer's mounting medium

In order to better understand the spectra, and as a consequence, to find explanations for the described irregularities, we collected spectra of Hoyer's mounting medium, its components and Hoyer's mounting medium lacking chloral hydrate, which are shown in Figure 4.

Differences in the spectra enable to assign individual bands to constituents of this mounting medium. We found that in this case only the aqueous solution, not the pure powder of chloral hydrate can be spectroscopically compared to Hoyer's mounting medium, because all vibrational modes, which include –OH groups shift due to strong hydrogen bonding in aqueous solution (for the analog behavior of a chemically similar fluorinated geminal dihydroxy compound, see Murto (1975)). Most prominent are the shifts of the C–O bending and O–H bending vibrational modes from 516 cm^{-1} and 601 cm^{-1} in the powder sample to 575 cm^{-1} and 641 cm^{-1} in aqueous solution (Fig. 4a, b; band assignments based on the Raman spectrum of an aqueous solution of acetaldehyde (Matshushima, 1963)). Thus, these bands together with the strong modes at 273 cm^{-1} and 417 cm^{-1} , which were previously mentioned as marker bands for gum-chloral media (and polyvinyl lactophenol), can be assigned to chloral hydrate. In analogy with the vibrational spectrum of chloroform, we tentatively assign the most prominent modes of chloral hydrate at 273 cm^{-1} and 417 cm^{-1} to bending modes of C–Cl bonds (Ruoff and Bürger, 1970). By having identified the vibrational modes of chloral hydrate, we can explain one of the exceptions described above. Obviously, the polyvinyl lactophenol mixture we obtained from Museum für Naturkunde, in contrast to the recipes found in the literature (Neuhaus and Schmid, 2016), contained chloral hydrate as clearing agent. Based on the relative band intensities we conclude that only relatively small amounts of this compound were present in our samples of polyvinyl lactophenol and Berlese's mounting medium as compared to Hoyer's mounting medium. Interestingly, relatively weak

bands of chloral hydrate are present in the polyvinyl lactophenol and Berlese's spectra acquired at 785 nm (Fig. 3a), which vanish in the corresponding measurements performed at 632.8 nm (Fig. 3b). We explain this with the relatively high vapor pressure of chloral hydrate of 4.7 kPa (20°C) causing it to slowly evaporate when exposed to air (O'Neill et al., 2006) and with the order in which the measurements were performed. The 785-nm measurements were performed first and the measurements at 632.8 nm some days later after storing the media samples (without cover slips on top) in normal lab atmosphere allowing the chloral hydrate to evaporate. The generally low intensity of the chloral hydrate peaks at 273 cm^{-1} and 417 cm^{-1} may originate from storage of Berlese's medium over an extended period of time in a container without a tight lid. We found a similar effect for microscope slide samples with Hoyer's mounting medium and a cover slip not covering all of the medium. In such samples, we detected the whole Raman spectrum corresponding to Hoyer's mounting medium when measuring below the cover slip, but on uncovered areas on most spots the chloral hydrate signals were missing in the spectra. This demonstrates that Raman spectroscopy not only allows to acquire very specific data for the unambiguous determination of mounting media (or groups of media), it also allows to check the actual status of the medium in different parts of a sample. Thus, for example, the method enables to check if volatile substances, such as solvents, are still present in the matrix. We will come back to this point in section 4.3.

Assignment of the mentioned bands to chloral hydrate is confirmed by the spectrum of Hoyer's mounting medium without chloral hydrate, in which all chloral hydrate bands are missing (Fig. 4a, b). This spectrum mostly matches the Raman signals of glycerol, because the signals of gum arabic are relatively weak and partly overlap with signals from glycerol. By coincidence, there are overlapping bands at 417 cm^{-1} from both, chloral hydrate and glycerol, but both, widths and relative intensities of these bands allow an unambiguous

1 assignment. The chloral hydrate C–Cl bending mode at 417 cm^{-1} is sharper (narrower) and
2 more intense than the C–C–O bending vibration of glycerol (Bishambhar, 1939) observed at
3
4 the same spectral position. Relative intensity can be checked by comparing this band with its
5
6 neighboring band at 273 cm^{-1} (chloral hydrate) and 488 cm^{-1} (glycerol), respectively, showing
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8 that the chloral hydrate band is approx. 4 times as strong as its neighbor, whereas the glycerol
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10 band is only twice as strong as the band at 488 cm^{-1} . This example shows the importance of
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12 using the whole spectrum for comparison and not only single bands.
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19 Figure 4 also shows that the bands, which are specific for all water-soluble media investigated
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21 in this study (as mentioned, between 750 cm^{-1} and 1500 cm^{-1} , or in the whole spectral range
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23 for the media without chloral hydrate) are predominantly due to glycerol.
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4.2.3 Water-insoluble media

Coming back to Fig. 3, we now look at the spectra of mounting media soluble in organic solvents. Also here, a group of media revealing very similar Raman spectra can be identified. The spectra of Pertex, Eukitt and Entellan show a high resemblance throughout the whole range shown here. There are only small deviations for Pertex at around 850 cm^{-1} . Eukitt, Entellan and Pertex are known to be part of the polyacrylate media (Neuhaus and Schmid, 2016). Generally, these media consist of various types of polyacrylates dissolved in xylene. We tentatively explain the small spectral difference in the case of Pertex by either different acrylate monomers or different remaining amounts of the solvent xylene still present in the samples. All other bands, including the C=O stretching vibrational modes at 1728 cm^{-1} (Thomas et al., 2008) are in perfect accordance.

Furthermore, Fig. 3 contains Raman spectra of 3 mounting media, which are soluble in organic solvents and cannot be assigned to any group. Canada balsam is a natural balsam made from the resin of the balsam fir tree (*Abies balsamea*), which is dissolved in xylene. Permout consists of a polyterpene based on α -pinene, β -pinene, dipentene and β -phellandrene dissolved in toluene. DPX contains the polystyrene 'distrene 80', the plasticizer dibutylphthalate and the solvent xylene (Tab. 2; Neuhaus and Schmid, 2016). All strong and sharp bands in the spectra of Permout and DPX can be assigned to vibrational modes of aromatic rings and are explained in section 4.3. The spectra of 5 additional media, completing the actual status of our database, can be found in Figure 5. These are: Euparal, Araldite, Glyceel (a coverslip seal), Technovit 7100, and Roti-Histokitt II.

4.3 Identification of mounting media by Raman spectroscopy

Figure 6 shows examples of one water-soluble (Hoyer's mounting medium) and two water-insoluble media (Permout and DPX), which were unambiguously identified in 7 different samples taken from museum collections.

4.3.1 Hoyer's mounting medium

In Fig. 6a, b the spectra of the samples ZMB (*Pycnophyes* sp.) and USNM 234015 collected at 785 nm and 632.8 nm, respectively, are compared to the reference spectra of Hoyer's mounting medium revealing perfect matches in terms of band positions and shapes. As described above, in contrast to the spectra of other glycerol media, the Raman spectrum of Hoyer's mounting medium is dominated by the bands of chloral hydrate at 273 cm^{-1} and 417 cm^{-1} . Weaker bands between 750 cm^{-1} and 1500 cm^{-1} can mainly be assigned to glycerol. The spectra in Fig. 6a, b acquired at the two different laser wavelengths show a typical behavior. All microscope slide samples investigated in this study showed fluorescence to some extent. Most often, spectra collected at 632.8 nm are located on the rising edge of a fluorescence band, visible as a baseline increasing towards higher wavenumbers, or approximately on top of it, revealing an almost flat but significantly elevated baseline, as it is the case in this example. Even with the Raman spectrum acquired in the range of maximum fluorescence emission, in this and many other cases, the Raman bands were clearly identifiable after applying the local fluorescence reduction method described in section 4.1. Like in many other cases, the Raman spectrum collected at 785 nm is located on the falling edge of a fluorescence signal and thus, shows a much less elevated baseline, which decreases towards higher wavenumbers. In this case no significant differences are seen between the spectra collected at the two different laser wavelengths. Thus, both wavelengths are suited to

1 identify the media. The perfect match of spectra for both lasers is as an additional proof of the
2 correctness of the assignment and the robustness of the general approach.
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7 **4.3.2 Permout**

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9 In the case of the samples USNM 1226533, USNM 1226547, USNM 1226553 and
10 USNM 1225554 we found very significant differences between spectra collected at 785 nm
11 and at 632.8 nm (Fig. 6c, d), but still, an unambiguous identification of the medium Permout
12 was possible. The main difference is the presence of a very pronounced band at approx.
13 1370 cm^{-1} in the spectra excited at 785 nm, which is a contribution from the microscope glass
14 slide and cover slip. Such strong glass bands are typical for samples consisting of a very thin
15 layer of mounting medium between glass slide and cover slip making it almost impossible to
16 avoid overlap of the measurement volume with glass. This example nicely shows that glass
17 bands can be strongly reduced or even avoided when switching to 632.8 nm wavelength. This
18 has mainly two reasons. First, as fluorescence occurs on the wavelength scale, fluorescence
19 bands necessarily appear on different Raman shift positions when spectra are excited with
20 different laser wavelengths (for an explanation, see section 3). Thus, a different part of the
21 glass fluorescence spectrum, presumably having weaker bands, overlaps with the Raman
22 spectrum at 632.8 nm. Second, as described in section 3, both the lateral and depth resolutions
23 of a microscope are proportional to the wavelength of light. Therefore, the smaller focus of
24 light at 632.8 nm can be located more precisely within the mounting medium and has less
25 overlap with glass. In this example, some of the Raman bands coinciding with the broad glass
26 bands in the range of $> 1000 \text{ cm}^{-1}$ can still be identified, but vanish in case of the strongest
27 glass band (sample USNM 1226533), because they are overwhelmed by the much stronger
28 fluorescence. Also for other samples, very strong glass bands made Raman measurements in
29 the range of $> 1000 \text{ cm}^{-1}$ impossible, and in such cases also another, weaker glass band at
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1 approx. 370 cm^{-1} interferes with the Raman spectrum. To our experience, reduction of glass
2 bands by spatial filtering (see section 3), *i.e.* by reducing the size of an adjustable pinhole
3 aperture placed in front of the detector of the Raman microscope, was only very limited but
4 very significantly reduced the spectral intensity. Therefore, we propose to switch to another
5 laser wavelength to reduce the influence of interfering glass bands.
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12 The spectrum of Permout can quite easily be understood, as most strong and sharp bands are
13 due to the solvent toluene, whereas the broad bands are obviously due to the polyterpene this
14 medium also consists of (see section 4.2.3 and Table 2). In Figure 7, the Permout spectrum is
15 compared to the Raman signature of toluene, clearly revealing that all sharp bands in
16 Permout, some of them overlapping with broad bands, are due to the solvent. In addition to
17 the partly evaporated chloral hydrate mentioned in section 4.2, this is another example of how
18 the Raman method not only can identify the mounting medium that was employed to prepare
19 a sample, but also enables to check the current composition of the medium for the presence of
20 volatile compounds. In all samples included in Fig. 6c, d, the solvent was still present, which
21 is not the case for the solvent xylene in the DPX samples described in the following section.
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41 4.3.3 DPX

42 In Fig. 6e, f we demonstrate the identification of the medium in sample ZMB F.217a as DPX.
43 Interestingly, also this medium shows sharp bands appearing at the same positions as in the
44 toluene spectrum, but here, these are not due to the solvent, but can be assigned to
45 polystyrene. The high similarity of their spectra, which are dominated by vibrations of
46 aromatic rings, can be explained by the high chemical similarity of the aromatic ring and its
47 neighboring groups, which is a monoalkylated benzene in both cases. Nevertheless, Permout
48 and DPX can be distinguished based on their Raman spectra. For a more concise comparison
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of the two individual spectra please refer to Fig. 7. A comparison of the spectra of pure toluene and pure polystyrene reveals that only the bands at 1003 cm^{-1} , 1032 cm^{-1} , 1583 cm^{-1} and 1603 cm^{-1} are present in both spectra. Toluene has additional bands at, for example, 523 cm^{-1} , 624 cm^{-1} , 788 cm^{-1} , 1211 cm^{-1} and 1381 cm^{-1} , which are missing in the polystyrene spectrum, and *vice versa*, bands at, for example, 621 cm^{-1} and 1451 cm^{-1} are specific for polystyrene. When looking at the spectra of the mounting media, there are further bands allowing to distinguish them. As mentioned above, Permout in addition to the sharp aromatic modes, reveals several broad bands which can be assigned to the terpene polymer and are not present in the DPX spectrum. DPX shows additional bands at, for example, 652 cm^{-1} and 1730 cm^{-1} , which are not due to polystyrene and are not present in the Permout spectrum. They can tentatively be assigned to the plasticizer dibutylphthalate with the band at 1730 cm^{-1} being the C=O stretching vibrational mode of the carbonyl groups. In contrast to all Permout samples described above, we found that the solvent was (almost) completely evaporated, because xylene would give rise to additional Raman modes, which we were unable to detect in both, the sample ZMB F.217a and the pure, hardened medium.

5. Conclusion

A novel procedure for the discrimination of different mounting media was established. It exploits the fact that Raman microscopy allows for a direct applicability of microscopic preparations without any sample pretreatment or sample destruction/depletion. The Raman spectra of several commonly used preparatory agents reveal the chemical fingerprint of the used media, resulting in the formation of clusters or groups of chemically similar agents. Excitation with 632.8 nm and with 785 nm yield very similar spectra, indicating that the approach is very versatile, and identification of the samples can be attained with either of the

1 two wavelengths. A stepwise deconstruction of the spectra with respect to the individual
2 constituents shows that not only can the reagent mixture originally used for embedding but
3 also its current composition be identified. The method is proven to be capable of assigning
4 “real life” samples from museum collections to a catalogue of media-specific Raman spectra.
5 This contribution also serves as a starting point of a spectral database of embedding media in
6 biology and pathology. The library will empower a wide range of users to an unambiguous
7 material characterization and be expanded by external users data input.
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Figure captions

Fig. 1. Light micrographs of some slides used in this study. (a) ZMB F.217, *Dugesia milloti*, embedded in DPX in the 1980ies. (b) ZMB (*Pycnophyes* sp.), embedded in Hoyer's mounting medium about 1999. (c)–(d) USNM 1226547 (c) and USNM 1226553 (d), *Cateria gerlachi*, embedded in Permout according to our measurements between 1964–1967; embedding medium with peripheral white crystals (c) or still clear (d). (e) ZMB (Crustacea), probably embedded in Canada balsam about 1887. (f) Diptera, embedded in Canada balsam : phenol 1:1 about 2010. (g) ZMB Q.5732, Rotatoria, embedded about 1900, medium unknown.

Fig. 2. Schematic of the instrumental setup and measurement procedure.

Fig. 3. Raman spectra of water-soluble mounting media (blue graphs) and water-insoluble mounting media (green, black, orange and red graphs) collected at a laser wavelength of 785 nm (a) and 632.8 nm (b), respectively.

Fig. 4. Raman spectra of Hoyer's mounting medium in varying compositions and of pure constituents, allowing to assign Raman bands to individual compounds.

Fig. 5. Raman spectra of further water-insoluble mounting media collected at a laser wavelength of 785 nm (a) and 632.8 nm (b), respectively.

Fig. 6 Examples for the identification of mounting media (coloured traces) by comparison of their Raman spectra with reference spectra from our database (black traces).

Please notice that the huge peak at about 1370 cm^{-1} in (c) measured at 785 nm originates from glass fluorescence and does not become apparent at 632.8 nm (d).

Fig. 7 Comparison of the Raman spectra of Permout and its solvent toluene as well as the polymer constituent polystyrene and its complete medium DPX.

Table 1.

Microscope slide samples used in this study.

Catalogue number	Species	(Potential) embedding medium	Time of embedding
USNM 234015	<i>Pycnophyes borealis</i>	Hoyer's mounting medium	1986–1988
USNM 1226533	<i>Cateria gerlachi</i>	Permout (our measurements)	1964–1967
USNM 1226547	<i>Cateria gerlachi</i>	Permout (our measurements)	1964–1967
USNM 1226553	<i>Cateria gerlachi</i>	Permout (our measurements)	1964–1967
USNM 1226554	<i>Cateria gerlachi</i>	Permout (our measurements)	1964–1967
ZMB F.217a	<i>Dugesia milloti</i>	DPX	1980ies
ZMB Q.5732	Rotatoria	unknown	about 1900
ZMB (<i>Pycnophyes</i> sp.)	<i>Pycnophyes</i> sp.	Hoyer's mounting medium	1996–1999
ZMB (Crustacea)	Crustacea	probably Canada balsam	about 1887
ZMB (Diptera)	Diptera	Canada balsam : phenol 1:1	about 2010

Table 2.

(Potential) contents of selected embedding media. Composition of media from published sources, probably not all ingredients mentioned because proprietary of manufacturer.

Composition of natural resins like Canada balsam, gum arabic, and sandarac based on analysis of original ingredients or of raw (!) resins; final products may contain fewer or different chemicals (Lillie et al., 1953).

(Trade) name	Ingredients	References
Araldite resin	bisphenol A epoxy resin (pre-polymer), butylphenyl glycidyl ether, dibutyl phthalate	Adam & Czihak, 1964; Horie, 2010
neutral Canada balsam	abienol, abietic acid, Δ^3 -carene, dehydroabietic acid, isopimaric acid, laevopimaric/palustric acid, limonene, myrcene, neoabietic acid, β -phellandrene, α -pinene, β -pinene, potassium carbonate, sandaracopimaric acid, xylene	Gray & Mills, 1964; Bender, 1967; Böck, 1989; Mills & White, 1994
DPX	distrene 80 (a polystyrene), dibutyl phthalate, tri- <i>o</i> -cresyl phosphate (older formulation), xylene	EMS ² ; Kirkpatrick & Lendrum, 1939, 1941; Roe et al., 1991; Kiernan, 1999; Ravikumar et al., 2014
Entellan neu	poly- <i>n</i> -butyl methacrylate, xylene	EMS ²
Eukitt	poly(butyl methacrylate-co-methyl methacrylate), xylene	EMS ² ; Sigma-Aldrich ³
Euparal grün	camsal (= camphor, phenyl salicylate), copper salt, eucalyptol, limonene, manool, paraldehyde, α -pinene, β -pinene, sandarac (communic acid, polycommunic acid), sandaracopimaric acid, 12-acetoxy-sandaracopimaric acid, sugiol, torulosic acid, torulosol, totarol	Gilson, 1906; Shepherd, 1918; Lillie et al., 1953; Gough, 1964; Böck, 1989; Mills & White, 1994; Scalarone et al., 2003
Fluoromount G	glycerol, disodium hydrogen phosphate, polyvinyl alcohol, sodium azide, water	EMS ² ; Witte, 1976
Glyceel	butyl acetate, cellulose dinitrate, industrial methylated spirit (85–90% ethanol, 0–5% methanol), polymerized linseed oil, sulphur-free toluene	Bates, 1997
Hoyer's mounting medium, Berlese's mounting medium	chloral hydrate, glycerol, gum arabic (polysaccharide with arabinose, galactose, rhamnose, glucuronic acid as salt of calcium, magnesium, potassium + 2 glycoproteins + galacturonic acid), water, occasionally: iodine & potassium iodide	Higgins, 1977, 1983; Upton, 1993; Mills & White, 1994; Dror et al., 2006; Faraji & Bakker, 2008
Kaiser's glycerol gelatin	gelatin, glycerol, phenol, water	Merck ¹ ; Adam & Czihak, 1964; Steedman, 1976
Permout	dipentene, α -pinene, β -pinene, β -phellandrene, toluene	EMS ² , Bioworld ⁴ ; Greco, 1950
Pertex	different polybutylacrylates, xylene	TAAB ⁵
polyvinyl lactophenol	lactic acid, phenol, polyvinyl alcohol, water	Downs, 1943; Salmon, 1947, 1951a, 1951b, 1954
Roti-Histokitt II	toluene	Roth ⁶
Technovit 7100 & hardener II	liquid: 2-hydroxyethyl methacrylate; hardener: 5-barbituric acid, dimethyl sulfoxide	Kulzer ⁷

Vectashield mounting citric acid, glycerol, sodium phosphate
medium

Sigma-Aldrich⁸

¹ Merck: http://www.merckmillipore.com/DE/de/product/Aquatex%C2%AE,MDA_CHEM-108562#anchor_BRO, accessed 21.05.2015.

² EMS: = Electron Microscopy Sciences,
http://www.emsdiasum.com/microscopy/products/histology/mounting_media.aspx, accessed 21.05.2015.

³ Sigma-Aldrich: <http://www.sigmaaldrich.com/catalog/product/fluka/03989?lang=de®ion=DE>, accessed 21.05.2015.

⁴ Bioworld: <http://www.bio-world.com/msds/21750009/Permount-mounting-medium.html>, accessed 17.06.2015.

⁵ TAAB: http://www.taab.co.uk/pdf-products.php?cat_id=2&sub_cat_id=29&pdf_id=214&prod_added=1,
accessed 30.10.2015

⁶ Roth: https://www.carlroth.com/en/en/Life-Science/Histology-Microscopy/Mounting-Media/Hydrophobic-Mounting-Media/Roti%3Csup%3E%C2%AE%3C-sup%3E-Histokitt-II/p/000000010003cb0c00020023_en

⁷ Kulzer>: http://kulzer-technik.de/de_kt/kt/maerkte/metallographie/produktbereiche/verbrauchsmaterialien_einbetten_1/technovit_7100.aspx

⁸ Sigma-Aldrich:
<http://www.sigmaaldrich.com/catalog/product/sigma/m1289?lang=de®ion=DE&gclid=CPOL8rrv58gCFQoKwwodV4ED8g>

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7