

Ultrasensitive chemiluminescent immunochemical identification and localization of protein components in painting cross-sections by microscope low-light imaging

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Abstract The characterization and localization of proteins and other organic components in the complex stratigraphy of paintings is crucial for authentication and studies of painting techniques. With this aim we have developed a new ultrasensitive immunochemical procedure for the detection of the protein ovalbumin (chicken egg white albumin), present in binding media or varnishes, in painting cross-sections. The technique is based on chemiluminescence imaging detection combined with optical microscopy, and allowed the sensitive localization of the target protein in cross-sections with high spatial resolution. In order to evaluate its performance, the method was first applied to standard samples (also containing different common pigments), then used for the localization of ovalbumin in samples obtained from a Renaissance wood painting.

Keywords Binding media · Chemiluminescence · Imaging · Immunoassay · Ovalbumin · Painting cross-section

Introduction

Characterization and localization of organic and inorganic components in the complex structure of paintings is particularly useful for authentication and studies of painting

techniques. Various techniques, including FTIR [1] and Raman microscopy [2], SEM-EDX [3], and static secondary-ion mass spectrometry (SIMS) [4], are available for characterization of inorganic materials, allowing information to be obtained on both their chemical nature and localization within the painting's layers. Each layer could also contain many organic substances (proteins, oils, waxes, gums, resins, etc.), alone or mixed with other materials, as components of binding media and varnishes. The analytical procedures required for identification of such compounds need substantial improvements. Chromatographic techniques are sensitive and selective but require extraction of the analyte from the sample [5–13], thus losing the spatial information. On the other hand, micro infrared spectrometry and staining tests [14] allow localization of organics in painting cross-sections, but they are not specific, since molecules are recognized via their functional groups (for example, it is possible to detect protein amide groups but not distinguish between different proteins). Recently, proteomics techniques have been also used, allowing the identification of proteins from ancient paintings [15].

Immunological techniques were proposed many years ago as an alternative method for identification and localization of proteins in paint media [16, 17] taking advantage of the high specificity of the antigen–antibody reaction, which would allow different proteins to be distinguished and the biological source of a protein (e.g., bovine vs. rabbit collagen) to be determined. Such techniques are widely employed in bioanalytical and clinical chemistry [18], from microtiter plate-based quantitative assays to immunohistochemistry techniques. Nevertheless, only a few data have been reported in the literature concerning the use of immunochemical techniques for characterization of organic components in paintings [19–21], and therefore the potential of such techniques are still

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widely unexplored. In addition, the previously reported applications were based on fluorescence detection, and in many cases significant interferences were observed due to sample fluorescence [19]. In fact, painting materials can show intense autofluorescence due to the presence of pigments and/or binding media, which could hamper the use of immunofluorescence techniques. In an attempt to overcome this drawback we have developed a new immunochemical method for the localization of ovalbumin (a protein often present in binding media and varnishes) in paint cross-sections based on optical microscopy and employing, for the first time, chemiluminescence (CL) imaging detection. Chemiluminescence detection is widely used in analytical chemistry [22], and CL imaging has already proved to be more sensitive than colorimetric and fluorescence detection techniques, allowing the localization of target molecules in cells and tissues with good spatial resolution and easy quantitative evaluation of the signal [23–25]. Most interestingly, due to the absence of an excitation source, CL imaging detection is not affected by interferences due to the autofluorescence of the sample components.

The CL immunochemical method for the localization of ovalbumin relied on the binding to the target protein of a specific primary antibody, which was then detected by a horseradish peroxidase (HRP)-labeled secondary antibody and a CL enzyme substrate. The imaging of the CL signal produced by the enzyme-catalyzed reaction allowed the detection and stratigraphic localization of the target protein. To evaluate the performance of the method, whole-egg tempera standard samples (with or without pigments) were used as models, then the immunolocalization assay was used for detection of ovalbumin in samples obtained from a Renaissance wood painting.

Materials and methods

Reagents

Anti-chicken egg albumin antibody (whole antiserum, produced in rabbit), horseradish peroxidase (HRP)-conjugate polyclonal anti-rabbit IgG antibody (produced in goat), albumin from chicken egg white (ovalbumin), bovine serum albumin, gelatin (type A, from porcine skin), and bovine nonfat dried milk, were purchased from Sigma–Aldrich (St Louis, MO, USA). The luminol-based HRP CL detection reagent Westar Supernova was from Cyanagen (Bologna, Italy). The smalt (ground glass colored with cobalt(II) salts), azurite, and malachite ($\text{Cu}_3(\text{CO}_3)_2(\text{OH})_2$), hematite (Fe_2O_3), cinnabar (HgS), and minium (Pb_3O_4) pigments were obtained from Zecchi (Florence, Italy). Polyester resin for sample embedding (SeriFix Resin and

SeriFix Hardener) was purchased from Struers (Ballerup, Denmark). All other chemicals were of analytical grade. Experiments on membranes were performed on Protran nitrocellulose membranes (Whatman, Maidstone, UK).

Instruments

Chemiluminescence imaging of membranes was performed with a Night Owl LB 981 low-light luminograph (Berthold Technologies, Bad Wildbad, Germany) equipped with a thermoelectrically-cooled, back-illuminated CCD camera.

For CL microscopy imaging a BX 60 epifluorescence microscope (Olympus Optical, Tokyo, Japan) connected to a liquid-nitrogen-cooled ultrasensitive CCD camera (LN/CCD Princeton Instruments, Roper Scientific, Trenton, NJ, USA) was used. The microscope was enclosed in a dark box to avoid interference from ambient light and equipped with an OptiScan ES103 motorized microscope stage (Prior Scientific Instruments, Fulbourn, UK) for sample positioning. Live color images of the samples were obtained by acquiring separate grayscale images for each primary RGB color using an RGB filter (CRI, Woburn, MA, USA), which were then combined into a single color image. Image processing and quantitative analysis were performed using Metamorph v. 4.5 image analysis software (Universal Imaging Corporation, Downingtown, PA, USA).

Visible fluorescence images of cross-sections under UV irradiation were obtained by means of a BX 51 M optical microscope (Olympus Optical) connected to a DP70 color digital camera (Olympus Optical). Sample cross-sections were excited at 313 nm, while the fluorescence emission was acquired in the whole visible spectrum.

Preliminary characterization of standard and unknown samples was carried out by micro infrared spectrometry in reflectance mode with a silicon μ -ATR crystal [1]. Spectra were obtained using a Continuum μ m infrared microscope connected with a Nicolet Avatar 370 FT-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a liquid-nitrogen-cooled MCT detector. Spectra acquisition and processing were performed with Omnic v 7.3 software, supplied by the manufacturer of the spectrometer.

Standard samples

Standard samples were prepared according to traditional painting techniques [26]. Briefly, a layer of whole-egg tempera, i.e., a mixture of egg white, yolk, and water in a 1:1:1 (v/v) ratio, was applied on a preparation layer made of gypsum (obtained from Zecchi) and rabbit glue (purchased from Phase, Bologna, Italy). To evaluate the effect of pigments on the CL immunolocalization procedure, standard samples were also prepared using of a mixture of pigments and whole-egg tempera. The relative ratio

between binding medium and pigments depended on their nature. To obtain homogenous mixtures suitable for application as thin layers on the preparation ground, the following pigment/egg (*w/w*) ratios were adopted, in accordance with artists' traditional recipes: 4:1 for azurite, malachite, smalt and hematite; 3:1 for cinnabar and minium.

Experimental procedure

Cross-sections were prepared by embedding the samples in polyester resin and polishing them according to standard procedures. A systematic dry polishing method employing 4,000 to 12,000-grade silica abrasive papers (purchased from Micro-Surface, Wilton, IA, USA) was employed to obtain a high-quality surface (i.e., to reduce the surface porosity of the cross-section) [27].

Sample cross-sections were treated for 1 h at room temperature with the blocking solution (5% dried milk in water) then, after washing (3×) with PBS/milk (10 mmol L⁻¹ phosphate buffer saline, pH 7.4, containing 1.25% dried milk), they were incubated overnight at 4° C with the anti-chicken egg albumin antibody (primary antibody) diluted 1:4,000 (*v/v*) in PBS/milk. Afterwards, the samples were washed (5×) with PBS/milk and incubated for 4 h at 4° C with the HRP-labeled anti-rabbit IgG antibody (secondary antibody) diluted 1:2,000 (*v/v*) in PBS/milk. Samples were washed again (5×) with PBS, then the HRP CL detection reagent was added to cover the cross-section and the CL images were acquired using an integration time of 120 s. For each CL image, a live image of the sample was also acquired to assess the localization of the CL signal (therefore of the ovalbumin protein) in the sample by comparison of the CL and live images.

Results and discussion

Optimization of experimental conditions

Optimization of the experimental conditions of the immunoreactions was performed by preliminary measurements on ovalbumin samples spotted on nitrocellulose membranes. In order to select the most suitable concentrations of the antibodies, different dilutions of the primary and secondary antibodies were employed for detection of ovalbumin (data not shown). The highest CL signal/background ratios were obtained using 1:4,000 (*v/v*) and 1:2,000 (*v/v*) dilution factors for the primary and the secondary antibodies, respectively. Even though higher concentrations of the primary antibody led to stronger CL signals from the ovalbumin spots, the CL signal/background ratios were lower due to the concurrent increase of

the background signal, thus such concentrations were not employed in the assay. Experiments on membranes also allowed selection of the most suitable blocking agent to reduce the non-specific adsorption of the immunoreagents on the surface of the cross-section. Among the tested substances (bovine serum albumin, gelatin, and dried milk) the highest signal/background ratios were obtained using a 5% solution of non-fat dried milk in water (Fig. 1). Interestingly, bovine serum albumin was the least efficient blocking agent and led to a diffuse background signal over the whole membrane, which might be attributed to small cross-reactivity of the protein with the anti-ovalbumin antibody. To evaluate the detectability of ovalbumin by using the CL immunolocalization procedure, a calibration curve was obtained in the optimized experimental conditions for amounts of protein ranging from 0.1 to 100 ng/spot. The CL signal showed good correlation with the amount of protein, as shown in Eq. 1 where *Y* is the mean CL signal of the spot (RLU) and *X* is the amount of ovalbumin (ng/spot).

$$Y = (128.6 \pm 4.3) \log X + (132.2 \pm 4.9) \quad (n = 7, r^2 = 0.996) \quad (1)$$

Under these experimental conditions, the detection limit of the assay, estimated as the amount of protein giving a CL signal corresponding to the background signal plus three times its standard deviation, was about 0.2 ng/spot (or 0.03 ng mm⁻²). Even though the amounts of ovalbumin spotted on the membrane could not easily be compared with those present in painting cross-sections, the low value of the detection limit suggested that the CL immunolocalization procedure was sensitive enough to allow the detection of the protein in egg tempera paintings.

Due to the porosity of the cross-sections, optimization of blocking and incubation steps was critical to avoid non-specific adsorption of the immunoreagents, which would result in high background signals and reduce the detectability of the target protein. Indeed, when the assay was performed in cross-sections of standard samples with a layer of whole-egg tempera using the same experimental protocol employed for nitrocellulose membranes (1-h incubations at room temperature with the blocking agent and the two antibodies) the CL signal/background ratio was low, due to the non-specific adsorption of the antibodies on the ground layer (Fig. 2a). However, the non-specific binding of the immunoreagents could be controlled, other than by careful preparation of the cross-section with a dry polishing method to reduce the heterogeneity of the surface, by reducing the incubation temperature. In fact, the signal/background ratio significantly improved when incubations with the primary and secondary antibodies were performed

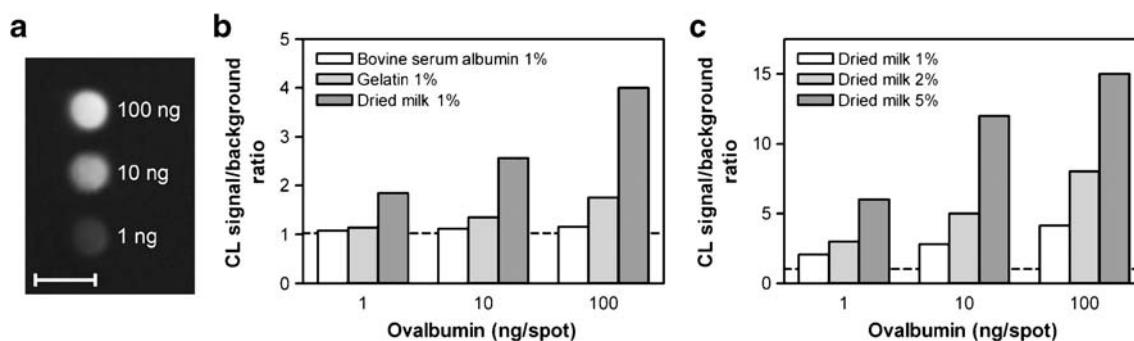


Fig. 1 Selection of (b) the blocking agent and (c) its optimal concentration performed on nitrocellulose membranes on which different amounts (1, 10, and 100 ng) of ovalbumin were spotted. The cross-sections were incubated for 1 h at room temperature with both the blocking agent and the primary and secondary antibodies

(used at their optimal concentrations). Data are reported as ratios between the CL signals of the spots and the background signal of the membrane. Panel (a) shows the CL image of a nitrocellulose membrane. Bar represents 5 mm

for longer times (overnight and 4 h for the primary and secondary antibody, respectively) and at 4° C rather than at room temperature (Fig. 2b). As shown by the CL profiles (Fig. 2c), under such incubation conditions the CL signal from the tempera layer increased while the background was much less affected, thus resulting in a higher signal/background ratio. We also investigated the effect of different incubation times with the blocking agent, but no significant improvement was obtained with incubations longer than 1 h (data not shown). Thus, it appeared that a 1-h incubation at room temperature with a 5% solution of non-fat dried milk was sufficient to saturate the non-specific binding sites of the sample.

Assay specificity

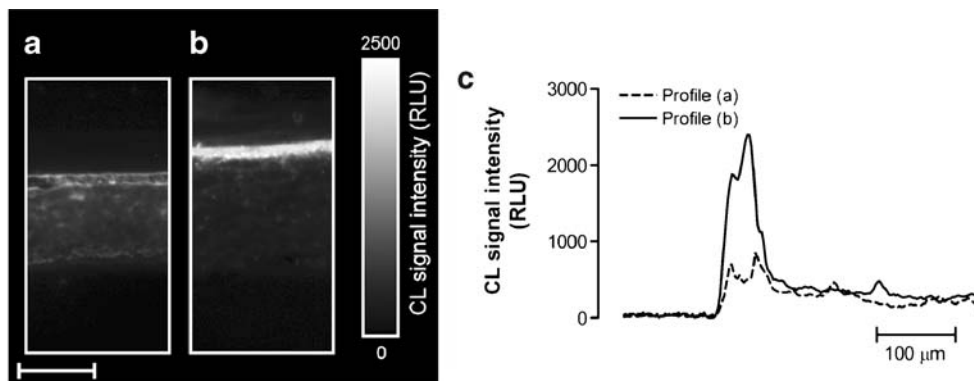
The specificity of the assay was assessed by performing the immunolocalization of ovalbumin in standard samples with a layer of whole-egg tempera either with or without the primary antibody. When the assay was performed using the anti-ovalbumin antibody, a sharp, intense CL signal was obtained corresponding to the tempera layer (Fig. 3a), while in the absence of the antibody this CL signal was not detected (Fig. 3b). Nevertheless, much weaker emission

(the intensity of which was about a factor of 20 lower than that of the ovalbumin-specific CL signal) was still observed in the whole cross-section (Fig. 3c), which could be attributed to the non-specific binding of the secondary antibody. As expected, no specific CL signals were also observed in cross-sections of standard samples obtained using other organic binding media (fish glue, oil). The spatial association of the CL signal with the binding medium was also clearly demonstrated by the results obtained in standard samples with a layer of tempera containing the smalt pigment (Figs. 4c,d). In such samples the relatively large size of the pigment particles allowed confirmation of the localization of the CL signal only corresponding to the binding medium.

Effect of pigments

To assess the suitability of the assay for the localization of ovalbumin in real paint cross-sections we investigated possible interferences due to painting pigments. Indeed, several metal ions contained in pigments, especially the Co^{2+} , Cu^{2+} , Fe^{3+} , Mn^{2+} , and Pb^{2+} ions, are known inhibitors of HRP [28]. Thus, release of such ions during the CL detection step could affect the enzyme activity, leading to

Fig. 2 Chemiluminescence images obtained for the immunolocalization of ovalbumin in cross-sections of standard samples with a layer of whole-egg tempera, performed (a) with 1-h incubations at room temperature with the primary and secondary antibodies and (b) according to the optimized experimental protocol. Panel (c) shows the profiles of the CL signal across the cross-sections. Bar represents 200 μm



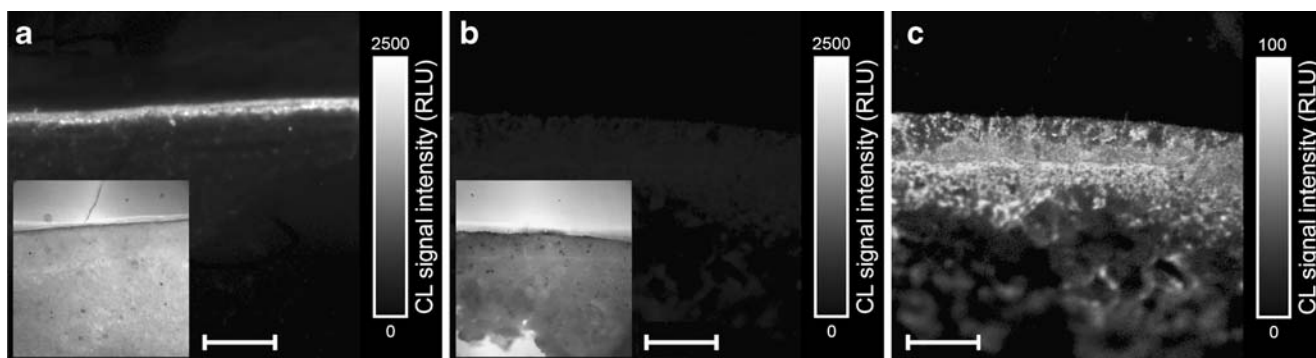


Fig. 3 Chemiluminescence images obtained for the immunolocalization of ovalbumin in cross-sections of standard samples with a layer of whole-egg tempera performed either with (a) or without (b) the primary antibody. The CL images in panels (a) and (b) are shown using the same grayscale, corresponding to the different CL

intensities. The CL image obtained without the primary antibody is also shown in panel (c) using a different grayscale to highlight the weak CL signal due to the non-specific adsorption of the secondary antibody (*insets*: live images of the samples). *Bars* represent 200 μm

enzyme inhibition and a weak—or even absent—CL signal. In addition, metal ions can also directly influence the CL luminol reaction, either by acting as catalysts (the Co^{2+} ion is the most sensitive catalyst of the luminol– H_2O_2 reaction [29]) or inhibiting the CL process (for example, the Cu^{2+} ion [30]). In order to study such interferences we analyzed standard samples of whole-egg tempera containing different common inorganic pigments (smalt, azurite, malachite, hematite, cinnabar, and minium). We analyzed the cross-

sections either following the protocol described above to assess negative effects on the HRP enzyme activity and/or the CL process or by omitting the secondary antibody to detect possible catalysis of the CL oxidation of luminol. The experiments performed in the absence of the immunoreagents did not show any catalysis of the CL reaction, and for all the pigments we were able to observe the CL emission from the tempera layer (see, for example, Fig. 4), even though the CL signal was significantly weaker for the

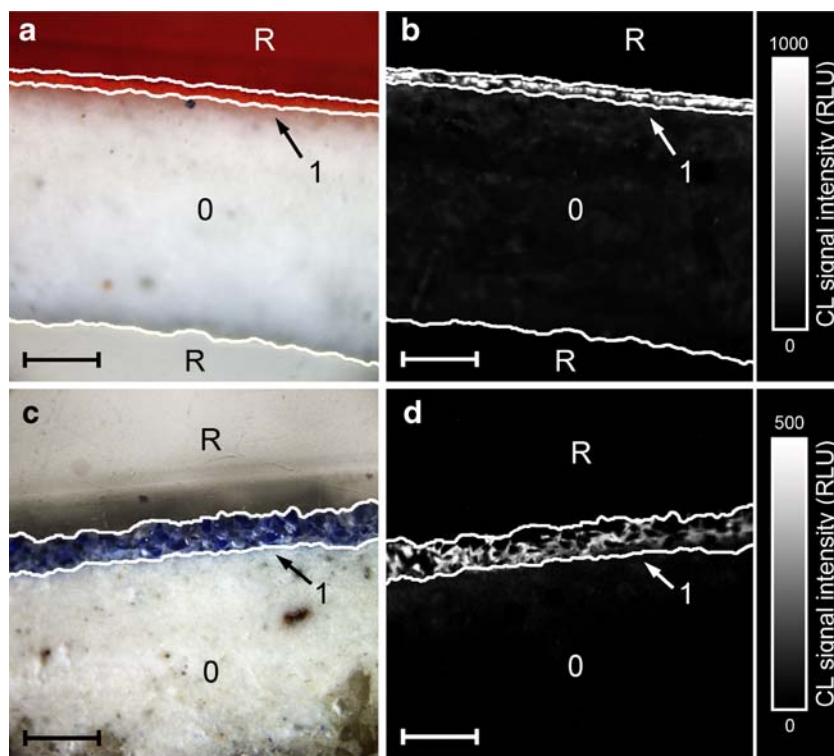


Fig. 4 Chemiluminescence immunolocalization of ovalbumin in cross-sections of standard samples with a layer of whole-egg tempera containing cinnabar (*top*) or smalt (*bottom*) pigments. Panels (a) and (c) show the live images of the cross-sections, panels (b) and (d) show

the CL images, confirming the localization of the CL signal in the egg tempera layer corresponding to the binding medium. The different parts of the cross-sections are indicated (*R*=resin, *0*=ground layer, *1*=egg tempera layer with pigments). *Bars* represent 200 μm

hematite-containing samples. We attributed the absence of detectable effects for the other pigments containing metal ions known to affect the CL reaction (e.g., the Co^{2+} ion) to low release of metal ions in the solution or, possibly, to loss of the soluble metal ion fraction during the sample processing steps prior to the measurement. We plan to include in further experiments other inorganic and organic pigments of historical interest, also performing investigations on artificially aged samples to assess the effect of degradation processes.

Case study

The CL method for the immunolocalization of ovalbumin was applied to cross-sections of samples taken from a wood painting by Nicolò Rondinelli (c. 1450–c. 1510), an Italian painter of the Renaissance period, active mainly in Ravenna. The CL images obtained for such samples (Fig. 5a) showed strong emission indicating the presence of ovalbumin in the uppermost layer ($\sim 15\text{--}30\ \mu\text{m}$) of the painting, even though the layer appeared discontinuous. This result was confirmed by IR reflectance spectroscopy and by observation of the sample fluorescence under UV excitation. In fact, the IR reflectance spectra of the cross-section (Fig. 5b) showed absorption at 1655 and $1545\ \text{cm}^{-1}$ in the upper painting layer, which can be assigned to the protein amide I (C=O stretching) and amide II (NH bending) bands. In addition, a bluish fluorescence emission under UV excitation (typical of proteins) with a spatial distribution strongly resembling that of the CL signal was observed in the top layer of the painting (Fig. 5c). Therefore, all the experimental findings suggested the presence of a proteinaceous material in the uppermost painting layer, but only the CL immunolocalization method

gave information about its nature, allowing us to hypothesize the use of an egg-based varnish.

Conclusions

The developed CL immunolocalization procedure allowed detection and localization of ovalbumin in aged painting cross-sections with a spatial resolution of the order of micrometers (i.e., within single painting layers) and with little or no interference from several common pigments used in paintings. Since this analytical approach could be extended to detection of other proteinaceous components, such as animal collagen, casein, etc, it can be complementary to the other analytical techniques used for analysis of the organic components of paintings, in particular in studies of painting techniques. In addition, the possibility of determining the biological source of a given protein (e.g., fish or rabbit glue) would make the immunolocalization method relevant also for authentication studies, for example by allowing determination of the production period and the origin of the painting materials.

Different proteins could be also detected in the same cross-section, either sequentially by polishing the sample before each experiment to obtain a fresh surface or simultaneously by employing a mixture of analyte-specific primary antibodies. In the latter case, analyte-specific primary antibodies should be raised in different species, thus being detectable by species-specific secondary antibodies labeled with different enzymes (such as HRP and alkaline phosphatase), each one detectable with its own CL substrate. In addition, the possibility of using different enzyme labels would allow the same assay to be performed with two independent CL detection systems in order to avoid

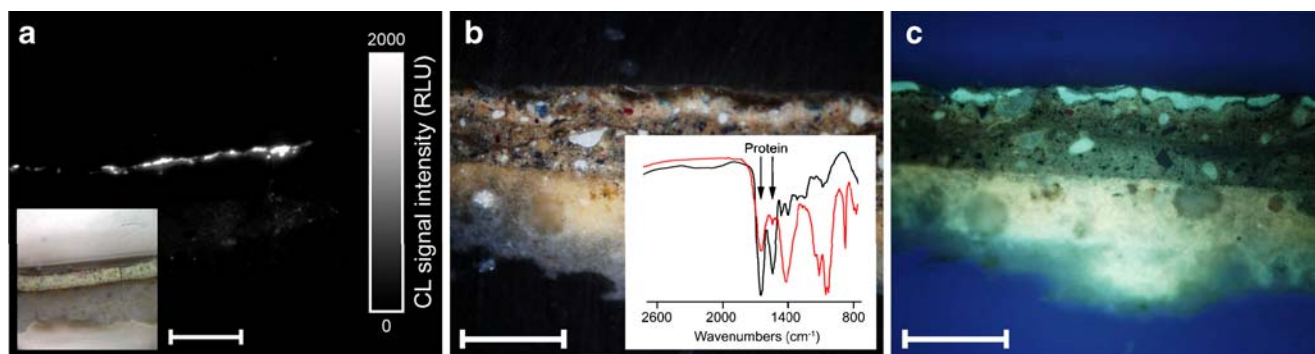


Fig. 5 Localization of organic components in cross-sections of samples from the wood painting of Nicolò Rondinelli. (a) Chemiluminescence image of a cross-section showing the localization of the CL signal in the upper painting layer (inset: live image of the sample); (b) live image of another cross-section from the same portion of the

painting and (inset) comparison of the IR reflectance spectrum of the upper painting layer (red) with a reference IR spectrum obtained from an aged egg-white sample (black); (c) fluorescence image of the same cross-section under UV irradiation. Bars represent $200\ \mu\text{m}$

any possible interference from painting components, or even to select the most suitable enzyme label for a given sample.

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