ACCOUNTS
of chemical research

Immunodetection of Proteins in Ancient Paint Media

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RECEIVED ON NOVEMBER 27, 2009

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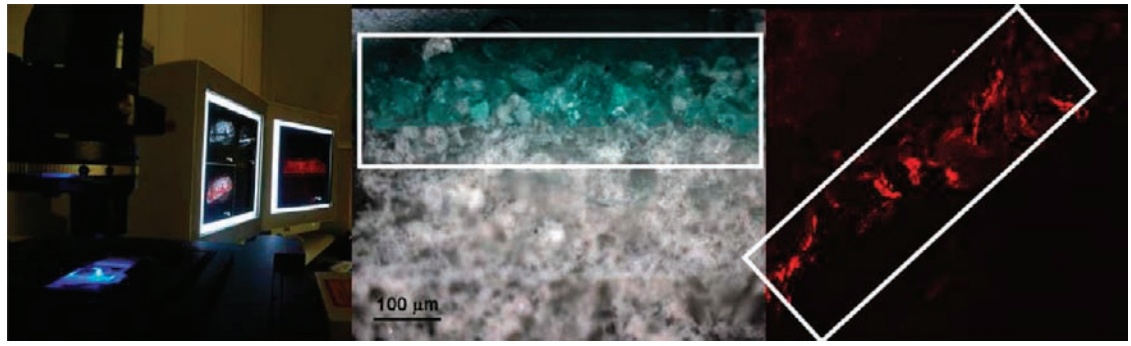

Diagnostic immunology is a powerful tool, widely used in clinical and biochemical laboratories for detecting molecules. In recent years, the technique has been adaptated to materials sciences as a result of the extensive advances achieved in immunology. Today, many companies supply custom antibodies as well as new high-performance bioprobes for virtually any use.

The idea of using immunodetection in the field of conservation science is not new. This analytical methodology is, in fact, particularly attractive for investigating biopolymers in painting materials; it is highly sensitive and selective with respect to the biological source of the target molecules. Among biopolymers, proteins have been widely used in the past as painting binders, adhesives, and additives in coating layers. An accurate assessment of these materials is necessary to obtain deeper insights into an artist's technique as well as to design proper restoration and conservation methods. In spite of the diagnostic potential offered by immunodetection-based techniques, some analytical drawbacks had, until recently, limited their use in routine applications in conservation science.

In this Account, we highlight the most important results achieved in our research on the development of analytical methodologies based on the use of enzyme-linked immunosorbent assay (ELISA) and immuno-fluorescence microscopy (IFM) techniques for the highly sensitive and specific identification of proteins in artistic and archeological materials. ELISA and IFM offer two alternative analytical routes to this final goal: ELISA provides a fast, cost-effective, quantitative analysis of microsamples put in solution, whereas IFM combines the immunodetection of the targeted molecules with the characterization of their spatial distribution. The latter approach is of great value in the stratigraphic investigation of paintings. We discuss the limits and strengths of these methodologies in the context of the complex matrixes usually found in the investigated materials and the prolonged aging that they have undergone.

Immunology is a relatively new technique in conservation science, providing a rich new field for innovation. We see two areas that are particularly ripe for future contributions. The commercial manufacture of antibodies specifically tailored for use in cultural heritage studies holds enormous potential. Moreover, the need for further refinement of detection systems in immuno-fluorescence techniques, especially the suppression of the autofluorescence background in painting materials, offers an abundance of opportunities for researchers. Immunology is a relatively new technique in conservation science, providing a rich new field for innovation.

Published on the Web 05/03/2010 www.pubs.acs.org/acr
10.1021/ar900279d

Introduction

Until recently, scientific studies in the field of cultural heritage focusing on paintings have primarily concerned themselves with inorganic compounds.[1,2] The investigation of organic components has been subjected to increased interest only in the past decade thanks to the development of new nondestructive and microdestructive techniques.[3,4] Even so, a detailed characterization of natural organic materials in ancient paintings is still a challenging issue because of their intrinsic chemical complexity and their tendency to easily undergo degradation over long periods of time.[5]

Among naturally occurring organic substances, proteins have been widely used not only as binders but also as adhesives or as additives in coating layers. In particular, animal glue, egg (both yolk and albumen), and milk (or its byproduct casein) are mostly encountered in paintings. They contain collagen, ovalbumin (egg white), and casein, respectively, as the main distinctive proteins.

Until now chromatography analysis seems to be the best established approach to provide detailed information on the wide class of natural organic compounds found in paintings (proteins but also oils, resins, waxes, and plant gums).[6] In regard to proteins, procedures for both high-performance liquid chromatography (HPLC) and gas chromatography (GC) techniques, mainly combined with mass spectrometric (MS) detection, have been developed to distinguish among egg, glue, and milk/casein on the basis of the quantitative determination of their amino acidic profiles.[7,8] This approach can be affected by the alteration of amino acid relative amounts induced by sample contamination. Furthermore, protein mixtures are difficult to be analytically resolved, and different biological sources of the same protein cannot be established. Recently, several new methods based on the proteomic approach have been developed, but they still need further experimentation for routine applications.[9,10] Therefore, an alternative analytical approach that is simple, cost-effective, has minimal sample manipulation, and that can possibly resolve a complex mixture while being selective with respect to the biological source is highly desirable for protein recognition in artworks.

Immunological techniques have the potential to become a powerful diagnostic tool in cultural heritage for highly specific and sensitive identification of proteins in microsamples of art and archeological materials.[11−17] To that end, enzyme-linked immunosorbent assay (ELISA),[18,19] immuno-fluorescence microscopy (IFM),[20] and immuno-chemiluminescence microscopy[21] have all begun to be systematically used to develop analytical methodologies for applications in conservation science.

This Account provides an overview of our research on the application of ELISA and IFM to the investigation of proteins in painting materials. ELISA is an accurate and sensitive immunochemical assay that can be used for the fast, easy, and cost-effective measurement of targeted antigens in a painting sample brought to solution. ELISA typically uses antibodies conjugated to enzymes as detection reagents. These enzymes act on chromogenic or fluorogenic substrates that produce an amplified detectable signal. In the case of IFM, immuno-fluorescent probes are used to obtain a highly sensitive and specific detection of the targeted molecule and, at the same time, to image its spatial distribution. This is a particularly valuable approach for the stratigraphic investigation of paintings, although at the expense of sample preparation time.

Our research work based on both methodologies is presented here in two separate sections where the main experimental aspects are described, and the most relevant results are reported and discussed for each approach.

ELISA

Direct ELISA is the simplest among various methods,[22] as the protein (antigen) is attached to a plastic solid phase (normally a 96-well microtiter plate) and an enzyme-labeled detection antibody is added. The detection antibody (named "primary antibody") binds specifically to antigens in a recognized molecular sequence: the epitope of the antigen. In indirect ELISA, the primary antibody is not conjugated with enzymes but instead is targeted by enzyme-conjugated secondary antibodies specific to the primary antibody class (IgG, IgM) of the primary antibody. The use of secondary antibodies increases the specificity of ELISA and reduces the unspecific background due to the linkage of antibodies on the plastic surface of the well plate.

The ELISA technique is used at the Getty Conservation Institute (GCI) to detect egg, mammal glue, casein, and plant gum in paint samples from works of art. It is a modified procedure based on literature[17,23] and uses the indirect method; the procedure[19] is summarized in Figure 1.

A paint sample (between 100 and 300 μg, while wall painting samples should be larger, up to 1 mg, as they are likely to have less binding media) is first dissolved in a elution buffer and then diluted and added to wells of an ELISA well plate. The binder containing the antigen to be detected attaches to the plastic of the well and is processed for immunologic recognition.

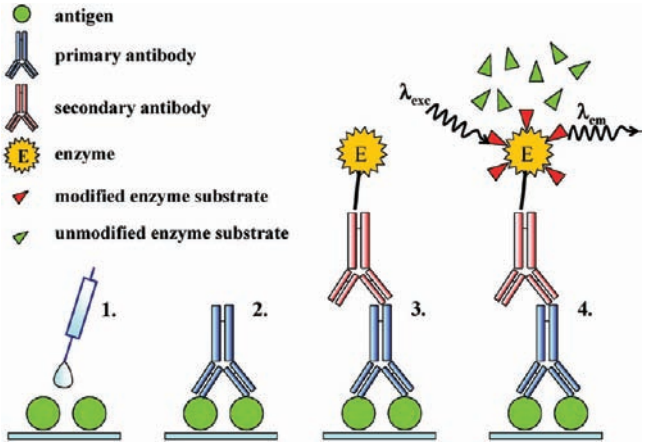

FIGURE 1. Sketch of the ELISA methodology adopted for protein detection in painting samples: (1) a paint sample is first dissolved in an elution buffer and then diluted and added to wells of an ELISA well plate; (2) a primary antibody targeted to the particular bound antigen is added to the well (2 h, room temperature); (3) an enzyme-conjugated secondary antibody is added (2 h room temperature). Both steps 2 and 3 are followed by sample rinsing with water to remove any unbound antibody; (4) p-NPP is added to the wells, and a reaction stop buffer (0.75 M NaOH) is added. Absorbance is read at 405 nm using a multiwell spectrophotometric plate reader.

The antibody conjugated enzyme alkaline phosphatase is used for colorimetric detection by enzymatic conversion of the substrate *p*-nitrophenyl phosphate (*p*-NPP) to *p*-nitrophenol, which is a yellow dye at pH 8.5. The reaction color development depends on the amount of antigen present in the well and the amount of time the enzyme is left to be active. The addition of a buffer solution to stop the reaction allows for comparison of quantitative results from different ELISA assays.

Specific research strategies at the GCI have focused on finding suitable antibodies to detect proteins in paint. Experimental efforts have focused primarily on (i) testing antibody specificity to recognize denatured aged proteins in paints; (ii) evaluating detection limits of the technique to estimate the appropriate sample quantity to be collected; (iii) decreasing contamination events (false positives) and investigating cases of false negatives; and (iv) selecting a suitable blocking solution able to reduce background due to antibody unspecific binding, or to its cross-reactivity with different proteins having similar epitopes. Finding optimal ELISA conditions requires extensive testing for each type of binding media to identify in one assay all those present in a sample, with the lowest background levels and the highest detection sensitivity. "Sea Block" (Pierce Chemical) in phosphate buffer solution (PBS) was chosen as the blocking agent giving the lowest background levels. The primary antibodies, corresponding secondary antibodies, dilutions, and detection limits are shown in Table 1. Sample absorbance is expressed as optical density at 405 nm (OD_{405nm}). For each antibody pair, background levels were evaluated in 20 blank wells; they gave OD_{405nm} averaged values ranging between 0.11 and 0.16. The threshold values for binder detection in Table 1 were estimated as the blank average OD_{405nm} plus three times the blank $OD_{405\ nm}$ standard deviation.

Each antibody chosen for use in ELISA was tested on several animal sources of glue, egg, and casein.[19] Eggs from several species of bird were tested for ovalbumin in whole egg, egg yolk, and egg white; all were positive except for egg yolk, as it lacks significant amounts of ovalbumin. Milk was tested from human, cow, goat, and buffalo, and all reacted positive for casein except human milk.

The limit of detection is dependent on the conservation degree of the binder as shown in Table 1 by the increase of the detection limit in the ELISA assays performed on 47 day artificially aged binders. Aging and pigments adversely affect the sensitivity of ELISA due to loss of antigenic sites, cross-linking, and nonsolubility.[8,25] The GCI's research initially focused on nonpigmented samples exposed to different conditions of UV light and humidity: exposure to UV light with high relative humidity (69−75% RH) consistently had the most dramatic effect on all binders, while exposure to UV light at low humidity (20−25% RH) or to fluctuating humidity only (12−85% RH) had a much less effect. More recently, ELISA was used to evaluate aging degradation effects on binders in the presence of pigments. Paint samples were produced by mixing three common proteinaceous binders with ten pigments usually found in wall paintings. They were prepared using traditional recipes,[26] applied to glass slides, and artificially aged for 47 days. The decrease of the detectable amount of binding media after artificial aging is shown in Figure 2. The measurement is based on known wt % values of binder in paint before aging and measured values after aging. Quantitation of the detectable amount of binder was obtained by calibration curves from 47 day artificially aged unpigmented standards.

As evidenced by Figure 2, pigments interacted differently with binding media. Results in charcoal/egg, charcoal/glue, red ochre/casein, red ochre/glue, and vermilion/egg exhibited no measurable change after aging. Even though a few of the paints showed up to a 100-fold decrease in the expected values, ELISA was still able to identify casein and egg in all aged paints. Only the animal glue antibody turned out to be very sensitive to binder aging, so that half of the samples gave a complete loss of signal. The specific role played by different pigments in the degradation of binders is still not clear.[5,8,25]

TABLE 1. Primary Antibodies, Corresponding Secondary Antibodies, Dilutions, and Detection Limits of Binders (Fresh and after 47 Days of Artificial Aging) Tested in ELISA

primary antibody catalogue # (company)	binder tested	species reaction	dilution[a]	secondary antibody (dilution[a])	threshold values (mean OD_{405nm} + 3 SD)[b]	detection limit (ng[c])	
						0 day	47 days
# AB1225 (Chemicon)	ovalbumin, whole egg	most birds (not yolk)	1:800	rabbit IgG (1:500)	0.21	0.6	1
#RCAS-10° (Immunology Consultants)	casein, milk curd	buffalo, cow, and goat (not human)	1:800	rabbit IgG (1:500)	0.15	0.3	4
#T40113R (Gentaur)	collagen, animal glue	most mammals (not rabbit)	1:400	rabbit IgG (1:500)	0.19	3	12
#JIM13 (Carbo Research Univ. of Georgia)	plant gum, gum arabic	most Acacia spp. and fruit tree (not Tragacanth)	1:50	rat IgG (1:500)	0.17	7	12

[a] Primary and secondary antibodies were diluted in a solution of Sea Block in PBS (1:10 in volume). [b] Values are the blank mean OD_{405nm} + 3 SD of 20 independent determinations. [c] ELISA was performed on solutions of pure binder in an elution buffer (see Figure 1). The detection limit was calculated by dividing the weight of the binder by the highest dilution factor that still yielded a positive result.

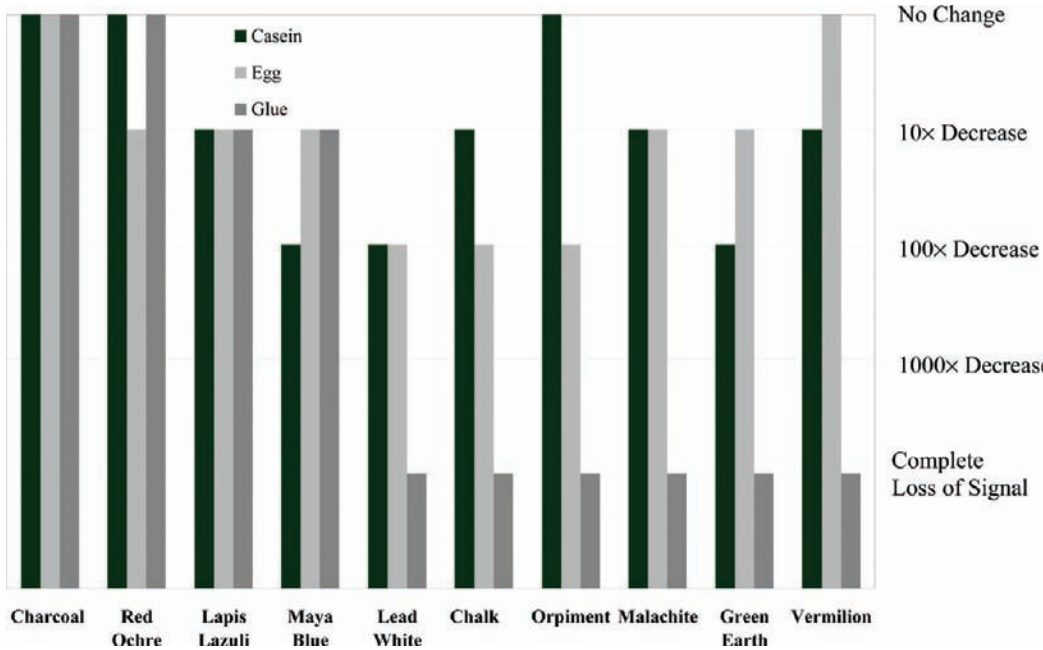

FIGURE 2. Changes in detectable amounts of binding media when exposed to pigments after 47 days of artificial aging. A Xenon-arc Ci400 weatherometer, with filters to stop far UV-light, irradiance of 0.5 w/m^2, RH of 60−80%, 40 °C chamber temperature, was used. *X*-axis shows the pigments and their binding media: black = casein, light gray = egg, and dark gray = animal glue. The *Y*-axis shows the ratio between the measured concentration of binder (expressed as wt % binder in the paint) to the known binder wt % concentration before artificial aging. No change = no significant decrease in % binder; 10×, 100×, and 1000× decrease = 10, 100, and 1000 times decrease in % binder, respectively; complete loss of signal = no binder detected.

Recently, we also tested an antibody (see Table 1) for the detection of gums. Their recognition is a challenging task even for chromatographic techniques.[6] ELISA results from several plant sources showed positive for gum Arabic and fruit tree gums, while gum Tragacanth was negative. Pigment and aging degradation effects were also evaluated after 47 days of artificial aging of pigmented plant gums (same conditions of Figure 2), obtaining positive results for half of the samples. These highlighted the need to test all new antibodies both for aged gums and species reactivity.

During the course of research, various works of art were tested by ELISA;[19] the results are summarized in Table 2.

GC-MS[24,25] was used to support ELISA results, and it proved that the assay is valid for ancient materials. Yet, in some cases, ELISA provided discrimination of different proteinaceous components, not distinguished by GC-MS (Table 2). The oldest successfully analyzed paint sample to date is from the Tomb of Nefertari, nearly 3000 years old; it tested positive for plant gums. Other Egyptian objects dating from 200 B.C. to 50 A.D. tested positive for mammal glue and egg as the binder. Overall, the results were very encouraging, but false negatives were obtained for few samples.

More recently, the focus of our research has been to eliminate false negatives when testing artificially aged pigmented samples. For example, robust extraction procedures such as sonication and heat were tried in an attempt to increase the solubility of cross-linked binders. Most likely, new antibodies need to be made specifically to identify aged binders.[27] Of course, those

TABLE 2. Results of Various Artworks Tested by ELISA

			ELISA		GC/MS[a]	
artwork	origin	pigment	identified binder	OD_{405}	identified binder	wt %
Tempera portraits on wood, Getty museum #74.AP.20	Egypt, c. 200 A.D.	black	glue	0.6	animal glue	2
		red	glue	0.4	animal glue	2
			egg	0.5		
Jacob Lawrence, *Paper Boats*	American, 1949	black	egg	0.6	NT[c]	–
			casein	0.8		
Potala Palace wall paintings	Tibet 17th C. to present	copper green	glue	0.9	animal glue	2
		yellow	glue	0.6	animal glue	1.5
		lead red	NR[b]	–	animal glue	0.8
model of the plaza (maquetas) with inlayed and painted wood	Tomb of Huaca de la Luna, Peru c.14th C.	red	plant gum	0.6	polysaccharide	1
		gold	plant gum	0.4	polysaccharide	1
		white	plant gum	0.2	polysaccharide	1
Maria, das Kind anbetend by Bevilacqua Gemäldegalerie Alte Meister in Dresden	1480 A.D.	brown	glue	0.6	protein	7
			egg	0.6		
		gold leaf on blue	egg	0.4	NR[b]	–
			plant gum	0.6		
		dark green	egg	0.7	protein	2
Wall painting, Church of the Mission of St. Frances by Andrea Pozzo	Italy c.17th C.	copper green	egg	0.8	egg	–
Nefertari tomb	Egypt c. 1000 B.C.	red iron oxide	plant gum	0.7	*Acacia* gum	4
		Egyptian green	plant gum	1	NT[c]	–
		charcoal black	plant gum	1	NT[c]	–
		Egyptian blue	plant gum	0.5	NT[c]	–
		yellow ochre	NR[b]	–	*Acacia* gum	2
Cartonnage, #79374 and #79385 Petrie Museum of Egyptian Archaeology	Egypt c. 200 B.C.	Green Earth	egg	2.0	NT[c]	–
			glue	2.0		
painted wood head HUCSM A0939 Skirball Center	Egypt c. 200 A.D.	red	glue	2.0	Animal glue	9

[a] Paint samples analyzed by GC-MS are reported as wt % of binder in paint. GC-MS sample compositions were compared to those of standard reference binding media using the method of correlation coefficients.[25] [b] No result: the analysis failed to yield data or no binder was detected. [c] Not tested: analysis not done.

antibodies that are readily commercially available and applicable to binding media are highly desirable, but the need for tailored antibodies for cultural heritage is compelling, yet expensive.

IFM

We focused on the development of an IFM protocol to identify and localize proteins in painting stratigraphy based on the indirect method. Similarly to indirect ELISA, in this approach, the primary antibody forming the immuno-complex with the targeted protein is detected by a secondary antibody which is conjugated with a fluorophore (Figure 3).

The analytical protocol has been optimized for recognition of egg white, bovine casein,[20,28] and, recently, animal glue by comparing different conditions for antibody dilution, incubation time, and temperature. From one microsample (<1 mg), a few thin cross sections are obtained and processed for immunodetection of proteins. Details about the adopted method and materials are reported in Table 3.[20]

Pictorial models of easel paintings and *secco* paintings on dried plaster were used as benchmarks to evaluate limits and

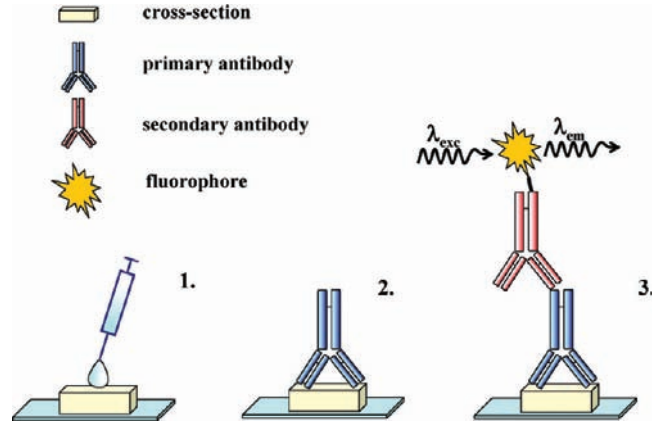

FIGURE 3. Sketch of the IFM methodology adopted for protein detection in painting cross sections: (1) addition of the blocking solution (serum goat 5 vol % in PBS) and incubation at 37 °C for 30 min; (2) addition of the primary antibody and incubation at 37 °C for 2 h. Both steps 1 and 2 are followed by multiple sample rinsing with PBS with final drying in air; (3) addition of the secondary antibody and incubation at 37 °C for 2 h. Samples are read for specific fluorescence under the microscope.

strengths of IFM for the investigation of proteins which have undergone prolonged aging and are enclosed in predomi-

TABLE 3. Reagents used for Immunodetection of Egg White, Casein/Milk, and Animal Glue by IFM

binders	detected protein	primary antibody	secondary antibody
egg white	chicken egg albumin	mouse monoclonal to ovalbumin (ab17293), ABCAM plc, U.K. (1:250 diluted in PBS)	QDot 605 goat anti-mouse IgG conjugate (Q11002MP), Invitrogen S.R.L, Italy (1:100 diluted in PBS)
casein, milk	bovine β-casein	mouse anti-β-casein α-AH4[28] (1:50 diluted in PBS)	
animal glue	collagen I from mammals	mouse monoclonal to collagen I (ab23446), ABCAM plc, U.K. (1:50 diluted in PBS)	

nantly inorganic matrixes. We aged the binders mixed with hematite, giallorino, malachite, minium, and smalt in the presence of high humidity (85% RH at 40 °C for 3 months). By comparison of the results for related and unrelated antibodies, the IFM method was assessed to work properly for positive and negative tests in all the records. No evidence of aging effects and pigment interferences was shown, although these phenomena may affect IFM analysis due to loss of antigenic sites. Differently from ELISA, protein cross-linking and nonsolubility are not limiting factors.

In addition to immunologic issues, further experimental clues of IFM with respect to ELISA relate to fluorescence detection. In fact, unspecific fluorescence is the most important drawback of IFM that delayed its application to the study of painting materials.[15−17] For these samples, an intense interfering background emission, mainly originating from the inorganic substrates, may hinder data interpretation.

For example, Figure 4 reports the optical microscopy and IFM images obtained by tagging casein contained in a microsample of a mural painting with the fluorophore fluorescein isothiocyanate (FITC). The sample was collected from painting fragments recovered from the frescos attributed to Giotto, decorating the vaults of the upper church of the Basilica of San Francesco in Assisi that collapsed after the earthquake of 1997. Immuno-fluorescence was used to assess the presence of proteinaceous binders for the supplemental use of the *secco* painting technique on the frescos. The comparison of the images of the untreated and treated samples under the fluorescence microscope (Figure 4b and c, respectively) shows how unspecific fluorescence prevails over the specific fluores-

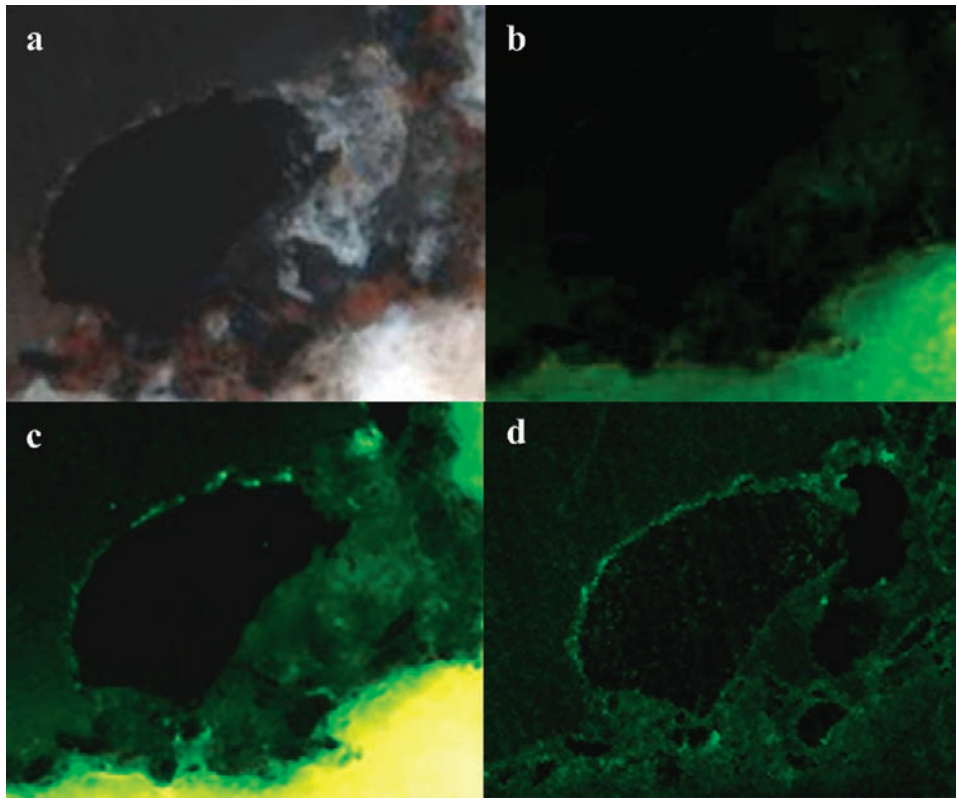

FIGURE 4. Cross-sectional images of a microsample from frescos attributed to Giotto. (a) Normal light 100× image; fluorescence 100× images (b) before and (c,d) after the immunological assay for casein by labeling with FITC fluorophore. Immuno-fluorescence emission of the binder is distinctly detected with the fluorescence microscope (c); drastic background suppression is obtained at the confocal microscope (laser excitation wavelength of 488 nm, band-pass detection 505−550 nm) (d).

cence of casein that is, however, clearly visible in the paint layer and around the big dark grain.

The problem of unspecific emission is well-known in fluorescence microscopy.[29] As this aspect is critical for successful IFM essays, our research was particularly focused on it. When autofluorescence is negligible (absence of lakes[30]), the most important contribution to unspecific fluorescence arising from painting cross sections comes from the preparation layer (plaster or gypsum) which is characterized by a prevailing porous inorganic matrix. A fluorescence background is produced according to two primary mechanisms: light scattering phenomena at the sample surface and unspecific adsorption of antibodies on the porosity of the samples. While the latter phenomenon can be effectively inhibited by sample treatment with a proper blocking solution as for ELISA, light scattering suppression is more difficult and can be achieved following different strategies as suggested by literature in biological and medical research.[29]

As a first option, confocal fluorescence microscopy is widely used to obtain an easy and satisfying reduction of nonspecific background emission.[31] In laser-scanning confocal microscopy, a monochromatic laser source is used to scan across a defined sample area, while the use of a very small aperture (pinhole) in the optical path allows to detect light emitted within the focal plane at different sample depths and, at the same time, to discard the out-of-focus light. Moreover, the LeicaSP2-AOBS confocal microscope used for this work is equipped with a spectral detection system (acousto-optical beam splitter) in which the light emitted from the sample is spectrally decomposed by a prism before reaching the detectors. Sliders in front of the photomultiplier tubes allow a very precise selection (1 nm steps) of the wavelength range to be detected (to a minimum of 5 nm). This detection system gives much more flexibility compared to the standard filter-based fluorescence microscopes and allows for spectral scans within the focus plane.

An example of the use of the confocal setup for immunofluorescence detection in painting cross sections is shown in Figure 4c and d where light emission from the same sample is observed under a conventional fluorescence microscope and the confocal one, respectively. The image collected with the confocal microscope clearly shows a drastic attenuation of the background emission.

A further improvement in terms of quality of images and selectivity of the IFM method can be achieved by using new high performance fluorescent labels specifically developed for imaging in cell biology; these show increased quantum yield and photostability.[32] In particular, a new technology based on the peculiar optical properties of quantum dots (QDs, nanoparticles of semiconductors composed of groups II−VI and III−V elements) has shown to have great potential in the application of these fluorophores as bioprobes for molecular imaging. QDs offer several advantages with respect to other common fluorophores: they have high fluorescence yield, a long lifetime, broadband absorption, and sharp and intense band emission, whose position can be controlled by changing QD composition and dimension.[33,34] In particular, tunability of QD emission enables one to increase the Stokes shift between absorption and emission wavelengths, thus achieving a major gain in suppressing light scattering background. For example, Figure 5 shows the image of the immuno-fluorescence emission of casein in another microsample from the frescos cycle of Assisi obtained by protein labeling with quantum dots emitting at 605 nm (QD605). Furthermore by taking advantage of the optical properties of QDs, it is possible to exploit the capability of confocal microscopy of completing imaging analysis with punctual measurements of sample emission spectra. As an example, Figure 5b shows the typical fluorescence emission of quantum dots QD605 from the tagged proteins as recorded on the sample cross-section.

Thanks to the recent technological development of light sources and detection systems for fluorescence imaging,[35] a further improvement in background suppression in IFM can be obtained by exploiting the long lifetime of the QDs. Common organic dyes typically show a fast fluorescence emission decay of few nanoseconds[36] that overlaps with the short-lived autofluorescence background. Conversely, QD fluorescence emission decays in a time span of a few tens of nanoseconds (10−100 ns) at room temperature.[37] This offers the opportunity of performing time-gated analysis to enhance the signal-to-noise ratio by delayed collection of the immuno-fluorescence signal after background fluorescence decay.[34] An example of preliminary results obtained by combining the use of QDs as bioprobes with time-resolved immuno-fluorescence detection is shown in Figure 6.

Here, the fluorescence emission of QD605 fluorophore was used to tag ovalbumin in an artificially aged painting layer of lead tin yellow in egg. Figure 6c shows the sample emission collected by time-resolved fluorescence imaging[38] at 9 ns of time delay in the area evidenced by red contours in Figure 6a and b. At 9 ns after excitation, light emission is ascribable only to QD fluorescence, and although experimental conditions were not optimized, protein distribution is clearly visible. These preliminary results of the study are a prelude to interesting future applications of this advanced analytical approach of IFM in cultural heritage by exploitation of the QDs' properties.

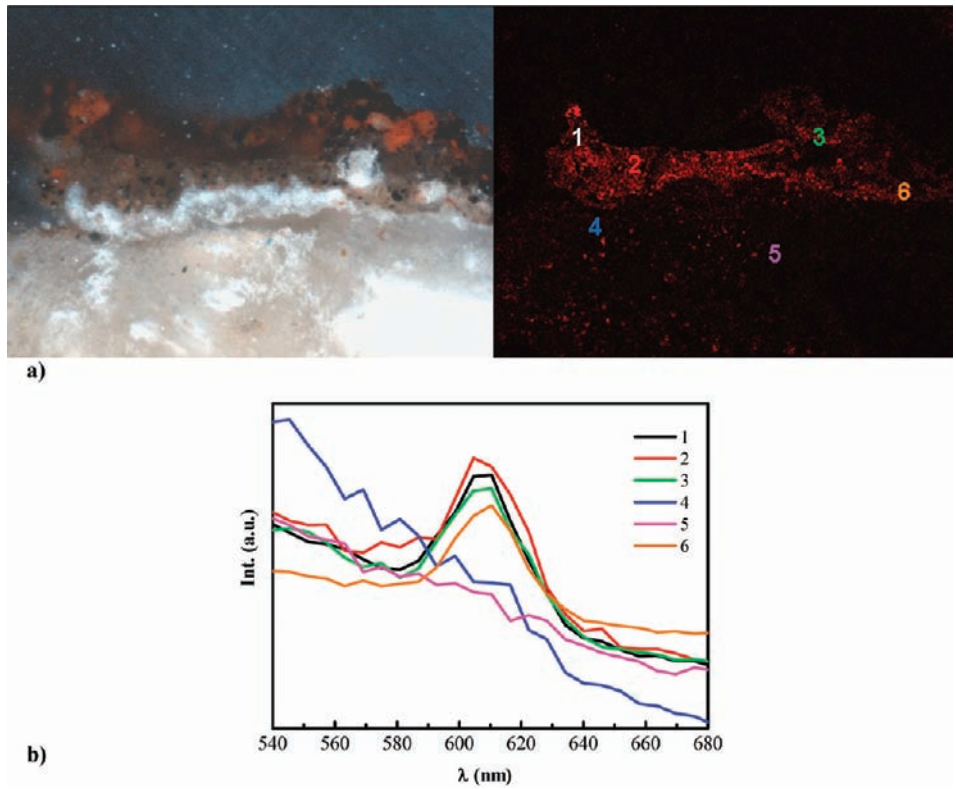

FIGURE 5. (a) Normal light (left) and fluorescence confocal microscope (right) cross-sectional images (160×) of a microsample from the fresco cycle of Assisi attributed to Giotto. Casein tagging was obtained by using QD605 fluorophore. Excitation wavelength was 458 nm. (b) Emission spectra collected in different fluorescent areas of the sample labeled by numbers. Light emission was collected by using a spectral scan window of 5 nm from 540 to 680 nm and sampling at 2.5 nm intervals. The typical QD emission at 605 nm allows for the unambiguous distinction between specific fluorescence and background.

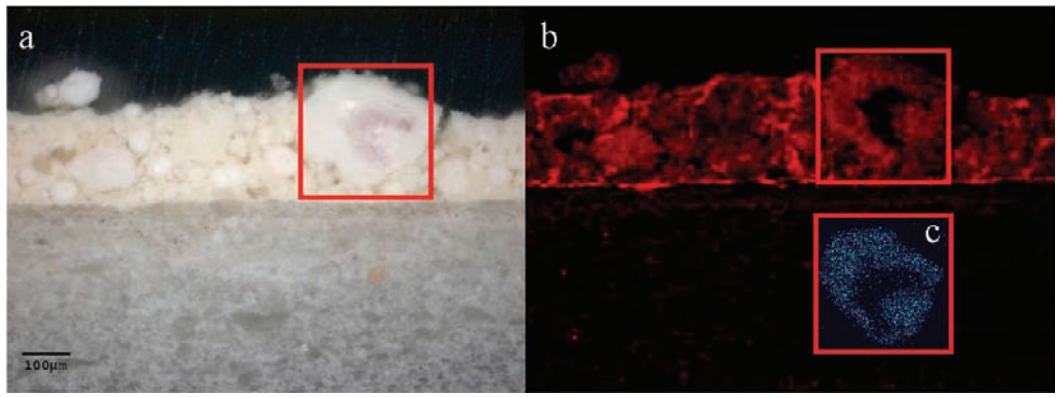

FIGURE 6. (a) Normal light and (b) fluorescence confocal microscope images (100×) of a cross section of an artificially aged painting layer of lead tin yellow in egg on a gypsum/glue preparation. Inset (c) shows the laser scanning time-resolved immuno-fluorescence image corresponding to the area evidenced by red contours in (a) and (b). QD605 fluorophore was used to tag egg ovalbumin. The fluorescence confocal microscope image was acquired by exciting at 458 nm and collecting from 595 to 620 nm. The time-resolved immuno-fluorescence image was obtained at 400 nm of excitation wavelength at 9 ns of time delay (laser pulse width of 60 ps). The image is corrected for the background in order to avoid light spurious effects.

Conclusions and Outlook

The current state of our research on the unconventional use of immuno-detection based techniques applied to the field of science of cultural heritage is summarized here. Our results emphasize the analytical potentials that ELISA and IFM techniques offer for the high specific and sensitive identification of proteins in ancient materials. ELISA is particularly suitable for fast, routine analyses, while IFM can be applied for stratigraphic studies. Both are microdestructive; however, samples of truly micrometric size are sufficient for analysis.

From our experience, in spite of the numerous positive results obtained for both laboratory standards and real samples, further experimentation of the analytical methodologies is mandatory. Two main research routes have to be fully explored: The first is strictly immunologic and concerns the production of tailored antibodies for cultural heritage. Manufactured antibodies should respond to the specific needs of this field, providing high sensitive recognition of aged, denatured protein in inorganic matrix and distinguishing their biological sources (possibly within the same biological family). To achieve multiple protein recognition in the same sample, antibody cross-reactivity should be also tested. The availability of commercially manufactured antibodies would give each laboratory equal access to the same reagents and comparable data, and quality control and batch-to-batch consistency would be far superior. The second route is related to the development of the detection system in immuno-fluorescence based techniques, with the suppression of autofluorescence background as the most challenging issue. In this Account, we report different solutions to the problem; however, much experimental work is ongoing in this subject.

Immunology can be considered a relatively new field in conservation science, and it requires significant efforts on basic issues that have been already solved for applications in other scientific disciplines. However, the potential developments are exciting. One of this is the "multiplex" determination to screen for multiple targets in the same sample. "Multiplexing" detection in fluorescence imaging[39,40] can be obtained by combining multicolor imaging of different fluorescence probes to localize different proteins. To this end, QDs offer unprecedented properties in terms of emission tunability and fluorescence decay rates, allowing to discriminate different QDs' signals within the same specimen.

The financial support provided by Eu-ARTECH (contract RII3-CT-2004-506171), a project of the sixth FP of the European Union within the program of Research Infrastructure, is acknowledged. The Soprintendenza ai Beni A.P.P.SAD dell'Umbria, Italy, is gratefully acknowledged. Authors are also grateful to Prof. Loredana Latterini of the Chemistry Department of the University of Perugia for her assistance with time-resolved fluorescence measurements.

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Joy Mazurek was born on July 2, 1970 in Laguna Beach, California. She is Assistant Scientist at the Getty Conservation Institute, USA, since 1998. She obtained her masters degree in biology from California State University Northridge in 2007; her thesis was "Antibody Assay to Identify Binding Media in Paint".

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FOOTNOTES

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