The Use of Immunofluorescence Microscopy (IFM) and Enzyme-linked Immunosorbent Assay (ELISA) as Complementary Techniques for Protein Identification in Artists' Materials

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Abstract

Antibody-based immunological approaches to identifying protein-based materials offer several advantages over the traditional methods used for analyzing works of art. These techniques are able to distinguish with ease between different protein types (i.e., collagen vs. albumen vs. casein) and also to determine unambiguously the biological source of the protein (i.e., bovine collagen vs. rabbit collagen vs. sheep collagen). The technique of Enzyme-linked Immunosorbent Assay (ELISA) is highly sensitive (detection limits below one nanogram) and is relatively simple and cost-effective. In addition, immunofluorescence microscopy (IFM) offers the possibility of spatially resolving target proteins in embedded cross-sections. This paper presents part one of a case study in which these complementary methods were successfully employed for the identification of egg albumin in the analysis of an important 17th-century French cabinet by André-Charles Boulle in the collection of the J. Paul Getty Museum. The authors suggest that ELISA and IFM have the potential to become routine analytical tools in conservation science laboratories.

1. Introduction

The recent technical study of a 17th-century French cabinet in the collection of the J. Paul Getty Museum has afforded an excellent opportunity to demonstrate the efficacy of immunological or antibody-based approaches for the identification of protein-based binding media in art works. While the potential of immunological techniques in art conservation has been discussed and investigated sporadically over the last several decades, these techniques have found little practical application, particularly in the United States. In recent years, the technology and scientific infrastructure related to immunology and antibodies has advanced significantly. Today, the techniques and materials necessary to conduct highly specific and sensitive immunological protein analysis on art materials are both reliable and affordable. Two methods in particular, Enzyme-linked Immunosorbent Assay (ELISA) and immunofluorescence microscopy (IFM) have the potential to become routine analytical tools in conservation science laboratories. These methods offer several advantages over the traditional methods used for protein analysis in conservation; they are able to distinguish with ease between different protein types (i.e., collagen vs. albumen vs. casein) and also to determine unambiguously the species source of the protein (i.e., bovine collagen vs. rabbit collagen vs. sheep collagen). ELISA is highly sensitive (detection limits below one nanogram) and is also a relatively simple, fast and cost-effective technique, while IFM offers the possibility of spatially resolving target proteins

Figure 4. The Getty cabinet, attributed to André-Charles Boulle, as it appears today.

in embedded cross-sections. These two techniques are particularly powerful analytical tools for the conservator and conservation scientist when they are used in tandem. This case study briefly describes relevant immunochemical principles and techniques, provides an overview of the conservation-related literature and presents the results of ELISA and IFM analysis for the identification of a very thin egg white or glair layer in paint samples taken from sculptural elements of the Getty's cabinet.

2. Background

2.1 General immunochemical principles

The rationale for using immunological approaches to identify materials used in art samples is straightforward, and the biological basis for this approach is well understood (for review, see Goldsby et al., 2003). The concept is based upon the immune response that organisms generate in response to the presence of foreign molecules, such as a virus or a bacterium. The immune system of higher organisms has evolved to be able to recognize components on the surface of these foreign molecules. These components are called antigens, and are typically proteins, but can also be lipids or carbohydrates. Once the antigen is recognized, the immune system mounts an attack by generating antibodies that bind to the antigen. The site upon which the antibody binds to the antigen is called the antigenic site, or epitope. Antibody binding to the antigen epitope is one of the most specific interactions known in biology, and this specificity is one of the major advantages of this approach, as will be detailed below.

This immune response has been harnessed for laboratory use. Experimental animals (typically rabbits or mice) can be injected with any foreign molecule antigen (e.g., hen's egg albumin, sheep collagen). The animal generates an immune response and produces antibodies. Each antibody has a variable region (which is the part that binds to the antigen) and a constant region that determines the general class of which the antibody is a member. There are several different classes, all of the immunoglobulin (Ig) protein family. The general antibody classes include IgA, IgE, IgG and IgM. Typically, these antibodies are polyclonal. That is, they represent many different antibodies that recognize different epitopes within the antigen. The experimental advantage of polyclonal antibodies is that one effectively has multiple chances to identify a given protein because multiple epitopes are being probed. This could be important, for example, in cases where antigens are degrading over time, and thus any one given epitope may be preferentially lost. The disadvantage of polyclonal antibodies is a potential loss of specificity. That is, a polyclonal antibody against sheep collagen may bind to epitopes that are common in all mammals, and thus one might be unable to conclude that a given collagen found in a sample was specific for a given species. One common way around this problem is to use a monoclonal antibody, which specifically recognizes one epitope. Thus, if one injects an experimental animal with a known small portion (a peptide fragment) of sheep collagen protein that is unique to sheep, then the likelihood of species specificity increases.

The use of antibodies for antigen labeling and identification is a routine laboratory tool in the biological and biomedical community not only because of their utility but because of their availability and their cost-effectiveness. A vast number of species-specific polyclonal and monoclonal antibodies are commercially available, and many of these are likely to be of interest to art conservation. These include antibodies against protein antigens found in egg white (albumin), skin glues (collagen, fibronectin, integrin), blood (ferritin) and milk products (casein), to cite just a few. Additionally, these antibodies are created from protein antigens of many species, including chickens, cows, fish, goats, horses, rabbits and sheep. Typical costs for such commercially available antibodies are from tens to hundreds of dollars; on average, this works out to be approximately one dollar per experiment. Applications are not limited to commercially available "off-the-shelf" antibodies. There are also antibody production companies that will generate user-requested antibodies. Although this is more expensive, it is theoretically possible to generate antibodies for any antigen (e.g., generating antibodies that would recognize isinglass, i.e., antibodies against sturgeon air-bladder antigens).

The antibody that recognizes an epitope on the antigen of interest is known as the primary antibody. One can engineer primary antibodies with a "tag" that is detectable by some experimental procedure in order to determine the presence of the antigen in a sample. However, for practical reasons, current immunological approaches use secondary antibodies as the read-out for the presence of antigen. The secondary antibody binds to a conserved region of the primary antibody; in other words, the primary antibody acts as an antigen for the secondary antibody. Therefore, the secondary antibody (which is tagged for detection) signals the presence of the primary antibody, which in turns signals the presence of the antigen of interest (fig. 1). For example, a primary monoclonal antibody specific for hen's egg albumin might be created by injecting a mouse with an albumin peptide corresponding to an epitope specific for albumin found in chickens. Upon injection of the albumin peptide antigen, the mouse would generate an immune response by creating mouse-specific antibodies of, say, the IgG

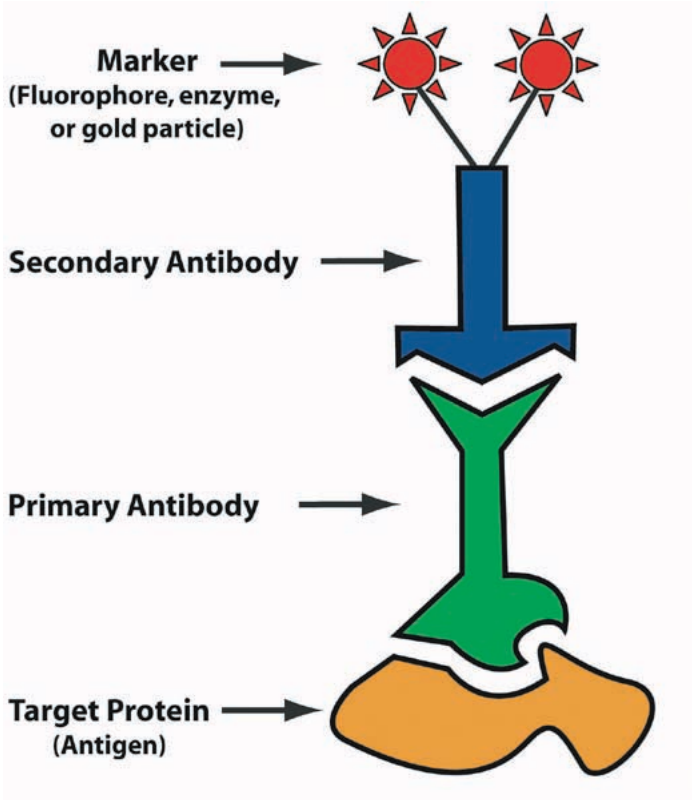

Figure 1. Typical current immuno-detection methods utilize a double antibody technique in which the secondary antibody is tagged for detection.

antibody family. In parallel with this, one could inject a rabbit with the constant region of mouse IgG to create a secondary antibody that non-specifically labels any mouse IgG. Thus, one would incubate the sample of interest with a mouse IgG antibody that recognizes hen's egg albumin. Then, one would label the primary antibody with the secondary antibody that specifically recognizes mouse IgG. This IgG molecule, prior to injection in the rabbit, is conjugated to a tag that permits laboratory detection. Common tags for secondary antibodies are: (1) enzymes that permit detection by any one of several methods including colorimetric assay (e.g., ELISA, see below) or chemilumnescence; (2) fluorochromes that permits detection by fluorescence microscopy; or (3) nano-particles of gold that permit detection by electron microscopy.

2.2 Enzyme-linked Immunosorbent Assay

2.2.1 Principles

The technique of Enzyme-linked Immunosorbent Assay (ELISA) is based on the use of an enzyme-conjugated secondary antibody which acts to substantially amplify the immunoreactive signal created by the primary antibody (Crowther 1995). Briefly, in an ELISA assay, proteins are extracted from a sample of interest into an aqueous solution and then placed in the well of an ELISA plate (fig. 2). Due to molecular interactions between proteins and the polystyrene of the plate, the proteins bind to the well walls. Primary antibodies are then placed within the well and allowed to bind to their antigen epitope(s). After removal of the primary antibody and rinsing of the well, a secondary antibody is applied which will bind to any reacted primary antibody; the secondary antibody is conjugated to an enzyme, usually alkaline phosphatase. Unbound secondary antibody is then washed out of the well and an enzyme substrate, such as the alkaline phosphatase substrate p-nitrophenyl phosphate (p-NPP), is added to the well. The result of the enzymatic reaction between antibody and substrate is a reaction product that can be detected by colorometric assay. For example, the reaction product of the alkaline phosphatase:p-NPP interaction is nitrophenol, which is yellow. Measured at the appropriate wavelength using spectrophotometry, the optical absorbance of this reaction product is proportional to the enzymatic activity of the secondary antibody, which in turn is proportional to the amount of primary antibody bound to the antigen (fig. 3).

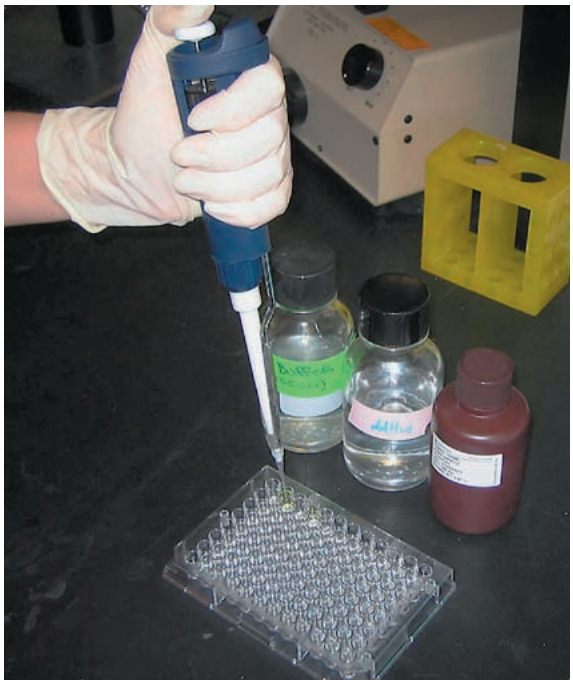

Figure 2. Eluted proteins and antibody solutions are added to the wells of an ELISA plate with a pipettor.

ELISA assays have several advantages for art conservation. First, a single extraction can be used in multiple assays, thus extending the usefulness of small sample sizes. For example, the extracted sample can be subdivided and applied to multiple wells of the ELISA plate. Or, a single volume of extracted sample

can be used to seed multiple wells. Alternatively, a single well can be probed multiple times with a succession of different primary antibodies until a positive sample is identified. Second, the assay is exquisitely sensitive to small amounts of antigen. This is due to the fact that a single enzyme molecule is able to catalyze the reaction of thousands of substrate molecules, thus amplifying the signal by several orders of magnitude. This sensitivity greatly minimizes the likelihood of obtaining a false negative result. These advantages are at the expense of a loss of spatial resolution. That is, the extraction procedure effectively destroys any ability to localize a given protein to a particular layer or position in the sample.

2.2.2 History of use in conservation

To date, the conservation-related uses of ELISA have been largely confined to the realm of archaeometry. Since about 1990, a number of studies have used this technique to identify the species origin of archaeologically recovered bone, as well as of traces of blood, either found on artifacts, or even as a binder in paint (for example, Cattaneo et al., 1991; Hyland et al., 1990; Scott et al., 1996). In addition, ELISA has been used for such varied purposes as identifying microbes associated with deterioration on metal, stone and wood (Gaylarde, 1990; Tayler and May, 1994; Clausen, 1997). Studies such as that by Schweiter et al. demonstrate that ELISA is sufficiently sensitive to work with extremely aged proteins; this study found immuno-reactive collagen-derived peptides in a 100,000 to 300,000 year old fossil mammoth skull (Schweiter et al., 2002).

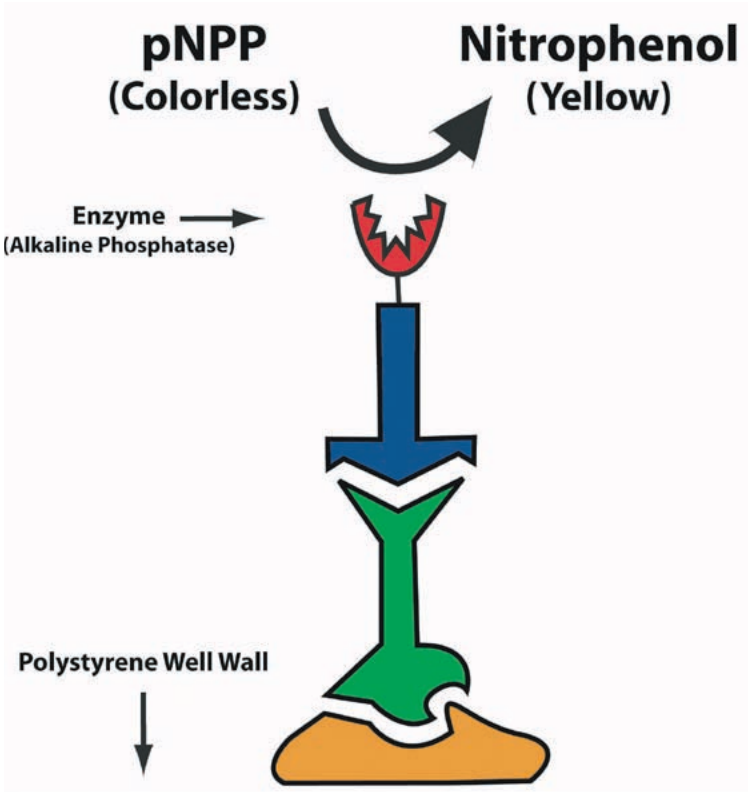

Figure 3. The ELISA technique utilizes an enzyme-conjugated secondary antibody for antigen detection.

Hodgins and Hedges have recently used the ELISA technique to investigate the abilities and limitations of antibodies to discriminate between collagens of different species origins, both in archaeological bone and in historic glues. Their work suggests that while the technique is fundamentally sound, care must be taken to test specific batches of commercially produced antibodies for possible species cross-reactivity, particularly among bovids (Hodgins and Hedges, 1999). These authors have used ELISA to identify historic collagen-based glues as well as parchments and even violin strings. Their work also reaffirms the persistent nature of collagen immuno-reactivity, even with

highly artificially aged samples where dramatic alteration of the protein structure has occurred. (Hodgins and Hedges, 1998). Hodgins and Hedges have also investigated the effect of pigment-protein interactions on the deterioration of immunoreactive epitopes in aged collagen. Working with seven different pigments, they noted little effect from pigment interaction when using polyclonal antibodies for non-species specific collagen detection, with the exception of verdigris. However, when the authors used bovine-specific antibodies for the same test, the results were much more varied; three pigments accelerated the deterioration of immunoreactivity, two had no effect and two actually appeared to stabilize the immunoreactivity of the collagen sample (Hodgins and Hedges, 2000). These results and others point to variable stability among individual epitopes within a given protein molecule. Their work may mean that quantitative analysis of proportional composition in aged material of mixed origin may be difficult to achieve.

2.3 Immuno-fluorescence Microscopy (IFM)

2.3.1 Principles

The general principles underlying immuno-fluorescence microscopy, or IFM, are similar to those of ELISA; however, in the case of IFM, detection is achieved using a fluorescence microscope and secondary antibodies that are conjugated to a fluorophore. Upon excitation with a photon of light at a given wavelength, the fluorophore emits a photon of light at a wavelength that is proportional to the energy released after excitation. For example, the fluorophore rhodamine B, when excited with green light (525–540 nm) emits a red signal (615–630 nm), while the fluorophore FITC (fluorescein) is excited with blue light (490–495 nm) and emits a green signal (520–530 nm). Thus, following antibody treatment, the sample is imaged using a microscope that can emit and detect light at specific wavelengths.

Because of the magnitude of the signal amplification inherent in the ELISA assay, detection of antigen by IFM is likely not to be as sensitive. There is also the problem of bleaching of the fluorophore with repeated excitation, and thus the signal may degrade over repeated measurements. However, the advantages of IFM are substantial. Unlike with an ELISA assay, the sample remains intact. This permits one to localize the antigen to a specific region of the sample. In addition, it is possible to detect multiple antigens within the same sample; for example, one could simultaneously use a rhodamine-conjugated secondary antibody to label a sheep-specific collagen primary antibody and an FITC-conjugated secondary antibody to label a rabbit-specific collagen primary antibody. The commercial availability of dozens of different fluorophores conjugated to multiple different animal immunoglobulins permits an almost limitless set of imaging possibilities.

2.3.2 History of use in conservation

The first suggestion of using IFM in art conservation came from Meryl Johnson and Elisabeth Packard in a paper entitled “The identification of binding media in Italian paintings of the fifteenth and sixteenth centuries” which was presented at the Ninth Annual Meeting of the IIC-American Group in May of 1968 and

subsequently published in *Studies in Conservation* (Johnson and Packard, 1971). The authors reported encouraging results using a single antibody technique and improvised fluorescence microscopy equipment to identify egg proteins in cross-sections of aged tempera paintings. They noted that new types of dedicated fluorescence microscopes were then becoming commercially available which they felt would greatly increase the potential for application of this technique in art conservation. Keck and Peters agreed that IFM on cross-sections held great potential, and noted that it could be used as a complement to amino acid analysis (Keck and Peters, 1969).

In 1982, Robin Talbot, then a student at the Cooperstown training program, presented a paper at the Third Annual Conference of Art Conservation Training Programs entitled "The fluorescent antibody technique in the identification of proteinaceoous materials" (Talbot, 1982). In this paper, Talbot demonstrated success with a single antibody technique in identifying albumin layers in laboratory-prepared reference materials as well as in identifying casein in two artifacts undergoing conservation treatment. As was the case with Johnson and Packard a decade earlier, Talbot used improvised fluorescence microscopy equipment for the bulk of her work, though she was able to use a purpose-built fluorescence microscope at a nearby hospital as well. Talbot noted that while such equipment was unfortunately rarely available to conservators, the advantages of using "the proper instrument cannot be overemphasized." Wolbers, in a case study of "A Pair of New England Portaits by Richard and William Jennies," discussed the use of IFM on cross-sections taken from paintings dating to the turn of the 19th century (Wolbers, 1988). Wolbers was the first to document the use of the double antibody technique for IFM in conservation and was able to identify a thin albumin layer using a rhodamine-conjugated secondary antibody, though he felt that the possibility of non-specific staining required that the results be confirmed by other methods. In a paper presented in the general session of the 1987 AIC conference, he and Landrey expressed the additional concern that the prolonged exposure of sample material to aqueous solutions could cause unacceptable dissolution of water-soluble components in the sample (Wolbers and Landrey, 1987).

Kockaert et al., (1989) conducted the first systematic study of IFM for the identification of ovalbumin in binding media, applying the method to single cross-section samples taken from 13 paintings and 5 polychrome sculptures dating from the 12th to the 20th centuries. The authors concluded empirically that aging had little effect on the detectability of albumin, though they cited difficulties in visualizing positive results under several circumstances: where the fluorochrome (FITC) and natural fluorescence of the target layer were similar in color; where the fluorochrome and the pigment of the target layer were similar in color; and where porous layers were present (because of problems with non-specific staining). Significantly, the authors noted difficulties obtaining reproducible results with certain samples, and further noted that negative results could not be interpreted to mean that no albumin was present, only that it was not detected.

For over a decade after the publication of Kockaert, no further mention could be found in the conservation literature regarding IFM. Then in 2001, Ramírez-Barat et al. published a note on methodology for IFM in *Studies in Conservation* (Ramírez-Barat et al., 2001a; see also Ramírez-Barat, 2001b) in which they discussed results obtained using laboratory-prepared "model" samples. In this article, the authors offered several significant solutions for difficulties posed by previous investigators. To increase the detectability of aged proteins in cross-sections, they recommended a brief pre-treatment with trypsin, a protease enzyme which can help unmask aged proteins and make their epitopes more available for reaction with antibodies. In addition, they noted that the sensitivity of IFM can, in theory, also be increased by using a triple antibody technique, the signal being amplified with each successive step. In order to greatly reduce non-specific staining (noted by Wolbers and Kockaert), they also recommended the application of a "block" solution prior to incubation with the primary antibody. This is simply a solution of non-reactive protein which serves to fill up or "block" any non-specific protein binding sites in the sample. Ramírez-Barat et al. also noted that the difficulties of visualization cited by Kockaert could easily have been overcome by choosing fluorochromes of different colors, depending on the characteristics of the target layer. Finally, Ramírez-Barat et al. address the problem raised by Wolbers of possible dissolution of water-soluble material out of the sample during the lengthy incubation in aqueous solutions. They discuss attempts to solve this problem by fixation of the proteins as well as by total impregnation of the sample in a hydrophilic embedding medium; however they report no success to date with these methods (for further discussion of this issue, see Avenues for Further Research, section 4.1, below).

2.4 ELISA and IFM as complementary techniques

ELISA has been shown to be a remarkably effective tool for the analysis of proteins in both archaeological and art materials. It offers the possibility of exceptionally specific identification from very small samples. Because the sensitivity is so high, one can test for any number of specific proteins or protein classes by dividing the sample's elution product into smaller aliquots. In addition, its sensitivity appears to be only marginally affected by sample aging. The significant limitations of this technique are that it requires the irreversible dissolution of the sample material, and that the resolution of proteins in individual layers within a thinly-layered complex cross-section is very difficult (i.e., can only be accomplished by physically separating the layers prior to analysis).

IFM offers capabilities which nicely complement those of ELISA and redress its limitations. It is able to spatially resolve the target protein(s) within a thinly-layered complex cross-section sample, and it leaves the sample essentially intact for further examination in the future. IFM is not, however without shortcomings of its own. While Ramírez-Barat et al. offer significant solutions for many of the potential problems associated with IFM, they do not address the problem (as noted by Kockaert et al.) of interpretation when confronted with negative staining results, i.e., that absence of proof does not equal proof of absence. This is the

essential weakness of IFM that has led the authors of this paper to recommend that IFM be used in conjunction with ELISA.

If a small portion of the material sampled for cross-section examination can be reserved for analysis by ELISA, then a pre-screening of the sample can be made to identify proteins of interest present in the cross-section. Subsequent examination using IFM can then be targeted at proteins known to be present; this limits the number of incubations to which a cross-section must be subjected (particularly since several proteins may be probed simultaneously using different fluorescent tags) and also removes any ambiguity in the face of a negative staining result.

3. Case study

3.1 Description of the artifact

In 1977 the J. Paul Getty Museum acquired a truly extraordinary cabinet-on-stand attributed to André-Charles Boulle, cabinetmaker to Louis XIV of France (fig. 4, page 2). The cabinet is one of a pair of monumental pieces thought to have been made in the late 17th century as a tribute to the military victories of Louis XIV over the English and the Germans. The mate to the Getty cabinet survives at Drumlanrig Castle in Scotland. Among the most prominent and unusual features of these cabinets are the two carved figures supporting the front corners of the cabinet, said to represent Hercules and Hyppolita.

At the time the cabinet was purchased for the museum, the skin of the figures was a dark brown or nearly black color, thought to be in imitation of bronze. Prior to the shipment of the cabinet from Europe to California, a thorough restoration was undertaken in London. In the initial stages of the London restoration, it was determined that the dark skin tone of the figures was the result of numerous layers of restoration paint lying over the original surface. The restorers determined at that time that the first and original layer of paint on the figures was white, in imitation of marble, and thus, the figures were stripped down to their "original" white surface, which is how they are exhibited currently. Sadly, we now know that the white surface seen today is a chalk-based gesso which would have been a preparatory layer, not a finished surface. Since the overlying layers were removed during the restoration, no evidence now remains of the original appearance of these figures.

Recently, in an attempt to understand the original appearance of the Getty figures better, a detailed investigation has been carried out on the nearly identical figures of the Drumlanrig cabinet (fig. 5). These figures retain an appearance which is very similar to that of the Getty cabinet prior to 1978 and, it was hoped, might provide evidence as to the original appearance of both cabinets. With the kind permission of His Grace, The Duke of Buccleuch, owner of the Drumlanrig cabinet, five minute samples of paint were taken from the ungilded areas of the Drumlanrig cabinet figures for technical examination.

These samples were initially examined by traditional methods to identify any original layers of polychromy and to determine their composition. Of the six earli-

est layers found, one in particular (layer 4; a very thin, transparent, organic layer) defied analysis by these methods. It was our desire to positively identify this layer that led us to pursue immunological methods. This case study first describes the traditional scientific techniques used to analyze the cross-sections and discusses their limitations. This is followed by a more detailed description of the application of ELISA and IFM to the identification of layer 4.

3.2 Traditional methods of analysis

3.2.1 Microscopy

Examination of these samples in cross-section with optical and electron microscopy revealed that the six earliest layers present over the wood substrate on the non-gilded areas of the figures were (1) calcium carbonate-based gesso, (2) iron-based red pigment, (3) vermillion pigment, (4) transparent organic layer, (5) mixed lead white and vermillion pigments and (6) copper flake pigment (fig. 6). These layers were covered with at least three generations of over-paint. The early six-layer arrangement is in accord with descriptions in 17th and early 18th century French treatises of techniques for "bronzing" (Félibien, 1676; de la Rivière, 1721) but is quite different from later recipes. This led us to believe that these earliest layers might well represent the original surface decoration and might thus give us a good idea of the original appearance of the figures.

Figure 5. The Drumlanrig cabinet in Scotland, nearly identical to the Getty cabinet.

While the determination of the inorganic components of the original layers was relatively straightforward, understanding and identifying the organic components proved more difficult. The morphological characteristics of the lower three layers suggested that they were leanly bound and that they might be distemper layers. Layer 5 appeared to be more medium-rich, and based on the choice of pigments and the use of metal powder on the surface, we suspected that it might be a pigmented oil size applied to bind the copper powder of layer six.

The transparent organic layer in the middle of the layer structure was more of an enigma. Its function in the structure was not immediately apparent and its morphology was unusual, maintaining a very consis-

tent thickness of 4–5 µm as it closely conformed to the rough surface contour of the underlying vermillion layer. This layer was found to be weakly fluorescent under ultraviolet illumination. Initially, two cross-sections were stained with Rhodamine-B (Rho-B), triphenyltetrazolium chloride (TTC) and fluorecein isothiocyanate (FITC) and then viewed under the fluorescence microscope in order to give some preliminary indication of the nature of the binding media. The results of the staining suggested that layer 5 was indeed oil-bound (positive staining with Rho-B) but were ambiguous with regard to the lower four layers (weak or no staining with Rho-B, TTC or FITC).

3.2.2 Instrumental analyses for binder identification

In an attempt to further the identification of the organic media within the original six layers, we endeavored to analyze the layers individually by Fourier transform infrared spectroscopy (FT-IR) and gas chromatography with mass spectrometry (GC-MS). The small size of the sample material available and the thinness of the constituent layers made the application of these two techniques difficult, particularly with regard to layer 4, the transparent organic layer. Two flake samples of approximately 1 mm^2, containing the original six layers, were prepared for the analysis. Working under a stereomicroscope at high magnification, the samples were meticulously scraped down, layer by layer, with a microscalpel. The scrapings from each layer were collected and labeled for analysis. We expected that these scrapings would show some cross-layer contamination, but we hoped that some useful information could nonetheless be extracted from the sequence of analyses.

The first set of scrapings were analyzed by FT-IR microscopy in transmission mode using a diamond anvil cell. This analysis confirmed that layers 1–3 were bound in a protein medium and suggested that the medium might be animal glue, since the spectra showed no peak at 1740 cm^{-1} where egg yolk has a characteristic absorption band. The scrapings from layer 4, the organic sealer, yielded spectra characteristic of vegetable oils; no resin or protein-related peaks were detected. This result was apparently at odds with the previous negative staining result for oils using Rhodamine B. Analysis of layers 5 and 6 confirmed the initial hypothesis and the staining results, suggesting that layer 5

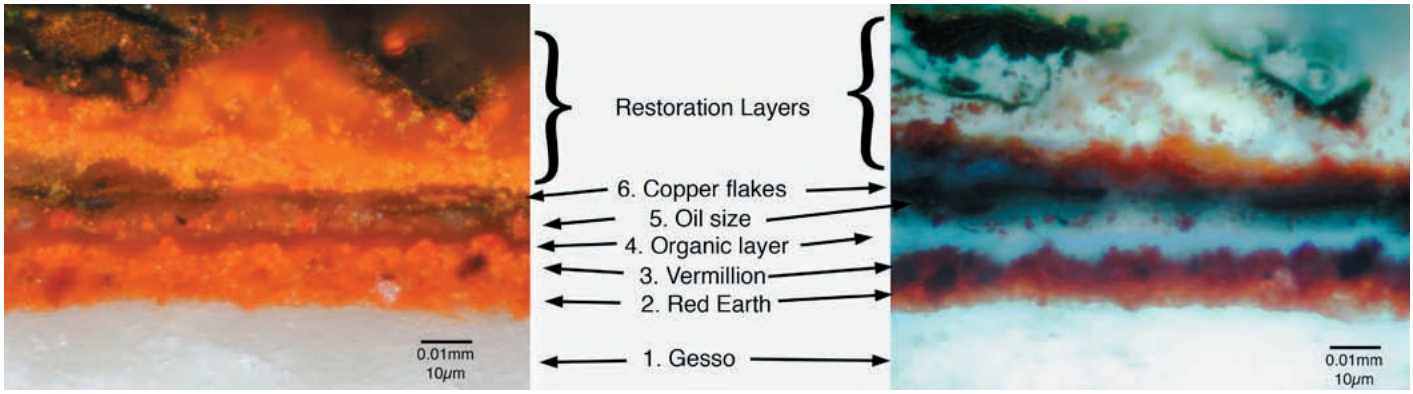

Figure 6. Photomicrographs in cross-section of samples taken from "bronzed" areas of the Drumlanrig cabinet. The sample is seen illuminated with visible light on the left and ultra-violet light on the right.

was an oil-bound pigmented size used for the application of dry copper flake pigment (layer 6).

In an effort to clarify the apparently contradictory results associated with the transparent, organic layer 4, prepared cross-sections were also examined by direct reflectance FT-IR microscopy with the hope of avoiding the problems associated with inter-layer contamination when analyzing scrapings. Thanks to the support of the National Synchrotron Light Source (NSLS) in Brookhaven, New York, we were able to conduct this analysis using IR synchrotron radiation (IRSR), which allows much more selective focusing of the incident IR radiation than standard FT-IR microscopy (Smith, 2003). Even using this advanced technique, the narrowest window we were able to work with successfully in reflectance mode was 15µm wide, or several times the thickness of our target layer. As a result, we were unable to collect individual spectra from discrete layers of the cross section; however, by creating a "line map" of sequential spectra collected every 4µm along a line perpendicular to the layers, we were able to get some indication of their composition. Within the series of spectra, the maximum absorbance in the 1550 cm^{-1}, 1650 cm^{-1} and 3310 cm^{-1} regions (associated with proteins) came when the analysis was centered on layer 4, suggesting that, contrary to the previous analysis of scrapings, this layer had a significant protein content.

The second set of scrapings were analyzed by GC-MS. The scrapings for each layer were too small to be weighed, so no estimate of binder percent by weight could be given. Furthermore, the small sample size mandated that the same material be used in the test for resins, oils and waxes as well as in the test for proteins; this meant that the second test (for proteins) could not be considered quantitative. Due to these limitations, and the presumed inter-layer contamination, the results of the GC-MS analysis were difficult to interpret. While proteins were found in the lower layers (1, 2 and 3), amino acid analysis yielded no good correlation to any reference artists' materials. In addition, an oil component was detected in layer 3, contradicting the results of staining and FT-IR. The scrapings from layer 4, the transparent organic layer, yielded both proteins and oil but no trace of resin; the protein and oil results were inconclusive with regard to the relative proportion of the two components, and again, amino acid analysis yielded no meaningful correlation to any reference artists' materials. Layers 5 and 6 showed the presence of oils but no protein or resin. This result was in accord with our findings using staining and FT-IR, again suggesting that layer 5 was an oil-bound pigmented size used for the application of dry copper flake pigment (layer 6). Unfortunately, fatty acid ratios from this analysis could not give a clear indication of oil type.

3.2.3 Conclusions regarding traditional analytical methods for organic binder identification

Analysis by staining, FT-IR and GC-MS of the sample material from the Drumlanrig cabinet gave a reasonably clear indication that the gesso and red ground (layers 1–3) were bound in a protein-based material, probably animal glue. In addition, these methods made it fairly clear that layer 5 is an oil-bound layer, presumably a

size for the subsequently-applied copper flake pigment. Unfortunately, these three methods yielded ambiguous results regarding the nature of layer 4, the transparent organic layer lying in the middle of the original layer structure. While natural resin could likely be ruled out as a component of this layer, little more could be said with certainty about its composition.

The difficulties encountered in the analysis of organic components in the Drumlanrig samples can be attributed to two primary factors: the small size of the sample material available and the thinness of the constituent layers. In this case, the limited sample material made it impossible to compare the relative proportions of protein and oil detected in the scrapings by GC-MS. This caused problems of interpretation, particularly in the analysis of layer 4. The thinness of the layers made successful isolation of sample material from any one layer practically impossible. The inevitable inter-layer contamination resulted in equivocal analytical results, again, particularly with regard to layer 4.

Several factors led us to suspect that the transparent organic material which made up layer 4 might be egg white. Perhaps the most significant of these was the layer's unusual morphology (referred to in section 3.2). A series of sample boards were prepared, sampled and examined microscopically in cross-section in an attempt to recreate this distinctive appearance. Four sample boards were prepared in total, using four different organic media to recreate layer 4: animal glue, mastic, drying oil and beaten egg white (glair). Of these, only the egg white reproduced the thin, coherent layer morphology of the original, maintaining a consistent thickness as it followed the irregular surface of the underlying distemper paint. In the course of preparing the sample boards, it was found that the egg white layer in particular acted as an effective sealer for the porous distemper ground, allowing the subsequent oil size to be applied thinly and evenly, without soaking into the ground.

The results of traditional analysis suggested that egg white was a possibility for layer 4 but seemingly could offer no hope of verifying this. Because we felt that a clear understanding of the original appearance of the cabinets' figures depended on a detailed understanding of the original materials and working techniques, we decided that further efforts should be made to positively identify this layer. It was at this point that we began to seriously pursue immunological techniques to confirm or reject our hypothesis that layer 4 was composed of egg white (albumin).

3.3 Immunological approaches

3.3.1 Introduction

From the outset, we decided to take a two-pronged approach to immunological testing for albumin in layer 4. The first step would be to test for the presence of albumin in the cross-section as a whole using ELISA with a polyclonal antibody and several known positive and negative controls. If no albumin were detected, our analysis would be concluded and, given demonstrated immuno-reactive stability of proteins and ELISA's high degree of sensitivity, it would be reasonable to conclude that layer 4 was not composed of egg white. If albumin *were* shown to be present somewhere in the sample, we would then attempt to use IFM to

resolve its location within the layer structure. Given the lower sensitivity of IFM, we felt that having a positive ELISA result prior to proceeding with IFM would greatly reduce our uncertainty if confronted with a negative IFM result; i.e., we would know that such a result did not imply that there was no albumin in the sample, but rather that our procedure had simply failed to resolve the albumin.

3.3.2 ELISA

3.3.2.1 Procedure

A very small flake of sample material from the Drumlanrig cabinet which had not been embedded was selected for analysis by ELISA. This flake measured approximately 0.5mm ^{2}. Working under a stereomicroscope, the bulk of the restoration layers were scraped off of the flake with a micro-scalpel and the remaining flake, containing all six of the original layers, was analyzed for the presence of egg albumin.

First, any proteins in the sample flake were extracted by placing the flake in a 1.5 ml Eppendorf tube with 20 µl of elution buffer (10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 6 M urea, 1% sodium dodecyl sulfate (SDS) and incubating for 30 minutes at room temperature (RT). Several µl of this solution (containing the extracted proteins) were then placed into one well of an ELISA plate along with double their volume of 100 mM $NaHCO_3$. The ELISA plate was then covered with Parafilm and incubated for 1 hour at 37°C to permit binding of eluted proteins to the walls of the well. Next, the well was washed; unless otherwise noted, all washing throughout the protocol was performed four times (two minutes each wash) using phosphate-buffered saline (PBS; 150 mM NaCl, 5.2 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4). Approximately 100 µl blocking solution (PBS, 0.02% Tween-20, 5% nonfat dry milk) was then added to the well and incubated for one hour at RT, followed by washing. The blocking solution, which floods the sample with inert protein, serves to minimize the possibility of non-specific binding of the antibodies.

For this procedure we acquired an anti-ovalbumin IgG (hen egg white) primary antibody generated in a rabbit, and an anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody generated in a goat. The antibodies were reconstituted per manufacturer's guidelines and diluted in blocking solution; typical dilutions ranged from 1:1000 to 1:5000. 50 µl of diluted primary antibody were then added to the well and incubated for one hour at 37°C to allow the antibody to bond to any ovalbumin present on the walls of the well. This was followed by washing to remove any unbound primary antibody (if no ovalbumin were present in the well, all of the primary antibody would be washed away at this point). 50 µl of alkaline phosphatase-conjugated secondary antibody were then added and incubated for one hour at 37°C to allow binding to any remaining primary antibody. Again, this was followed by washing to remove any unbound secondary antibody. Finally, the well was washed twice (two minutes each wash) in AP buffer (10 mM Tris base, pH 9.5, 10 mM NaCl, 0.5 mM $MgCl_2$).

Next, 100 µl of colorless p-nitrophenyl phosphate (p-NPP) were added to the

well and allowed to react for 1 h at RT. In the presence of alkaline phosphatase (conjugated to the secondary antibody), p-NPP is converted by enzymatic reaction to a yellow-colored liquid. After one hour in the well, the reaction product was removed, diluted in 200 µl water and placed in a spectrophotometer cuvette. The amount of enzymatic activity (directly proportional to the amount of target protein present in the well) was assessed using a spectrophotometer to measure changes in optical density at 405 nm.

A number of negative and positive controls were included in the experiment. The control wells were treated as described above except that the sample material was different. The three negative controls included wells in which (1) no sample material was included at all (blank), (2) the sample material was a flake of wood and gesso (containing collagen) from a sample board and (3) the sample material was composed of the restoration layers which had been removed from the actual Drumlanrig flake. Positive control material included (1) fresh egg white (diluted to 1 part per billion in water), (2) a 1mm^2 section of an albumin photograph dating to about 1860 and (3) a 1mm^2 flake taken from the replication sample board made using egg white.

3.3.2.2 Results

The results of the ELISA trials are shown in figure 7. The flake containing the original six layers from the Drumlanrig cabinet yielded a clear positive result for ovalbumin. The control wells all performed as expected, so our confidence in the reliability of the results was high. Knowing that egg white was present somewhere in the original six layers of the Drumlanrig polychromy, we decided to proceed with IFM and attempt to determine precisely where in the layer structure the albumin was present.

3.3.3 IFM

3.3.3.1 Procedure

There are a number of variables which need to be controlled in order to optimize the results of IFM. Principal among these are the concentration of both primary and secondary antibodies, the incubation time for each antibody and the temperature at which the samples are incubated. We optimized our protocol for IFM using cross-sections from the sample board made using egg white for layer 4. The sample board was first artificially aged at 40°C for three months in alternating high and low humidity in order to more closely simulate the response of the 325 year-old Drumlanrig sample material. Cross-sections were prepared using Technovit 2002 light-curing embedding resin (poly(methylmethacrylate)) and were dry-polished with successively finer grades of Micro-mesh abrasive cloths, ending with 12,000 grit.

After numerous trials with varied concentrations, times and temperatures, we settled on an optimized protocol as follows. First, the exposed cross-section was covered with a 50 µl drop of blocking solution for ten minutes at 4°C (all solutions were delivered by pipettor). As with ELISA, blocking or flooding the sample with an inert protein served to minimize the possibility of non-specific binding of the antibodies. It should be noted that Ramírez-Barat and de la Viña recommend pre-

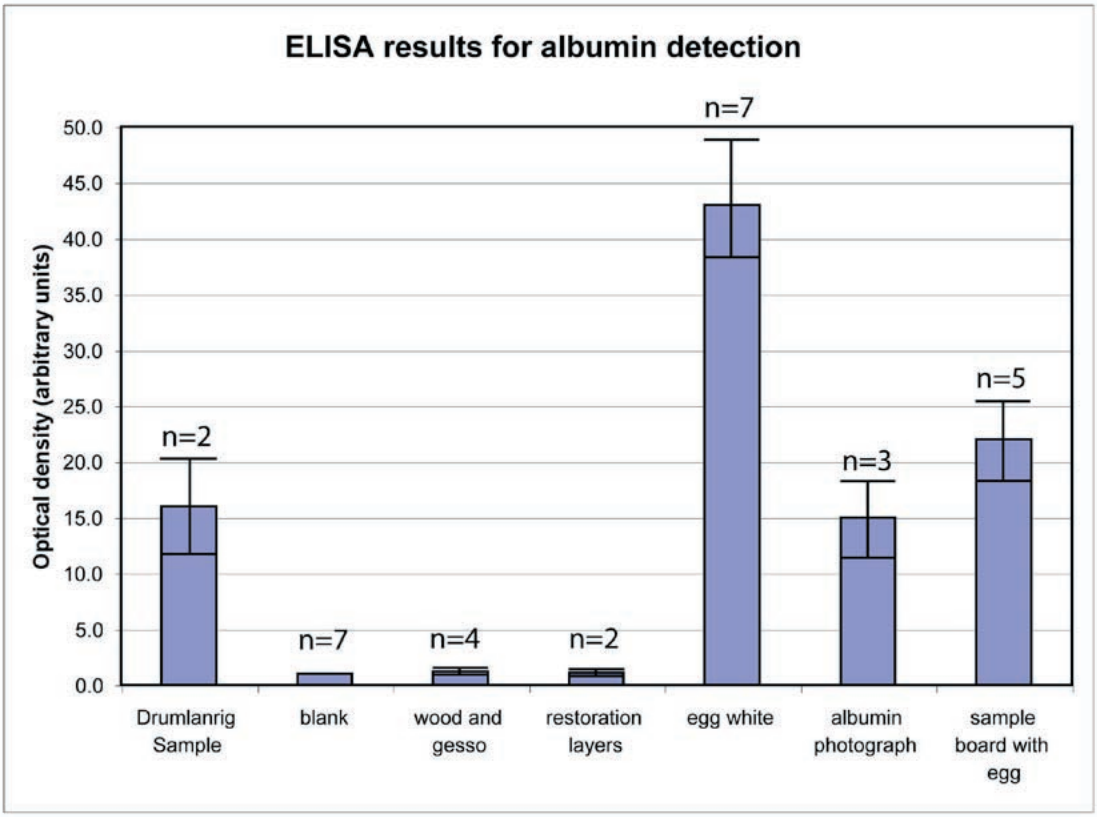

Figure 7. Results of ELISA testing: the mean optical density measured for the blank control was arbitrarily set to one and other measures are reported as multiples of that density; n= number of replicate measurements; error bars indicate the standard error of means.

treatment of the sample with trypsin prior to blocking, though we omitted this step. Next, the same primary albumin antibody used for ELISA was then diluted 1:2000 in blocking solution and a drop placed on the sample for two hours at 4°C. Any unbound primary antibody was then washed off by applying 100µl drops of PBS at 4°C to the sample, waiting 30 seconds and removing the drop with a pipettor. This washing process was repeated 4 times. Next, an anti-rabbit IgG rhodamine-conjugated secondary antibody generated in a goat was diluted 1:50 in blocking solution and a 50 µl drop was placed on the sample. The secondary antibody was allowed to sit on the sample for 30 minutes at 4°C and was then washed off as before (fig. 8).

Because the samples contained water-soluble material, they were very sensitive to mechanical damage when wet. Great care was taken not to touch the sample itself with the pipettor tip when applying or removing solutions. After the final washing, the sample was allowed to air dry for approximately 15 minutes before being cover-slipped with Stoddard's solvent.

The samples were then viewed with an Olympus BH-2 fluorescence microscope. In order to optimize the visualization, the sample was viewed using a "G filter set" designed for use with red-fluorescing rhodamine conjugates. This filter set causes the sample to be illuminated with green light (500–550nm) and allows flu-

oresced light in the 400–450 nm range to be transmitted back to the eyepieces. Other filter sets can be used in conjunction with different fluorophores, allowing for the use of yellow- or green-fluorescing conjugates of secondary antibodies.

3.3.3.2 Results

Once we were satisfied that our protocol produced repeatable positive results on the artificially aged sample board material, a minute sample from the Drumlanrig cabinet, analogous to that used for the ELISA test, was prepared as a polished cross-section. After staining according to the procedure outlined above and viewing with the "G-filter set" for rhodamine, the sample yielded a bright fluorescence, clearly concentrated in layer 4 (figure 9). This result unambiguously confirmed our hypothesis that layer 4 contains egg white. We now believe that this layer was added over the distemper ground to seal it and prevent the overlying oil size from soaking in irregularly.

3.4 Discussion

This study illustrates how common barriers to traditional analysis of protein-based artists' materials can be overcome using immunological methods. In this case, the techniques of ELISA and IFM allowed the positive identification of a very thin ovalbumin or egg-white layer in a 325 year-old sample of polychromy where traditional methods of analysis had failed even to demonstrate convincingly that the layer was composed of protein. Though rarely used, ELISA and IFM offer several significant advantages to the conservator and conservation scientist for the analysis of protein-based artists' materials. They are able to discriminate, not only between different protein types, but even between similar proteins of different biological origin. They are not compromised in this respect by contamination from adjacent layers, a problem that can greatly complicate or entirely confound amino acid analysis (Keck and Peters, 1969; Schilling and Khanjian, 1996) as well as FT-IR. ELISA offers the possibility of sub-nanogram detection limits for the analysis of bulk sample material and can also be effectively used as a screening test on minute flake samples prior to examination with IFM. In cases where little prior information exists about the nature of proteins expected in a sample, this screening function could be of particular utility. IFM allows the spatial resolution of target proteins, even in complex, thinly-layered cross-sections.

Figure 8. Applying antibodies to a polished cross-section by pipettor.

While the sensitivity of IFM is not as great as that of ELISA, our results, along with those of other investigators (see section 2.3.2) offer encouragement that this technique has the possibility to find widespread use in the field of conservation.

4. Selected avenues for further research and application

4.1 Degradation of cross-sections in aqueous solutions (IFM)

The vulnerability of cross-section samples to damage caused by prolonged exposure to aqueous solutions during IFM is an issue which has been raised by several investigators, principally Wolbers and Ramírez-Barat (see section 2.3.2). In our study, we encountered some difficulties when staining our laboratory-produced reference samples due to the swelling of the hydrophillic distemper layers (1–3) during incubation. The swelling was found to be permanent and resulted in the vertical displacement of these layers by 3 to 4 µm above the level of the oil-bound, hydrophobic layers (4–5), even after drying. This caused some disruption of our target layer, which lay directly in between. In an attempt to reduce this disruption, we tried several methods of sample pre-treatment, including pre-wetting with aliphatic solvent, impregnation with cyclododecane and impregnation with our poly(methylmethacrylate) embedding medium. None of these methods significantly reduced the swelling, and in fact, most seemed to increase it (fig. 10). In the end, our best results came from using a low temperature for incubation (4°C) which, though it did not reduce the swelling, did appear to reduce the disruption of the sample, possibly by reducing the solubility of the proteins and in particular the collagen-bound distemper layers. It is interesting to note that when IFM was applied to the historic sample, little or no disruption of the layers was noted, presumably due to the degradation and concomitant loss of hygroscopicity of the 325 year-old proteins. In any event, there clearly remains much work to be done toward minimizing the adverse effects of prolonged exposure to aqueous solutions on sample material used for IFM.

4.2 Electron microscopy

As mentioned in section 2.1, secondary antibodies are available which are conjugated to colloidal gold particles. After incubation on an embedded cross-section, these offer the potential for clear visualization of target proteins with scanning

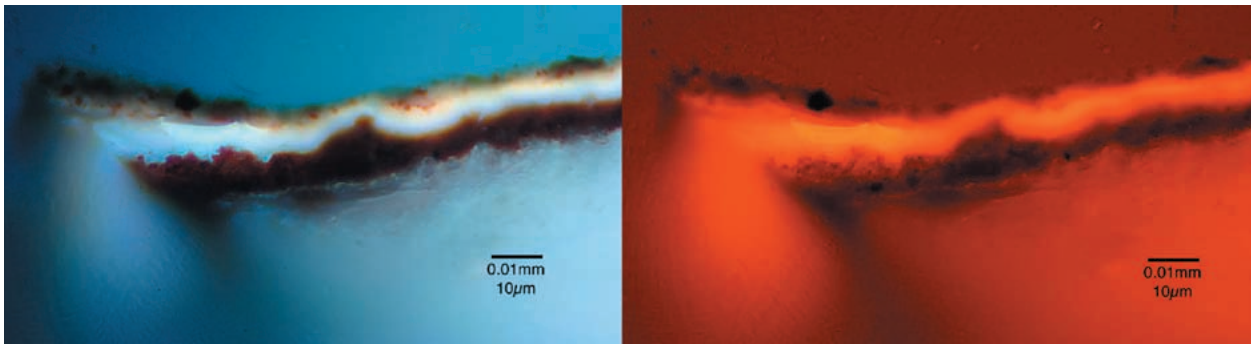

Figure 9. Positive staining for albumin in layer 4 of the Drumlanrig cabinet samples. The staining is not visible with a standard U.V. filter set (left), but is clearly present when viewed with a filter set designed for rhodamine (right).

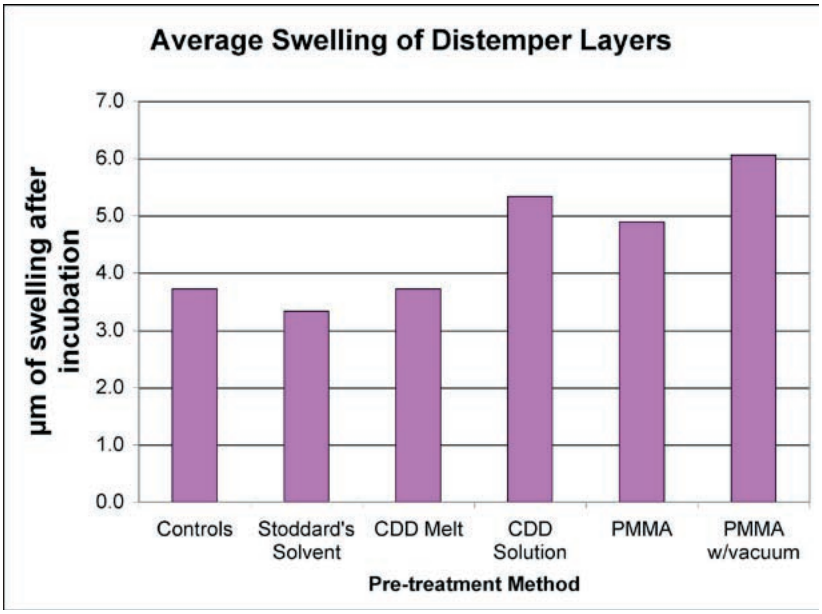

Figure 10. Measured swelling of distemper layers above the level of the embedding resin as a function of different methods of sample pre-treatment. Methods included pre-wetting with aliphatic solvent (Stoddard's), pre-consolidation with cyclododecane (CDD) and pre-consolidation with poly(methylmethacrylate) PMMA. Each bar represents the mean of nine measurements.

electron microscopy. The authors know of no instance where this or other related techniques have been used for the analysis of artworks. It remains to be seen if this approach offers the potential for enhanced sensitivity over IFM. (For an introduction to the subject see Polak and Varndell, 1984.)

4.3 Studies of the degradation in immuno-reactivity of proteins with age
Although interesting work on this subject has been undertaken, much remains to be done to understand the relative rates of degradation of the specific epitopes targeted by different antibodies. There may well be multiple monoclonal antibodies capable of discriminating any given species-specific protein, and prior studies indicate that the rate of degradation of their respective epitopes may vary significantly. It would certainly be valuable to have a coordinated evaluation of different batches of relevant antibodies from different manufacturers to determine the most stable antibody-epitope pairs useful for a range of artists' materials.

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Sources of Materials

Nunc-Immuno MaxiSorp Plates (ELISA plates)
Cat No: 62409-024 (0.4ml, flat bottom)

Primary Antibody (anti-Ovalbumin) [Hen Egg White] [Rabbit]
ROCKLAND (www.rockland-inc.com); Code: 200-4333

Alkaline Phosphatase-Conjugated Affinity Purified Secondary Antibody used for ELISA
CHEMICON International (www.chemicon.com)

p-Nitrophenyl Phosphate (p-NPP)
CHEMICON International (www.chemicon.com); Catalog Number: ES009-100ML

Rhodamine-Conjugated Secondary Antibody (anti-Rabbit) [Mouse] used for Immunofluorescence
PIERCE Biotechnology; Product Number: 31674

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