

UCLA

UCLA Electronic Theses and Dissertations

Title

Evaluation of Two Fluorescent Dyes used in Immunofluorescent Microscopy for the Detection of Proteinaceous Binding Media in Wall Paintings

Permalink

<https://escholarship.org/uc/item/6sp1z4gj>

Author

de Alarcon, Tessa

Publication Date

2012

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Evaluation of Two Fluorescent Dyes used in Immunofluorescent
Microscopy for the Detection of Proteinaceous Binding Media in Wall Paintings.

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Arts
in Conservation of Archaeological and Ethnographic Materials

by

Tessa de Alarcon

2012

ABSTRACT OF THE THESIS

Evaluation of Two Fluorescent Dyes used in Immunofluorescent
Microscopy for the Detection of Proteinaceous Binding Media in Wall Paintings.

by

Tessa de Alarcon

Master of Arts in Conservation of Archaeological and Ethnographic Materials

University of California, Los Angeles, 2012

Professor Ioanna Kakoulli, Chair

The following study sought to compare the efficacy of Qdot antibody conjugates to FITC antibody conjugates for secondary staining in the identification of proteinaceous binding media in wall paintings using immunofluorescence microscopy (IFM) and to further explore limitations of the technique as a result of pigment binder interactions. A protocol was developed for the identification of egg-based media in wall paintings using FITC for IFM, but no protocol was developed for the Qdot antibodies. Three pigments were used to explore pigment binder interactions and their effects on the results of IFM (vermilion, hematite, and lead white). Hematite combined with egg tempera was found to have reduced signal strength when compared to vermilion in the same binder. Lead white was found to be problematic due to enhanced autofluorescence of the organic binding media, making it difficult to differentiate positive and negative results.

The thesis of Tessa de Alarcon is approved.

Arlen Heginbotham

Abby Kavner

Sergey Prikhodko

David A. Scott

Ioanna Kakoulli, Committee Chair

University of California, Los Angeles

2012

TABLE OF CONTENTS

1. INTRODUCTION.....	1
2. IMPORTANCE OF ORGANIC BINDING MEDIA IN MEDIEVAL WALL PAINTINGS.....	4
3. ORGANIC BINDING MEDIA AND CONSERVATION TREATMENT CONSIDERATIONS.....	8
4. LITERATURE REVIEW: IDENTIFICATION OF PROTEINACEOUS BINDING MEDIA.....	11
4.1 CHROMATOGRAPHY, FTIR, AND HISTOCHEMICAL STAINING.....	11
4.2 IMMUNOCHEMISTRY: ELISA AND IFM.....	14
4.3 QUANTUM DOTS.....	18
5. METHODOLOGY: SAMPLE PREPARATION AND PHOTO-DOCUMENTATION.....	21
5.1 PREPARATION OF TEST TILES.....	21
5.2 PREPARATION OF CROSS-SECTIONS.....	24
5.3 PHOTO-DOCUMENTATION.....	26
6. PROTOCOL DEVELOPMENT.....	28
6.1 SECONDARY STAINING WITH FITC AND QDOTS.....	28
7. EXPERIMENTAL TRIALS: FITC.....	32
8. IMAGING THE QDOTS USING VPSEM.....	34
9. RESULTS.....	36
9.1 VERMILION AND HEMATITE TRIALS.....	36
9.2 LEAD WHITE TRIALS.....	39
10. DISCUSSION.....	42
11. CONCLUSION.....	47
12. APPENDIX A: TEST TILE PRODUCTION.....	49
13. APPENDIX B: PROTOCOL DEVELOPMENT.....	52

14. APPENDIX C: LIST OF MATERIALS.....	56
15. APPENDIX D: IMAGING OF THE DESICCATED QDOT SAMPLE.....	58
16. BIBLIOGRAPHY.....	60

LIST OF FIGURES

Figure 1: Chemical reaction showing the conversion of calcium hydroxide into calcium carbonate during the setting of a lime plaster.....	4
Figure 2: The above figure shows the differences between primary staining (a) secondary staining (b) and tertiary staining (c). Figure taken from Ramirez-Barat and de la Viña (2001: 283).....	15
Figure 3: Image of the FITC molecule and the antibody conjugation reaction (FITC1 2011).....	18
Figure 4: Schematic representation of the Qdots produced by Invitrogen (Structure of a Qdot Nanocrystal 2011).....	19
Figure 5: This image shows the set up used for viewing and documenting the cross-sections during testing.....	27
Figure 6: Examples of positive results through staining. The top images show one of the whole egg tempera cross-sections containing hematite under brightfield reflected light (top left) and when viewed with the FITC filter cube (top right). The bottom images show a whole egg tempera cross-section that contains vermilion under brightfield reflected light (bottom left) and when viewed with the FITC filter cube (bottom right). All four of the images were modified for publication by inserting a curve filter overall in Photoshop.....	37
Figure 7: The above image shows an example of one of the cross-sections (a whole egg tempera section with hematite) that presented positive staining below the paint layer. This image was modified for publication by applying an overall curve in Photoshop.....	37
Figure 8: Tile 1 right after fresco application completed. Note that the orpiment rapidly reacted with the alkaline limewater resulting in a color alteration to a greenish color. When the red lead was applied beneath the orpiment square, the red lead reacted with the orpiment (likely forming a lead sulfide) creating a dark area with a metallic luster along the junction of the two pigment zones.....	49
Figure 9: This image shows Tile 1 after it was completed. The 12 squares on the left side were applied using fresco and the 12 squares on the right half were applied a secco using distemper as the binding medium. Note that in the fresco side, the malachite has been affected by the alkalinity of the lime turning black and the overall lighter appearance of the pigments applied in fresco.....	49
Figure 10: This image shows tile the visible fluoresce of Tile 1 under UV radiation. The image was captured using a Nikon D90 and a long-pass filter to capture only in the visible range.....	50
Figure 11: This image shows Tile 2 after it was completed. The 12 squares on the left were applied using egg yolk tempera. The 12 squares on the left were applied using whole egg tempera. Note the patchy appearance of the orpiment, Egyptian blue, and Verona green earth with the whole egg. This resulted from poor affinity of these pigments to the binding media causing poor working properties. The lead white exhibited similar problems for both media as well, but is less apparent in this image.....	50

Figure 12: UV induced visible fluorescence image of Tile 2. The image was captured using a Nikon D90 and a long-pass filter to capture only in the visible range.....51

Figure 13: The top digital photomicrograph shows the desiccated Qdot sample under brightfield reflected light and the bottom digital photomicrograph shows the same sample when viewed using the FITC filter cube.....58

Figure 14: A shows the SEM montage map of the desiccated sample with the area of focus marked in red. B and C are details taken with the SEM of the area of focus while D shows that same region using IFM.....59

LIST OF TABLES

Table 1: Complete Pigment List.....	22
Table 2: XRD Confirmation of Pigment Identity.....	24
Table 3: Results from Testing on Cross-sections Containing Hematite.....	38
Table 4: Results from Testing on Cross-sections Containing Vermilion.....	39
Table 5: Results from Testing on Cross-sections Containing Lead White.....	40
Table 6: Results from Cross-sections Containing Lead White Exposed to Blocking Solution.....	41
Table 7: FITC Secondary Staining Protocols Tested Using Wet Grinding/ Polishing.....	52
Table 8: FITC Secondary Staining Protocols Tested with Dry Grinding/Polishing.....	53
Table 9: Qdot Secondary Staining Protocols Tested Using Wet Grinding/ Polishing.....	54
Table 10: Qdot Secondary Staining Protocols Tested with Dry Grinding/Polishing.....	55

ACKNOWLEDGEMENTS

I would like to thank my thesis committee, Dr. Ioanna Kakoulli, Dr. David Scott, Arlen Heginbotham, Dr. Sergey Prikhodko, and Dr. Abby Kavner, for all their help and input throughout this process. I would also like to thank Vanessa Muros for her help and moral support. Finally, I would like to thank my parents Dr. Pedro A. de Alarcon and Jill de Alarcon, as well as my fiancé Travis Martin, for helping me work through problems and questions and listening to me whenever I needed it.

1. Introduction

The identification and mapping of organic binding media in wall paintings is a critical step prior to conservation intervention. Ideally, a method used to identify organic components would not only provide material specific identification of all of the organic compounds but also their location within a wall painting's stratigraphy. Thus far, there is no single technique that meets these criteria especially in works of art with multiple paint layers.

Spatially resolved analysis of organic components in multi-layered structures is therefore very challenging. A method is needed that will be able to identify organic binding media within the individual paint layers in a multi-layered configuration while it will be minimally-invasive to the artifact analyzed. While, a few modern analytical techniques such as confocal Raman microscopy and micro-FTIR have shown potential at resolving small amounts of organic materials on cross-sections and thin-sections of paintings, these methods require scientific equipment and technical knowledge that is not available to most conservators. This research aims at exploring and improving a traditional method used by conservators, fluorescence microscopy, for the identification of binding media using immunofluorescent microscopy (IFM) on paint cross-sections.

More specifically, I will focus on the identification of egg tempera, a common proteinaceous organic binding medium, in wall painting. The method uses antibodies coupled with fluorescent stains to identify and localize proteins in cross-sections. It has great potential as it provides a high level of specificity, and sensitivity for the targeted protein and provides spatial resolution of the target compound within the wall painting structure. Previous authors have developed

protocols for this method (Cartechini et al. 2010 ; Heginbotham, Millay, and Quick 2006 ; Ramírez-Barat and de la Viña 2001), but there have remained a number of concerns and challenges that have hindered its wide-spread adoption within the conservation community (Heginbotham, Millay, and Quick 2006 ; Hodgins and Hedges 1999, 2000 ; Ramírez-Barat and de la Viña 2001).

Building upon pre-existing research in this area, I have developed a working protocol for fluorescein isothiocyanate (FITC), a well-known fluorescent stain in immunochemistry, taking into consideration:

- methods for sample preparations;
- pigment/binding medium interactions;
- nature, preparations and application of binding media in wall paintings and their possible effects on the analytical results;
- reliability (reproducibility) of results.

Quantum dots (photo-luminescent semi-conductor nano-crystals) were also tested as a fluorescent stain in the hopes of presenting an improved method for protein identification and localization in wall painting cross-sections. The intention was to assess the performance of the Qdots based on comparison with FITC, one of the fluorescent stains widely explored in previous studies. Although Cartechini et al. (2010) have established a working protocol for Qdot650, in their recent publication only parts of their protocol (such as their dilution times) were provided. In the present study it proved impossible to develop a successful procedure for the paint-cross

sections using Qdot525 and regrettably, no comparison could effectively be made to the results from Cartechini et al. (2010).

2. Importance of Organic Binding Media in Medieval Wall Paintings

Wall painting techniques are traditionally divided into two groups, fresco and secco. In a fresco painting normally no organic binding media are used as pigments are applied onto the surface of a fresh calcium hydroxide (lime)-rich plaster layer and fixed through the setting (drying and hardening) process of the lime. The setting of a lime plaster involves a chemical reaction that converts calcium hydroxide into calcium carbonate through a reaction with the carbon dioxide (CO₂) of the atmosphere (Figure 1).



Figure 1: *Chemical reaction showing the conversion of calcium hydroxide into calcium carbonate during the setting of a lime plaster.*

In a fresco application, the pigment particles are englobated within the calcium carbonate crystals as they form effectively, binding the pigment to the surface of the plaster. Secco (meaning dry in Italian), on the other hand, is the application of paint onto a dry surface. In a secco application, it is therefore important to use an organic binding medium with film-forming properties able to create a stable paint layer.

The medieval period (11th century to the 14th century AD) was a period of extensive experimentation and regional variation in both binding media employed, as well as, in substrate for wall painting (Cather, Park, and Williamson 1990: xii-xvi; Howard 2003: 3-8). Research by Howard (2003) on the materials used for English medieval wall painting, for example, has found that a much larger range of binding media and pigments were used in this time period and region than previously believed. Cather, Park, and Williamson (1990: xiv) have argued that, "the

division of wall painting technique into either fresco or secco is both oversimplified and unhelpful," as a result of the regional variation in technique of the medieval period, especially given that these were often used together.

Medieval wall paintings are typically divided into two periods based on the predominate styles: Romanesque and Gothic (Howard 2003: 3-8; Rosewell 2008: 7-30). Secco on lime plaster is a common feature of medieval wall paintings throughout Europe and can be seen in both the Romanesque and the Gothic periods (Howard 2003: 3-8; Koller, Leitner, and Paschinger 1990: 15). The conventional starting and ending dates of these periods vary depending on the region, although 1050 to 1200AD are the dates typically cited for the Romanesque. The Gothic began around 1140 in France and became the main style in Europe from the 13th to the 14th centuries (Kleiner and Mamiya 2005: 447-448, 479-486). In some regions it continued longer, as there are examples of gothic architecture in the UK dating to as late as the 15th century (Howard 2003: 10-12).

The wall paintings of both periods demonstrate regional variation in methods and technique and are characterized by increased experimentation with binding media to attain different effects. Technical studies of pigments and binding media have identified a huge range of organic binding media including oil, egg tempera, and glue tempera (distemper) (Howard 2003: 3-8; Koller, Leitner, and Paschinger 1990: 15; Mora, Mora, and Philippot 1984: 123). In addition, it was not uncommon for these to be used in combination with fresco techniques, particularly in the Romanesque period (Howard 2003: 3-6; Rosewell 2008: 16). Gothic wall paintings tended to be mainly secco (Howard 2003: 6-8; Mora, Mora, and Philippot 1984: 123). Due to the mixed

techniques and large number and variety of binding media that may have been used both in the Romanesque and Gothic periods, the identification of a period would not be sufficient to infer the presence or type of organic binding media.

Some information on the range of binding media employed can be gleaned from examination of medieval texts and treatises. During the medieval period, written works were transcribed to create new copies. In the case of artists' recipe books the transcriber would often add to or change the text based on information from other texts, contemporary knowledge, and in some cases the personal experience of the transcriber (Howard 2003: 16-17). As a result, these texts can be, "regarded as compilations of compilations which have undergone alteration and addition over a long period" (Howard 2003: 17). Given the nature of these texts to condense and combine information from many previous sources, they often also suffer from the limitation of providing information on solely the regional traditions rather than providing a larger picture of trends and techniques throughout medieval Europe (Howard 2003: 16-19).

One commonly cited manuscript is the 14th century text, *Il Libro dell' Arte*, by Cennino d'Andrea Cennini (1960). Although this text has the limitation of providing predominantly information on southern European techniques, with an emphasis on fresco painting techniques, it is also one of the most detailed. This is particularly true for its descriptions of materials and techniques used for wall painting (Howard 2003: 17, 19). Despite Cennini's bias toward fresco painting, this text does include information on secco application counting two recipes for egg-based tempera (whole egg and egg yolk); methods for using oils (linseed oil), as well as distemper (hide glue based) (Cennini 1960: 50-52, 59-60, 121-122). As a result, this text also demonstrates the variety

of organic binding media employed for wall painting in the medieval period, even in areas of southern Europe where fresco was favored.

3. Organic Binding Media and Conservation Treatment Considerations

The identification of binding media in wall paintings conservation is a critical step prior to treatment. Due to their nature, organic binding media are prone to deterioration and alteration over time. They are also extremely sensitive to conservation treatments. Both these factors make their identification and conservation challenging (Piqué 2010: 12; de la Cruz Cañizares et al. 2004: 277). Their vulnerability has been recognized by numerous authors and it is commonly recommended that all materials both original and added be identified prior to treatment (Cather 1993: 84; Mora, Mora, and Philippot 1984: 19-20).

To further complicate these issues there are many different classes of organic materials that have been employed as binding media including: proteins (egg, casein, animal glue), fats, oils (mainly linseed oil, and walnut oil), waxes (bees wax, paraffin), polysaccharides (vegetable gums), and natural resins such as terpenoids (Mateo Castro et al. 1997: 373). Each of these classes can be detected using a range of techniques, but due to their differences some will be more readily identified by one technique over another. However, as proteinaceous binding media are the focus of this work, only this class of organic binding media will be discussed in detail here.

Proteins are common and important constituents of many animal products. They are complex organic materials that range in form, and function. In addition, proteins are always composed of a limited range of amino acids (Mills and White 1999: 84). However, some proteinaceous binding media contain other organic compounds as well. Egg, for example, also contains significant amounts of fat (Mateo Castro et al. 1997: 373).

The proteins under investigation in this study are those in egg and animal glue. Albumin is the general term used to refer to the class of proteins present in egg. These are water-soluble globular proteins, and different parts of the egg contain various proportions of these. Egg white is predominantly made of ovalbumin and glycoprotein with ovalbumin making up 50% of the protein. Egg yolk, on the other hand, is much more protein-rich but contains a wide variety of proteins rather than being made up of any one in particular (Mills and White 1999: 87-88). The major protein in distemper, or animal glue based paint, is collagen. Collagen is a fibrous protein that is insoluble in water (Mills and White 1999: 86). To make the hide glue used in distemper, the connective tissue (from skin, muscle, bone, or hide) of an animal or fish must be boiled for a significant length of time to partially degrade the protein converting it to gelatin (Mills and White 1999: 86-87; Cennini 1960: 67).

While many causes of deterioration in secco wall paintings (such as salt crystallization and fluctuations of relative humidity (RH) and temperature) are similar to those occurring on fresco wall paintings, the effects of deterioration may vary and be wall painting-specific. The materials that make up the wall painting will react in different ways with the environment and so determine the features of the deterioration. For example, secco wall paintings affected by active crystallization cycles of soluble salts in an environment with fluctuating relative humidity will most likely show different deterioration patterns from a fresco wall painting in the same environment. This is for the reason that the location where salts crystallize within the wall painting stratigraphy is dependent on a number of factors including properties of the materials in the wall itself and the wall painting (Arnold and Zehnder 1987: 116-120). Even without salts,

secco wall paintings in fluctuating temperatures and relative humidity will be more prone to flaking than will fresco wall paintings due to the differences in thermal and hygric expansion coefficients of the paint layer as compared to the plaster substrate. These differences in behavior between the paint layer and the plaster substrate can be exacerbated through inappropriate conservation treatments and materials added to the surface and subsurface (Cather and Howard 1986: 51; Sawdry 1994: 50-51).

Furthermore, owing to potential interaction between conservation products and original materials the presence of organic binding media will affect treatment design and materials selection. For example, while alkaline-based reagents ($\text{pH} \geq 9$) can be used both as consolidants and cleaning agents for fresco wall paintings (Brajer and Kalsbeek 1999: 145-147; Dei et al. 1998: 80-88), the same alkaline treatments are totally inappropriate for any wall painting containing proteinaceous binding media as proteins will deteriorate when exposed to high pH (Brajer and Kalsbeek 1999: 147). Exposure to an alkaline environment can lead to deterioration on many levels either breaking apart the protein into the amino acids that it is composed of, or by altering the amino acid components. The proteins can be broken apart by alkaline hydrolysis freeing the constituent amino acids in the protein. Further deterioration can be caused as the amino acids themselves may deteriorate through alkaline hydrolysis either causing epimerization (a change to one of the asymmetric centers of the amino acid), or the destruction of the amino acid (Mills and White 1999: 84-86). This will further cause severe and irreversible deterioration of the paint layers containing proteinaceous binding media.

4. Literature Review: Identification of Proteinaceous Binding Media

While the characterization of organic binding media in wall paintings has advanced significantly in the last decade, the identification and mapping of organic binding media in secco wall painting is an area where additional research is still needed. To some extent research models can be borrowed from the more extensive data on paintings on canvas and wood, however, there are significant differences between these techniques that critically affect the usefulness of borrowing from one and applying it to the other. One fundamental reason why organic materials in wall paintings are found in much lower concentrations than those on a panel or canvas painting is due to the highly porous nature of wall painting as well as differences in the techniques of execution. For this reason, analytical techniques used to detect binding media in wall paintings may require slight modification to the methodology used on other forms of painting and extra caution in the interpretation of the results. Moreover, when evaluating the results of a sensitive method, it is imperative to identify all possible sources of contamination (Silva 1963: 56).

4.1 Chromatography, FTIR, and Histochemical Staining

The complex nature of the proteinaceous binding media further complicates their identification and therefore, highly sophisticated analytical techniques and expertise is required for their precise characterization. This is commonly done through the identification of its constituent amino acids (Mills and White 1999: 89-91). A technique that has been routinely used for the identification of proteins is gas chromatography, however, this is destructive requiring the consumption of samples to yield results. In addition, with this method samples from each layer are need to be taken and treated individually, a practice that is often challenging as physical separation of paint layers is extremely difficult if not impossible.

Chromatographic techniques including paper chromatography, gas chromatography combined with mass spectroscopy (GC-MS), and high performance liquid chromatography (HPLC) have all successfully been used for the identification of organic binding media (Silva 1963: 64; Howard 2003: 25; Vallance et al. 1998: 294-311; Mateo Castro et al. 1997: 373-381; Rampazzi et al. 2002: 237-238, 239; de la Cruz Cañizares et al. 2004: 277-285; Grywacz 1994: 177-183). GC-MS has been found to be effective due to its ability to separate complex mixtures as well as its ability to detect organics even in very small quantities (Rampazzi et al. 2002: 237-238). However, GC-MS is limited in its use for the identification of proteinaceous binding media, as proteins do not volatilize. For proteins to be analyzed using GC-MS, derivatization is necessary as it allows the amino acids to volatilize (Mills and White 1999: 90; Gautier and Perla Colombini 2007: 97-98; de la Cruz Cañizares et al. 2004: 278-279; Mateo Castro et al. 1997: 373-375). Other inherent limitations to this method are that it does not provide visualization of the layers and in cases with complex layering, depends on extremely precise sampling from individual layers. This method may be potentially problematic for medieval wall paintings where multiple binding media are known to have been used in a single work with different media used for different pigments, resulting in a complex layered structure.

Amino acid composition analysis is another traditional technique used for protein identification in wall paintings. This method depends on the hydrolysis of a protein into its constituent amino acids, which are then identified quantitatively using ion-exchange chromatography and the color reaction of amino acids to ninhydrin. However, this method is more sensitive for certain proteins and less so for others. For example, ninhydrin reacts less readily with the major amino acid

component of gelatin, making its identification challenging (Mills and White 1999: 89-90). This method is also destructive and does not provide any spatial information on the location of the protein within the sample (Hodgins and Hedges 1999: 1798-1799).

Fourier transform infrared spectroscopy (FTIR) has also been used to identify organic binding media, specifically within the context of medieval wall paintings (Howard 2003: 25). However, IR based techniques are not as widely utilized as other techniques because the spectra can be difficult to interpret and the differentiation between types of proteinaceous binding media typically cannot be achieved (Ramírez-Barat and de la Viña 2001: 282). Instead, it has found the greatest use as a screening process to confirm the presence of organic constituents rather than as a method of identifying the binding media type (Rampazzi et al. 2002: 237).

Chemical stains and immunochemistry are also commonly used for the identification of general classes of organic binding media. These stains, although widely used since the 1960's are fraught with problems. Some of the main issues are the low ratio of binder to pigment in wall paintings as well as the numerous possible sources of contamination, particularly from previous conservation efforts and biological activity (Silva 1963: 56-57). Weathering action on wall paintings can also cause the break down of the components of the wall painting further complicating their detection (Rampazzi et al. 2002: 239). These factors can lead either to false positives, or false negatives.

4.2 Immunochemistry: ELISA and IFM

Immunological methods for the identification of proteinaceous organic binding media have great potential for their detection and localization. Research evaluating these methods has been conducted predominantly on panel and canvas painting cross-sections (Heginbotham, Millay, and Quick 2006 ; Ramírez-Barat and de la Viña 2001 ; Hodgins and Hedges 2000). These included enzyme linked immunoassay (ELISA) and immunofluorescent microscopy (IFM). IFM in particular, has the potential for high specificity and can simultaneously provide spatial resolution of the binding media within the cross-sections (Ramírez-Barat and de la Viña 2001 ; Heginbotham, Millay, and Quick 2006). While most studies focused on canvas and panel paintings, both IFM and ELISA have been successfully applied for the detection of binding media in cross-section samples from wall paintings as well (Cartechini et al. 2010).

Wolbers (1987, 1988), first introduced immunochemistry for the identification of binding media in paint cross-sections into the conservation literature. However, the method he proposed differs drastically from more recent work. Instead of depending on a highly specific chemical interaction between antibody and protein, he used the mutual attraction between the proteins and the immunochemical dyes to directly stain proteinaceous binding media in a non-specific reaction.

Since Wolbers' initial work, additional studies have been conducted using secondary antibodies and fluorescent dyes to detect binding media in paint cross-sections (Ramírez-Barat and de la Viña 2001 ; Heginbotham, Millay, and Quick 2006 ; Cartechini et al. 2010). Primary staining is a one step process where an antibody labeled with a fluorescent dye is used. The sample is

exposed to the labeled antibody, which binds to the antigen (Figure 1a). The label attached to the antibody allows for the detection of the antigen. This method is cheaper and faster than secondary staining, but can result in a weak signal (Ramírez-Barat and de la Viña 2001: 282-283; Heginbotham, Millay, and Quick 2006: 91).

In secondary staining the sample is exposed to the un-labeled antibody, which binds to the antigen. A secondary labeled antibody is then used to detect and label the primary antibody. Secondary antibodies are capable of binding to multiple sites on the primary antibody, which results in an amplification of the signal (Figure 1b). However, there is a greater risk of non-specific staining when using secondary antibodies (Ramírez-Barat and de la Viña 2001: 283; Heginbotham, Millay, and Quick 2006: 91; Cartechini et al. 2010: 871). Studies examining the resolution capabilities of this technique have found that it is possible to achieve sub-micron resolution (Hodgins and Hedges 2000: 76).

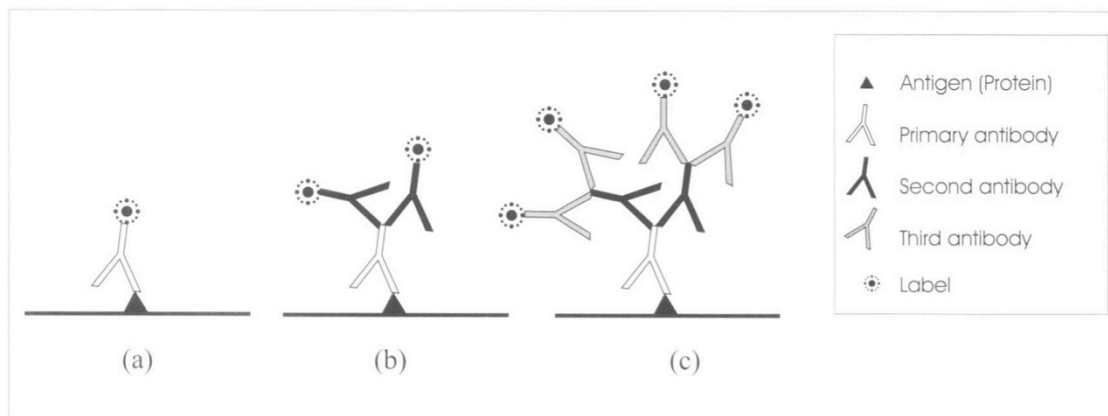


Figure 2: The above figure shows the differences between primary staining (a) secondary staining (b) and tertiary staining (c). Figure taken from Ramirez-Barat and de la Viña (2001: 283).

Previous studies investigating the potential of IFM for the detection and localization of proteins in paint cross-sections have encountered a number of challenges that have prevented the

technique from being widely applied. These problems included non-specific staining, the dissolution of water-soluble components, swelling of paint layers, weak fluorescence and photo bleaching, and difficulty in discriminating between natural fluorescence (auto-fluorescence) and the fluorescence emitted by the fluorescent dyes (Heginbotham, Millay, and Quick 2006: 93-94; Ramírez-Barat and de la Viña 2001: 285-286; Dolci et al. 2008: 30; Cartechini et al. 2010: 872-873). Non-specific staining is a product of two problems, either the antibodies are binding to proteins other than the one of interest, or are adsorbed by certain pigments or grounds (Ramírez-Barat and de la Viña 2001: 285-286). Some of these issues have been addressed with limited improvement by previous researchers (Heginbotham, Millay, and Quick 2006 ; Ramírez-Barat and de la Viña 2001 ; Cartechini et al. 2010). In an attempt to reduce the risk of distortion, swelling, and dissolution of paint layers in the cross-sections during IFM, Ramírez-Barat and de la Viña (2001: 284-286), tested and compared a range of embedding media but were unable to find a solution to this problem.

There have also been some concerns on the effects of aging and its relationship to the detectability of the protein of interest. Organic binding media are known to alter over time, causing shifts in solubility and, possibly altering the protein structure reducing available sites for binding with the primary antibody (Heginbotham, Millay, and Quick 2006: 99; Hodgins and Hedges 2000: 79-80; Ramírez-Barat and de la Viña 2001: 286). To further complicate these issues, the aging and deterioration of a paint film has been found to be affected by the pigments present due to pigment/binding medium interactions (Meilunas, Bentsen, and Steinberg 1990 ; Wouters, Van Bos, and Lamens 2000).

Owing to there concerns, many of the previous studies evaluating IFM have included a range of pigments and both aged and un-aged samples. Hodgins and Hedges found that photochemical aging of bovine-collagen paint films for example, did not affect the non-species specific collagen binding sites, but that species-specific collagen sites were affected (Hodgins and Hedges 2000: 77). Ramiriz and Barat, in their study on detection of egg white tempera found that positives were obtained on both the thermally aged and the un-aged samples with a general reduction in signal strength for aged samples irrespective of the pigment (Ramírez-Barat and de la Viña 2001: 285).

ELISA utilizes the same type of immunochemistry used in immunofluorescent microscopy, except that it is not preformed on a cross-section and so rather than using a stain that can be visualized an enzyme is used to label and detect the presence of the protein. Although it uses the same chemistry the proteins must be extracted from the sample to yield results and hence cannot be performed directly on paint cross-sections, which is the focus of this study. Heginbotham, Millay, and Quick (2006: 92-93, 100) have recommended ELISA for preliminary screening in the identification of proteinaceous materials in paintings using detached paint flakes, prior to immunofluorescence microscopy for the localization and mapping of the identified proteins within the different layers of the paint samples prepared as a polished section.

One of the most commonly used fluorescent stains for IFM is fluorescein isothiocyanate (FITC) (Figure 2). This is a fluorophore with an excitation at 492nm and emission at 520nm (Arslanoglu, Zaleski, and Loike 2011: 3002). Recently alternative staining methods have begun to be explored due to problems with this stain as it has low signal strength and tends to photo-

bleach. For example, DyLite 488 antibody conjugates behave similarly to FITC conjugates, but are much more intense and have less tendency to photobleach (Arslanoglu, Zaleski, and Loike 2011: 3002). There has been research into combining antibody chemistry with tags that can be detected using Raman micro-spectroscopy, known as surface enhanced Raman scattering (SERS) nanotags that have been complexed to antibodies as well. This can provide stratigraphic information through line scans of the sample, but is dependent on accesses to this type of equipment (Arslanoglu, Zaleski, and Loike 2011).

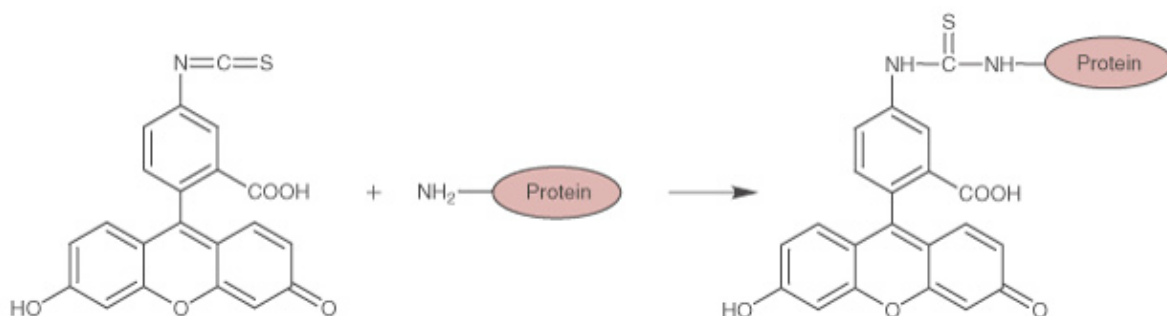


Figure 3: Image of the FITC molecule and the antibody conjugation reaction (FITC1 2011).

4.3 Quantum Dots

Quantum dots (semiconductor-based fluorescent nanocrystals) have been successfully used in the biomedical sciences and in materials engineering. Quantum dots can be made from a range of semi-conductor materials with different coatings to suit specific purposes in biological staining (Deerinck 2008: 112-113). The core of the Qdot is made of a semiconductor material. Currently cadmium combined with selenium or zinc and sulfur are the most common semi-conductors used in Qdots (Deerinck 2008: 112). The Qdots made by Invitrogen contain a semiconductor core of

cadmium mixed with selenium or tellurium. This core is then coated with an additional semiconductor shell, zinc sulfide (Figure 3). This shell helps improve the optical properties of the Qdot. Over the shell, there is a polymer coating, which facilitates the conjugation of the Qdot to the antibody, or other biomolecules (*Structure of a Qdot Nanocrystal* 2011). These nano-materials offer higher specificity in immunological chemistry to detect and localize proteins (Chan and Nie 1998: 2016-2017; Deerinck 2008: 113-114). The potential advantages of these nano-particles over traditional dyes used in biochemical assays include their stability with high resistance to photo-bleaching, high fluorescence yield due to their high extinction co-efficient, compositional contrast-based imaging at the nano scale using transmission electron microscopy (TEM), and higher specificity (Deerinck 2008: 113-116). These properties are extremely promising in conservation applications by providing accurate biolabeling and avoiding non-specific staining that could give false positive identification.

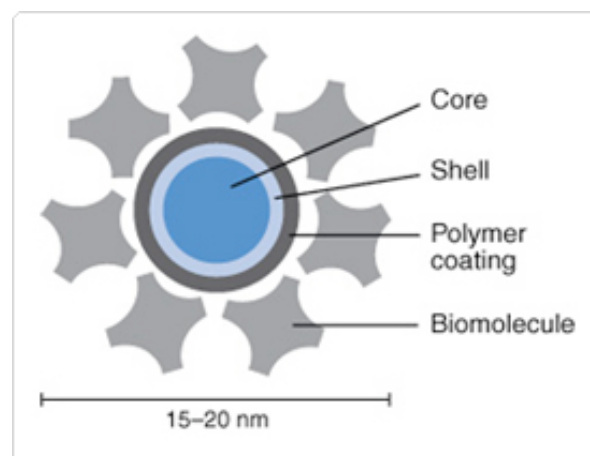


Figure 4: Schematic representation of the Qdots produced by Invitrogen (*Structure of a Qdot Nanocrystal* 2011).

Preliminary work by Cartechini et al. (2010) on the use of Qdots in IFM has been published with promising results. Thus far, the authors have developed protocols for three primary antibodies,

anti-chicken egg, anti-bovine β -casein, and anti-collagen I. All of these are compatible with the same Qdot secondary antibody (Qdot 605 goat anti-mouse IgG conjugate supplied by Invitrogen). As a result, successful methods were developed for the detection of egg white, casein, and animal glue. Furthermore, testing was conducted on both aged and un-aged samples with a range of pigments. The aging consisted of exposure to 85% RH at 40°C for three months, and the pigments were hematite, giallorino, malachite, minium, and smalt. These authors found that aging has no effect on the signal intensity of the staining. In addition, results using the Qdot protocol and imaging using a confocal microscope were compared to previous results obtained using FITC and imaging on a traditional fluorescent microscope both on samples from a fresco attributed to Giotto. The authors found notable improvement and reduction of non-specific staining with the Qdot protocol (Cartechini et al. 2010).

5. Methodology: Sample Preparation and Photo-Documentation

5.1 Preparation of Test Tiles

For the initial testing and for the development and refinement of the methodology for the detection of the organic binding media, three 12" x 12" ceramic test tiles were prepared (Appendix A). Each was soaked in water overnight. The tiles were then removed from the water and blotted dry. The rough side of each tile was then covered with a lime plaster of three parts sand to one part slaked lime.

Tile 1 was used for the controls, which consisted of distemper, a proteinaceous binding media with collagen rather than albumin as the primary protein, and fresco, which has no organic binding media. The plaster layer applied to Tile 1 was thicker than for Tile 2 so that the plaster would not set too quickly to allow the fresco application. After the plaster was applied, Tile 1 was divided into sections using rubber bands to create a grid consisting of 24 cells to accommodate 12 pigments using fresco and distemper application (see Table 1. for the complete pigment list). For fresco application, the pigments were mixed in distilled water and painted onto the wet plaster. This was done for all of the pigments except for lead white and orpiment, which were found to have a very poor affinity with the water necessitating the addition of limewater into the pigment water mixture before being painted onto the surface. During the setting of the plaster each cell was polished using a spatula to accelerate carbonation (the formation of calcium carbonate crystals) and ensure a good bond between the pigment and the plaster layer.

Table 1: Complete Pigment List

Pigments	Color	Chemical Formula	Supplier	ID number
Egyptian Blue	Blue	$\text{Ca CuSi}_4\text{O}_{10}$	Kremer Pigments	10060
Natural Malachite	Green	$\text{CuCO}_3 \cdot \text{Cu(OH)}_2$	Kremer Pigments	10300
Chrysocolla	Green	$(\text{Cu, Al})_2\text{H}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot n\text{H}_2\text{O}$	Kremer Pigments	10350
Verona Green Earth	Green	$\text{K}[(\text{Al, Fe}^{\text{III}}), (\text{Fe}^{\text{II}}, \text{Mg})](\text{AlSi}_3, \text{Si}_4)\text{O}_{10}(\text{OH})_2$	Kremer Pigments	1100
Hematite	Red	Fe_2O_3	Kremer Pigments	48551
Burgundy Yellow Ocher	Yellow	$\alpha\text{FeO-OH}$	Kremer Pigments	11573
Lead White	White	$2\text{PbCO}_3 \cdot \text{Pb(OH)}_2$	Unknown	
Cobalt Blue Medium	Blue	CoAl_2O_4	Kremer Pigments	45710
Ultramarine light	Blue	$(\text{Na, Ca})_8(\text{AlSiO}_4)_6(\text{SO}_4, \text{S, Cl})_2$	Unknown	
Orpiment	Yellow	As_2S_3	Kremer Pigments	10700
Red Lead	Red	Pb_3O_4	Unknown	
Vermilion	Red	HgS	Kremer Pigments	42000

Due to the thickness of Tile 1, it was allowed to set for one week until most of the calcium hydroxide in the plaster converted into calcium carbonate. After this period, the distemper was prepared using the method described by Massey (1967: 82). Commercially available rabbit skin glue was used to make the distemper. It should be kept in mind that previous studies using immunochemistry for the identification of collagen glues have found that despite its name, rabbits are not the source species for this product, and that although the actual source is unknown it is presumed to be primarily bovine (Hodgins and Hedges 1999). The hide glue was prepared by soaking about 25 mL of the hide glue pellets overnight in 250 mL of water. The next morning

the excess water was poured off. The hide glue was then heated in a hot water bath on a hotplate. Meanwhile the pigments were mixed with warm water into a thick paste, which was subsequently placed on a watch glass over a hot plate where the glue was slowly added. The mixture was thoroughly combined and additional pigment was added until it reached the desired consistency and immediately applied to the tile. As with the previous applications, the lead white did not mix well, leaving an irregular film.

As Tile 2 had a thinner plaster layer, it was allowed to set for 3 days prior to painting. Tile 2 was divided in the same manner as Tile 1, using rubber bands to create 24 squares of equal size. The same 12 pigments (Table 1) were used. Half of the tile was painted using egg yolk tempera and the other half using whole egg tempera. Both recipes were taken from Kakoulli (1994). The whole egg tempera was made by combining the following: one part egg yolk, one part egg white, one part white vinegar, and three parts distilled water. The egg yolk tempera was prepared by mixing one part egg yolk, one part vinegar, with three parts distilled water. The pigments were mixed with each of the binding media to create a good working texture and then applied onto the plaster surface. There were certain challenges observed during this process as well. Lead white was found to be incompatible with both and did not mix well leaving a patchy paint layer.

Tile 3 was prepared with the intention of being used for protocol development. Three coats of whole egg binding medium prepared using the recipe described above were applied to the dry plaster surface with no addition of any pigment. The goal in using this composition was to provide samples, which contained known positives that would present no interference, and so would be ideal for protocol development with both staining systems. However, when the whole

egg-binding medium was examined as a cross-section under the FITC filter cube, it was found to autofluoresce. As a result, these cross-sections were not utilized for protocol development.

5.2 Preparation of Cross-Sections

Testing focused on three of the pigments from each of the test tiles. The three were each chosen to represent different possible categories of pigment interference with the immunochemical analysis. Vermilion was selected as it neither blocks nor fluoresces and in previous studies by Hodgins and Hedges (2000: 77) was not found to reduce or enhance the signal from immunofluorescent staining. Hematite as an iron based pigment is a powerful quencher of fluorescence, and was chosen to evaluate the effects that quenching may have on the visualization of the protein (Kakoulli 2004). Lead white was chosen, as this pigment is known to have significant interaction with organic binding media. On its own lead white presents no fluorescence. However, when combined with an organic binding medium it enhances the fluorescence and broaden the emission response of the binding medium (Miyoshi et al. 1982: 5; Larson, Shin, and Zink 1991: 98-99). Prior to preparation of the cross-sections, the identity of these commercially available pigments was confirmed using X-ray diffraction (XRD). The results from the analysis of the three pigments of interest to this research are listed in Table 2.

Table 2: XRD Confirmation of Pigment Identity

Pigments	Supplier	ID number	Expected Phase	Phase 1 XRD	Phase 2 XRD
Lead White	Unknown	Unknown	Cerussite	Cerussite	Hydrocerussite
Vermilion	Kremer	42000	Cinnabar	Cinnabar	
Hematite	Kremer	48551	Hematite	Hematite	

For the preparation of the samples, small specimens were removed using a scalpel from each of the tiles. These were mounted in Buehler EpoxiCure two-part epoxy resin in a ratio of five parts resin to one part hardener (resin 20-8130-032 and hardener 20-8132-008) according to the following method to allow stratigraphic examination. A silicon rubber mold with four cells was filled halfway with the resin and allowed to set overnight. A sample was then placed into each cell of the mold along with a label and a fresh batch of resin was poured over the sample to fill the cells of the silicon mold. The mold was then placed under vacuum for at least 5 min. to remove any trapped air in the porous structure and to enable good impregnation of the samples with the resin. Once removed from the vacuum, the specimen was adjusted carefully using a needle. The samples were removed from the mold when the resin had fully cured.

Two different grinding and polishing methods were used on the cross-sections during protocol development. Initially, the samples were wet ground and polished. Each cross-section was ground to expose the sample using progressively finer grits of sand paper wheels on a Spectrum System 1000 electric grinder/polisher with water as a lubricant. After the grinding was complete, the samples were polished using a polishing pad and a 6 μ m polishing solution followed by a 1 μ m polishing solution also on the electric grinder/polisher.

However, wet polishing was found to adversely affect staining and so this method was substituted with dry grinding and polishing. Each of the cross-sections was ground using water and sandpaper on the electric grinder/polisher to remove the bulk of the epoxy but once the sample approached the surface only dry grinding using progressive grits of sandpaper with no

solvent of any kind was used to expose the sample. The sample was then polished by hand using progressively finer grits of Micro-mesh (cushioned abrasive cloth).

5.3 Photo-Documentation

The test tiles with the pigments and various binding media were documented using digital photography. UV fluorescence photography, and normal light photography using a Nikon D90. A UV light (excitation of 365nm) was used for the UV-induced visible fluorescence images. To ensure that they were captured only in the visible range a Kodak WRATTEN gelatin filter (No.2E) was used in front of the camera lens to block unwanted UV radiation. A QPcard 101 v2 was included in all images to facilitate comparison. The images were color corrected using Bridge CS4 and Adobe Photoshop CS and CS4 as indicated in the manual by R-Pozeilov (2008: 82-94) (see Appendix A for these images).

The cross-sections were photographed using brightfield reflected light, as well as, using an FITC filter cube on an Olympus BX51 microscope. This filter excites in the blue region (460-500nm with a peak at 480nm) and allows for emission in the green region (510-560nm with a peak at 535nm). Raw images were captured using a Nikon D70 and a laptop computer equipped with Camera Control Pro2. These were color corrected in Bridge CS4 and converted to TIFF images in Photoshop CS4 according to the method in R-Pozeilov (2008: 82-94). Scales were inserted in Photoshop CS and the images were saved as JPEG files. Due to heavy light pollution resulting from the microscope's present location in the general lab, a black felt curtain to eliminate excess light was constructed to fit the microscope (Figure 4).



Figure 5: *This image shows the set up used for viewing and documenting the cross-sections during testing.*

6. Protocol Development

Both the FITC and the Quantum dots were used as secondary antibody conjugates in this study. Secondary staining methods were chosen based on the availability options rather than preference; ideally, primary staining would have been used because it is a one step process and has higher specificity, especially for the Qdots where the lower signal strength of primary staining is not an issue.

6.1 Secondary Staining with FITC and Qdots

Protocol development was conducted on the vermilion whole egg tempera cross-sections, since vermilion as also mentioned above, does not enhance or interfere with the immunochemical reaction between the stain and the binding medium (Hodgins and Hedges 2000: 77).

An anti-chicken egg albumin antibody produced in rabbit supplied by Sigma Aldrich was used as the primary antibody. The same primary antibody was used for all protocols and with both secondary antibody conjugates. FITC conjugated to anti-rabbit IgG produced in goat supplied by Sigma-Aldrich was used as the secondary stain for the FITC testing (peak emission at 520nm). Qdot 525 goat F(ab')₂ anti-rabbit IgG conjugate supplied by Invitrogen was used for the Qdot protocols (peak emission at 525nm). This Qdot was selected, as it was the only one compatible with the FITC filter cube. It is the smallest of the Qdot nanocrystals secondary antibody conjugates supplied by Invitrogen at about 10nm. The primary antibodies sold by Invitrogen were not used in this study due to budgetary limitations. The Qdot conjugate was the same type of secondary antibody as supplied by Sigma (anti-rabbit IgG) and therefore should have been compatible with the primary antibody.

All of the protocols tested followed the same general methodology that is based on those developed by Heginbotham, Millay, and Quick (2006: 99-100) and by Ramirez-Barat and de la Viña (2001: 284):

- 1) A diluted block of casein (powdered milk) solution in 10% phosphate buffered saline solution (PBS) was applied to the cross-section and allowed to sit for 10 min. The block was then removed with an absorbent paper. Care was taken not to touch the embedded sample directly with the absorbent paper. Both a 1% casein solution and a 10% casein solution in the PBS were used in the protocol development.
- 2) A diluted solution of the primary antibody was applied to the cross-section and incubated (see below and Appendix B for details). Incubation times ranged from 1 hour to 72 hours. Most testing was conducted at room temperature, but initially some were also conducted under refrigeration.
- 3) The cross-section was rinsed 4 times using 10% PBS solution in de-ionized water. For each rinse 100 µl of 10% PBS solution was applied to each cross section and then wicked away with adsorbent paper.
- 4) A diluted solution of either FITC or Qdot 525 secondary anti-body conjugate was applied to the cross-section and incubated (see below for details).
- 5) The cross-section was rinsed 4 times using 10% PBS solution in de-ionized water. For each rinse 100 µl of 10% PBS solution was applied to each cross section and then wicked away with adsorbent paper.
- 6) The cross-section was allowed to dry for a minimum of 15 min.
- 7) The cross-section was covered with a glass cover slip and Stoddard's solvent was added.

- 8) The sample was viewed under the microscope in both brightfield reflected light and under the FITC filter cube on a BX51 Olympus microscope. The results were recorded and documented photographically as described in section 5.3.

Variations in the protocols tested focused on changes to steps 1, 2, and 4. These included changing the concentration of the blocking solution, the primary antibody solution, and the secondary antibody solution. The incubation times and temperatures for the primary and secondary antibodies also varied. Due to initial challenges in protocol development, as mentioned in section 5.2 wet grinding and polishing of the samples was abandoned and substituted by dry methods. This change in sample preparation was found to be critical as no positive staining was obtained on wet ground/polished samples but using the same conditions positive results were obtained on dry ground/polished samples (see Appendix B for details on all of the protocols tested).

The best results were obtained with the FITC using a blocking solution of 1% casein in 10% PBS, a dilution of 1:200 (by volume) for the primary antibody incubated at room temperature for 3 hours, and a secondary antibody dilution of 1:50 (by volume) incubated for 30 min. Once a working protocol was selected it was tested on all of the other binding media as well, to ensure that there was no non-specific staining.

The initial Qdot525-secondary antibody conjugate concentrations tested here were based on the available information on the protocol developed by Cartechini et al. (2010: 872) for Qdot605-secondary antibody conjugates. As with the FITC, due to concerns that wet polishing was adversely affecting the staining process, dry grinding/polishing methods were used. However,

still no positive results were obtained even after changing to dry grinding/polishing. A final attempt at developing a working procedure for the Qdot525 used the successful FITC protocol as a model. As positive staining was obtained using FITC, it confirmed that the primary antibody was binding with a protocol of 1% casein in PBS and a dilution of 1:200 (by volume) for the primary antibody with a 6-hour incubation. Additional tests for the Qdots used this method for the primary antibody and varied only with the concentration and exposure of the secondary. The last protocol tested for the Qdots was intended to be extreme to guarantee staining. This method used a 1:1 (by volume) dilution of the Qdot secondary antibody conjugate in the block and was incubated for 68 hours at room temperature. This however, did not yield any positive staining on the whole egg cross-sections and no further testing of the Qdot stains was conducted (for full details on all of the protocol tested see Appendix B).

7. Experimental Trials: FITC

Two trial sets were conducted, one on vermilion cross-sections and one on hematite cross-sections. Each trial consisting of four cross-sections: fresco, distemper, whole egg tempera, and egg yolk tempera. The goal of the testing was to determine: 1) if the results were consistent and reproducible; 2) the effects of the pigment binder interaction of the hematite as compared to the vermilion; and 3) if the egg yolk tempera behaved differently than the whole egg tempera. All testing was conducted using the working protocol for staining with FITC developed in this study. Results were assessed visually under the microscope and documented photographically using a Nikon D70 digital camera connected to a laptop equipped with Camera Pro2 software.

A third trial set was also conducted on sets of four cross-sections of lead white (fresco, distemper, whole egg tempera, and egg yolk tempera). A different method for evaluating and documenting the results of staining were necessary for this round due to the enhanced autofluorescence of the binding medium in the presence of lead white. Images of each of the cross-sections before staining were captured using a Nikon D70 camera and Camera Pro2 software under brightfield reflected light using an Olympus BX51 microscope. Once the image was in focus, the filter was changed to the FITC filter cube and additional images were captured at a range of shutter speeds. These shutter speeds were each recorded. After staining, the sample was photographed under brightfield reflected light and also using the FITC filter cube in the same location as was documented before treatment and with the same series of conditions. The image producing optimal documentation of the fluorescence was noted and compared with the before staining image at that same shutter speed. An increase in the appearance of the

fluorescence in the after-testing image was recorded as a positive and no discernable difference was recorded as a negative. This method is qualitative only, and no attempt was made to quantify the results.

Due to anomalous results during this third trial, a forth trial was conducted on six cross-sections. This included samples of paints from three whole egg tempera and lead white and three of distemper and lead white. The goal of this additional trial was to determine if the blocking solution and rinsing solutions were interacting with the paint layer of cross-sections containing lead white. These were not treated with the protocol already described but were instead exposed to the block solution (1% by weight casein in PBS) for three hours and 40 min, then rinsed four times with PBS, allowed to dry for 15min, with Stoddard's solvent covered using a cover-slip before being viewed under the microscope.

8. Imaging the Qdots Using VPSEM

Although no working protocol was successfully developed for the Qdots in this study, an attempt was still made to explore the possibility that these fluorescent tags could be viewed on an SEM with nano resolution capabilities. To this end, a field emission gun variable pressure scanning electron microscope (FEG-VPSEM) enabling nano scale resolution was used to image the Qdot 525 goat F(ab')₂ anti-rabbit IgG conjugate. This instrument (an FEI Nova NanoSEM™ 230) is also coupled with a Thermo Scientific NORAN System 7 energy dispersive X-ray spectroscopy (EDS), used here for elemental chemical analysis and spatial distribution of elements by elemental maps. The remainder of the Qdot secondary antibody conjugate was diluted one to one in de-ionized water. One drop was placed on a polished silicon wafer and allowed to dry under ambient conditions, while the other was placed on a polished silicon wafer and desiccated using silica gel. Prior to imaging of the samples on the FEG-VPSEM the samples were each documented photographically using the Olympus BX51 both under brightfield reflected light and using the FITC filter cube. These images were used to select areas of focus where there was clear fluorescence, indicating the presence of the Qdots in a significant amount.

With the assistance of Dr. Sergey Prikhodko, in the Department of Materials Science and Engineering at UCLA, imaging began with the desiccated sample to avoid possible sample deformation that may occur under the vacuum of the VPSEM. The samples were not coated, as this form of sample preparation is not necessary when using a VPSEM. Various imaging detectors were tested in an attempt to image the sample and both high vacuum and low vacuum modes. However, under high vacuum there was a significant amount of charging prohibiting

good imaging, while in low vacuum the small size of the Qdots (10nm) was at the limits of the imaging capabilities of the instrument. As a result, no images of the Qdots were obtained (Appendix D).

In a final effort to determine if the SEM could be used to image the presence of the Qdots, elemental maps of one area of the sample, known to have Qdots was produced using the EDS detector. However, no significant amount of cadmium, or selenium (the core of the Qdots) was detected. A peak that corresponds to the $K\alpha$ line of sodium (1.040) was detected. This peak is very close to the $L\alpha$ line for zinc (1.012), however as no $K\alpha$ line for zinc (8.637) was detected, it is most likely that this peak corresponds to sodium, rather than zinc. As sodium is a component of the saline solution in which the Qdots are supplied by the manufacturer the presence of sodium is not surprising. In the elemental maps, the only element, which showed a higher density in the region known to have the Qdots based on the fluorescence images, was carbon. This is likely from the polymer coating of the Qdots. However, in this case carbon cannot be considered a useful element confirming the presence of the Qdots, as there is carbon present already from the organic constituents in the sample. It is possible that no significant amount of cadmium or selenium was detected, as each Qdot contains only a few hundred to a few thousand atoms of the semiconductor material and so may be beyond the detection limit of the EDS (*Structure of a Qdot Nanocrystal* 2011). While Qdots can be imaged using TEM, it appears that they cannot be imaged using a VPSEM even with nano scale resolution.

9. Results

9.1 Vermilion and Hematite Trials

The results of all rounds of testing on the cross-sections containing hematite and those containing vermillion were consistent (Table 3 and Table 4). The whole egg tempera cross-sections for both the vermillion tests and the hematite tests were consistently positive. However, the intensity of the response was generally lower for the hematite, with two of the three positives being weak positives. A similar trend is seen in the egg yolk tempera where the vermillion egg yolk tempera cross-sections all tested positive, but with two of the three presenting weak positive results, while the hematite egg yolk tempera set all tested negative. All of the controls for the hematite testing set were negative, and all of the vermillion controls were negative as well, except for one inconclusive result with a fresco cross-section due to heavy non-specific staining overall. Four of the whole egg cross-sections (two from the hematite set and two from the vermillion set) showed in addition to staining in the paint layer, an intense staining just below the paint layer. This feature was also seen on one of the egg yolk tempera samples from the vermillion testing set.

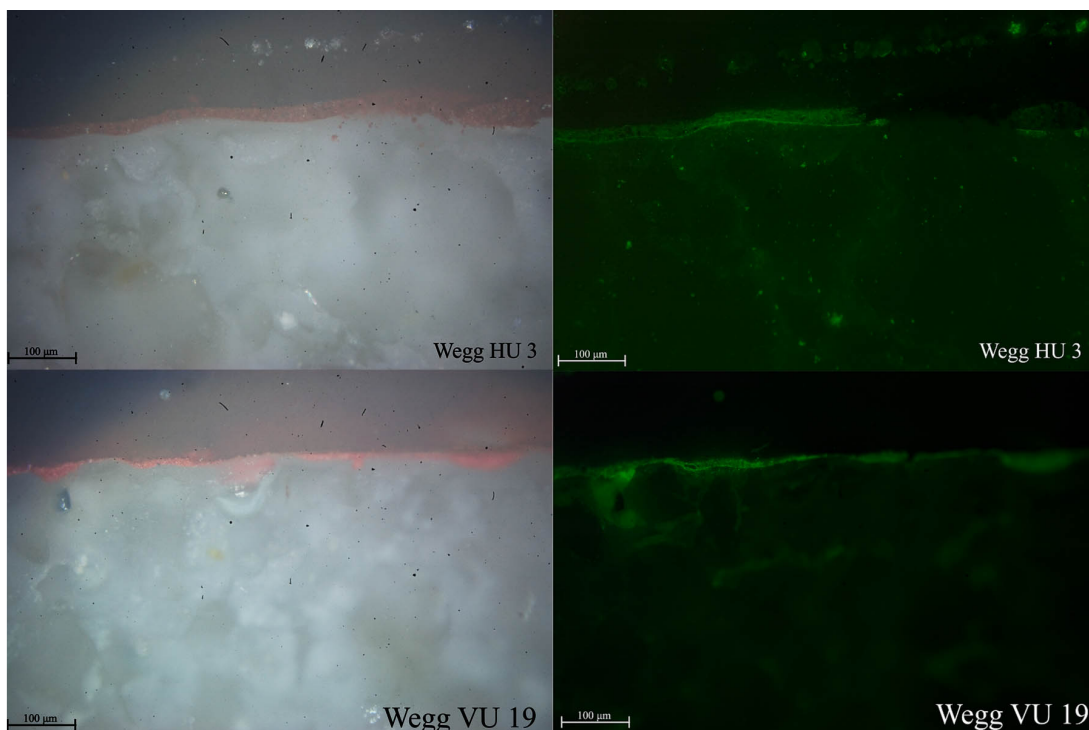


Figure 6: Examples of positive results through staining. The top images show one of the whole egg tempera cross-sections containing hematite under brightfield reflected light (top left) and when viewed with the FITC filter cube (top right). The bottom images show a whole egg tempera cross-section that contains vermilion under brightfield reflected light (bottom left) and when viewed with the FITC filter cube (bottom right). All four of the images were modified for publication by inserting a curve filter overall in Photoshop.

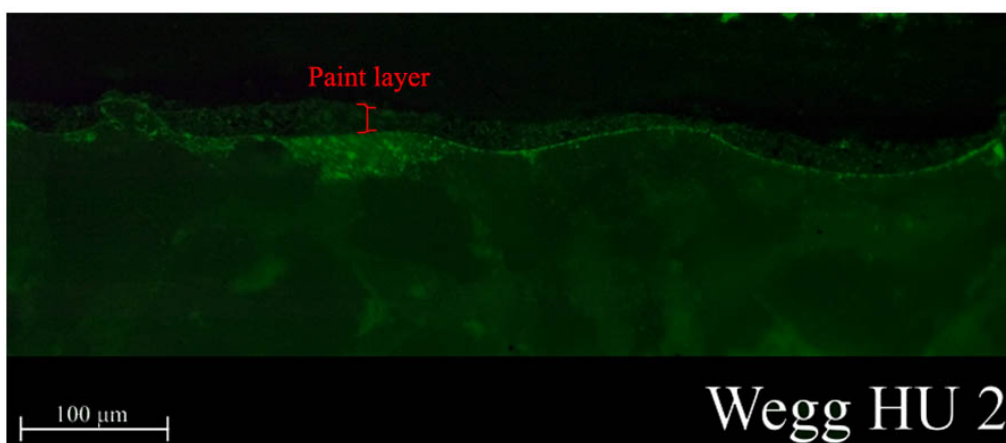


Figure 7: The above image shows an example of one of the cross-sections (a whole egg tempera section with hematite) that presented positive staining below the paint layer. This image was modified for publication by applying an overall curve in Photoshop.

Table 3: Results from Testing on Cross-sections Containing Hematite

Cross-section	Pigment	Binding medium	Results	Photography	Notes
Wegg_HU_2	Hematite	Whole egg tempera	Positive	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Heavy staining just below paint layer
Wegg_HU_3	Hematite	Whole egg tempera	Weak positive	ISO 200, white balance direct sunlight, shutter speed 15 seconds	
Wegg_HU_4	Hematite	Whole egg tempera	Weak positive	ISO 200, white balance direct sunlight, shutter speed 15 seconds	Staining below paint layer and non-specific staining in the ground
Yolk_HU_2	Hematite	Egg yolk tempera	Negative	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Only non-specific staining in areas with air bubbles
Yolk_HU_3	Hematite	Egg yolk tempera	Negative	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Non-specific staining in ground only
Yolk_HU_4	Hematite	Egg yolk tempera	Negative	ISO 200, white balance direct sunlight, shutter speed 15 seconds	Non-specific staining in ground only
Distemper_HU_2	Hematite	Distemper	Negative	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Some non-specific staining in areas with air bubbles
Distemper_HU_3	Hematite	Distemper	Negative	NA	
Distemper_HU_4	Hematite	Distemper	Negative	NA	
Fresco_HU_2	Hematite	Fresco	Negative	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Some non-specific staining in areas with air bubbles
Fresco_HU_3	Hematite	Fresco	Negative	NA	Only slight non-specific staining in areas with air bubbles, and could not be captured on camera
Fresco_HU_4	Hematite	Fresco	Negative	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Limited non-specific staining to air bubbles only

Table 4: Results from Testing on Cross-sections Containing Vermilion

Cross-section	Pigment	Binding medium	Results	Photography	Notes
Wegg_VU_19	Vermilion	Whole egg tempera	Positive	ISO 200, white balance direct sunlight, shutter speed 6 seconds	
Wegg_VU_23	Vermilion	Whole egg tempera	Positive	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Staining just below the paint layer and limited non specific staining in ground
Wegg_VU_22	Vermilion	Whole egg tempera	Positive	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Staining just below paint layer and some non-specific staining in ground
Yolk_VU_1	Vermilion	Egg yolk tempera	Weak positive	ISO 200, white balance direct sunlight, shutter speed 15 seconds	Could not capture any fluorescence at 6 seconds shutter speed
Yolk_VU_2	Vermilion	Egg yolk tempera	Weak positive	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Staining just below the paint layer
Yolk_VU_3	Vermilion	Egg yolk tempera	Positive	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Non-specific staining in ground
Distemper_VU_1	Vermilion	Distemper	Negative	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Some non-specific staining in the ground, no staining in the paint layer
Distemper_VU_2	Vermilion	Distemper	Negative	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Non-specific staining in the ground only
Distemper_VU_3	Vermilion	Distemper	Negative	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Non-specific staining in ground and in air bubbles
Fresco_VU_1	Vermilion	Fresco	Negative	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Some non-specific staining in areas with air bubbles
Fresco_VU_2	Vermilion	Fresco	Negative	ISO 200, white balance direct sunlight, shutter speed 6 seconds	Non-specific staining in the ground only
Fresco_VU_3	Vermilion	Fresco	Inconclusive	ISO 200, white balance direct sunlight, shutter speed 15 seconds	Non-specific staining over all

9.2 Lead White Trials

The lead white testing set proved to be more difficult to identify positive and negative results due to the intense auto-fluorescence of the lead white with the organic binding media (see Table 5).

This was not an issue for the fresco controls where there was minimal to no autofluorescence.

All of the whole egg tempera cross-sections appeared to have a more intense fluorescence after

staining than before staining, indicating a positive result. Only one of the egg yolk tempera cross-sections demonstrated the same type of apparent increase while the other two did not appear any different before staining as compared to after staining. All of the distemper controls appeared to increase in fluorescence, giving false positives. All of the controls with the fresco were negative. The results of exposure of the lead white with distemper and with whole egg tempera to solvent and blocking solution with no antibodies were no increase in fluorescence (Table 6).

Table 5: Results from Testing on Cross-sections Containing Lead White

Sample	Pigment	Binding Medium	White balance	ISO	Shutter speeds BT	Best Shutter speed AT	Results
Wegg_L U_13	Lead White	Whole egg tempera	Sunlight	200	1.6, 2, 2.5, 3, 6	1.6	Positive, appears to have increased fluorescence
Wegg_L U_14	Lead White	Whole egg tempera	Sunlight	200	1.6, 2, 2.5, 3	1.6	Positive, appears to have increased fluorescence
Wegg_L U_15	Lead White	Whole egg tempera	Sunlight	200	1.6, 2, 2.5, 3	1.6	Positive, appears to have increased fluorescence
Yolk_L U_1	Lead White	Egg yolk tempera	Sunlight	200	1.6, 2, 2.5, 3, 6	3	Negative, no discernable difference before and after staining
Yolk_L U_2	Lead White	Egg yolk tempera	Sunlight	200	1.6, 2, 2.5, 3	3	Negative, no discernable difference before and after staining
Yolk_L U_3	Lead White	Egg yolk tempera	Sunlight	200	1.6, 2, 2.5, 3	2.5	Positive, appears to have increased fluorescence
Distemper_LU_1	Lead White	Distemper	Sunlight	200	1.6, 2, 2.5, 3, 6	3	False positive, appears to have increased fluorescence
Distemper_LU_2	Lead White	Distemper	Sunlight	200	1.6, 2, 2.5, 3	2	False positive, appears to have increased fluorescence
Distemper_LU_3	Lead White	Distemper	Sunlight	200	1.6, 2, 2.5, 3	3	False positive, appears to have increased fluorescence
Fresco_L U_1	Lead White	Fresco	Sunlight	200	NA (minimal auto-fluorescence)	NA	Negative
Fresco_L U_2	Lead White	Fresco	Sunlight	200	NA (minimal auto-fluorescence)	NA	Negative, limited non specific staining in ground
Fresco_L U_3	Lead White	Fresco	Sunlight	200	NA (minimal auto-fluorescence)	NA	Negative, limited non specific staining in ground

Table 6: Results from Cross-sections Containing Lead White Exposed to Blocking Solution

Sample	Shutter speed Before exposure	Exposure time to 1% Casein in PBS	Best speed Shutter after exposure	Results
Distemper_LU_4	1.6, 2, 3	3 hr 40 min	3	Negative: decrease in fluorescence
Distemper_LU_5	1.6, 2, 3	3 hr 40 min	3	Negative: decrease in fluorescence
Distemper_LU_6	1.6, 2, 3	3 hr 40 min	3	Negative: decrease in fluorescence
Wegg_LU_16	1.6, 2, 3	3 hr 40 min	3	Negative: decrease in fluorescence
Wegg_LU_17	1.6, 2, 3	3 hr 40 min	3	Negative: decrease in fluorescence
Wegg_LU_18	1.6, 2, 3	3 hr 40 min	3	Negative: decrease in fluorescence

10. Discussion

During the protocol and methodology development, certain factors were found to be critical in this experiment and warrant further investigation and discussion. In particular aspects of sample preparation were found to significantly influence the results of IFM. The samples in this study were prepared using a two-part epoxy resin. While softer resins are more commonly used for the preparation of paint cross-sections, in this study none of the issues with dissolution, distortion, or swelling were observed in any of the samples. It is possible that the hardness of the epoxy and the impregnation of the samples under vacuum may have reduced the tendency of cross-sections to respond adversely to wet chemistry. However, wet polishing was found to remove or dissolve the proteins or otherwise interfere with IFM, despite there being no visual change to the cross-section, as no positive staining was obtained on wet polished samples. Only on dry polished samples were positive results obtained. Therefore, dry polishing should be the standard method of sample preparation when using IFM.

Testing on the various pigments and binders also showed that the pigment binder interaction affects the IFM results. The vermilion cross-sections proved ideal for protocol development, as the pigment was not found to enhance the signal or interfere with the signal strength. As a result, this pigment could be used as a baseline when compared to the other pigments, and revealed that while the whole egg tempera provided positive results across all three pigments, the relative strength of the fluorescence differed. Generally the samples prepared with hematite showed a reduction in fluorescence. This is not surprising, as this pigment is known to have natural quenching properties due to the iron ions in the hematite (Kakoulli 2004). It is also possible that

the reduced signal strength of the hematite cross-sections was caused by a pigment binder interaction the lead to fewer available binding sites on the protein. Hodgins and Hedges (2000: 77) observed this phenomena for collagen based paints made with verdigris and when combined with ultramarine. However, neither egg based tempera nor hematite were included in their study. The presence of positive staining below the paint layer in some of these samples may be the result of the absorption of the binding media into the porous ground during the manufacture of the test tiles.

The interaction of lead white with organic binding media, which has previously been found to both enhance and broaden the response of organic binding media when exposed to UV radiation (excitation of 356nm), has added a new challenge in the interpretation of the data (Larson, Shin, and Zink 1991: 98-99). The anomalous results were likely due to accidental contamination of the distemper samples with a reactive protein. The additional testing through exposure of the lead white with distemper and lead white with egg tempera demonstrated that the false negatives could not have been the result of an interaction of the lead white, and organic binding medium with the solvent and blocking solution used. It can be assumed then, that the false positive noted in the initial testing with the distemper indicated binding of the antibody tags to the cross-sections. As the rinsing protocol had not been changed, it should not have been the result of under rinsing and none specific staining from mutual attraction of proteins, but rather indicates a likely presence of contamination. The origin of this contamination is unknown, and appears to be limited to that round of testing as similar false positives were not observed in any of the other rounds of testing.

Although no working protocol for the use of Qdot antibody conjugates was developed as part of this study, the method may still have applications in the identification and localization of proteins in wall painting cross-sections. There are two possible reasons for why trials were unsuccessful. It is possible that the batch of antibodies used for testing was defective, or that the manufacturer has processed their secondary antibodies conjugates in such a way that they are only compatible with their proprietary primary antibodies. It is therefore recommended, that in any future study that the primary and secondary antibodies used in staining be from the same manufacturer.

Results from the three testing sets (vermilion, hematite, and lead white) demonstrate that the pigment/binder interaction, as well as the response of the pigment and binder to ultraviolet radiation affects the reliability and sensitivity of this method for the identification of organic binding media. Therefore, it is advisable to conduct pigment identification prior to staining samples from actual wall paintings to avoid areas containing pigments known to block fluorescence or enhance the auto-fluorescence of the binding media. As no testing was conducted on aged and un-aged samples it is difficult to determine if these pigments also accelerate or decrease degradation of the protein over time, a phenomenon that has been observed with other pigments and binders (Hodgins and Hedges 2000).

In addition, when interpreting results the possible implications from the method of production should also be considered, even if they cannot be fully anticipated or known. For example, in this experiment, the egg yolk tempera was shown to be less responsive to the stain than the whole egg tempera. The weaker response of these samples is likely the result of cross-sections

containing less of the target protein in the egg yolk tempera than the whole egg tempera either due to less binder being required to achieve a good working consistency or because less target protein was present in the egg yolk tempera recipe.

It is important to keep in mind that egg yolk while more protein-rich when compared to egg white, contains a wide variety of proteins rather than being made up of any one in particular. Egg white, on the other hand contains predominantly ovalbumin and glycoprotein (Mills and White 1999: 87-88). The primary antibody used was an anti chicken egg albumin antibody, which is not specific to any singular chicken egg protein, but should bind to all chicken egg albumins. As such, egg yolk should have contained more target protein. However, it is possible that the antibody bound preferentially to ovalbumin or the glycoprotein, the major constituent proteins in the egg white, or that the recipe for the egg yolk tempera resulted in a more dilute solution of protein. Either way, this demonstrates that method of paint preparation and not only the level of preservation can influence the strength of staining using IFM.

For this reason, a negative result for this method may not necessarily indicate there is no target protein present, only that there is too little to be detected or that it is sufficiently deteriorated as to prevent binding of the antibody to the target protein. This supports recommendations by other authors (Heginbotham, Millay, and Quick 2006 ; Arslanoglu, Zaleski, and Loike 2011) that a screening process should be conducted using another technique to confirm the presence of protein prior to using IFM, and that IFM should be used in cases where there is complex and or thin paint layers to localize the protein within the cross-section, rather than strictly for

identification. Screening with ELISA or amino acid analysis will also facilitate in determining what antibodies are needed for successful staining.

Currently, methods of staining are changing rapidly and there are ever more options for localizing the presence of proteinaceous binding media in cross-section. While Qdots deserve continued investigation so too do other options including SERS nanotag-complexed antibodies, which use surface enhanced Raman scattering nanoparticles coupled with antibodies to allow detection and visualization of the presence of the antibodies on a cross-section by taking a line scan using Raman spectroscopy which detects the SERS tags, and therefore the presence of the antibodies (Arslanoglu, Zaleski, and Loike 2011). Peroxidase staining techniques also warrant investigation, as these also use antibody chemistry but provide options such as colored reagent stains (immunohistochemistry), and fluorescent stains. They are also relatively inexpensive. Re-examination of primary antibody staining should also be conducted given that there are now numerous options for staining which may provide sufficient signal strength as to allow for this single step process to provide good results without the need for signal amplification of secondary staining.

11. Conclusion

This study demonstrates that the methods of sample preparation and pigment binder interactions both influence the signal strength and reliability of IFM. Dry polishing of cross-sections was found to be essential for obtaining positive staining results. Furthermore, Epoxy resins warrant further investigation as an imbedding medium as none of the distortion or dissolution of the cross-sections was observed in this study, which has been a problem for other researchers (Heginbotham, Millay, and Quick 2006 ; Ramírez-Barat and de la Viña 2001).

In addition, in this study the method of the paint preparation was found to affect the strength of staining. This should be taken into consideration when interpreting results as it is possible that on a single wall painting different colors prepared using the same binding medium, could present different signal strengths as a result not only of pigment binder interactions, but also through differences in the paint preparation from color to color or from batch to batch even of the same color. Therefore when using IFM on actual wall paintings results should always be carefully interpreted based on not only the considerations as recommended by other authors such as possible sources of contamination, possible deterioration of organic components, pigment binder interactions, but also possible variations in paint preparation techniques.

Pigment binder interactions were also observed to affect the strength of staining. Due to the complexity of the interactions between the binding media and pigments, it is advisable to screen samples before testing when conducting tests on actual unknowns. This was not done in this present study, as no unknowns were tested. Even so challenges were noted from pigment and

binder interactions, such as the enhanced autofluorescence of the organic binding media in the presence of lead white and the reduced signal strength of the samples containing hematite. To avoid such issues screening should include pigment identification as well as binding media identification to determine if and what types of proteins are present. Pigment ID can aid in avoiding problematic pigments (those that block fluorescence and those that enhance it, as well as those that accelerate deterioration), while the identification of the specific proteins present can confirm that an organic binding media is present. Furthermore, the technique used for the identification of binding media should be sufficiently sensitive to be able to determine what specific proteins are present so that the correct antibodies can be selected for staining with IFM.

12. Appendix A: Test Tile Production



Figure 8: Tile 1 right after fresco application completed. Note that the orpiment rapidly reacted with the alkaline limewater resulting in a color alteration to a greenish color. When the red lead was applied beneath the orpiment square, the red lead reacted with the orpiment (likely forming a lead sulfide) creating a dark area with a metallic luster along the junction of the two pigment zones.

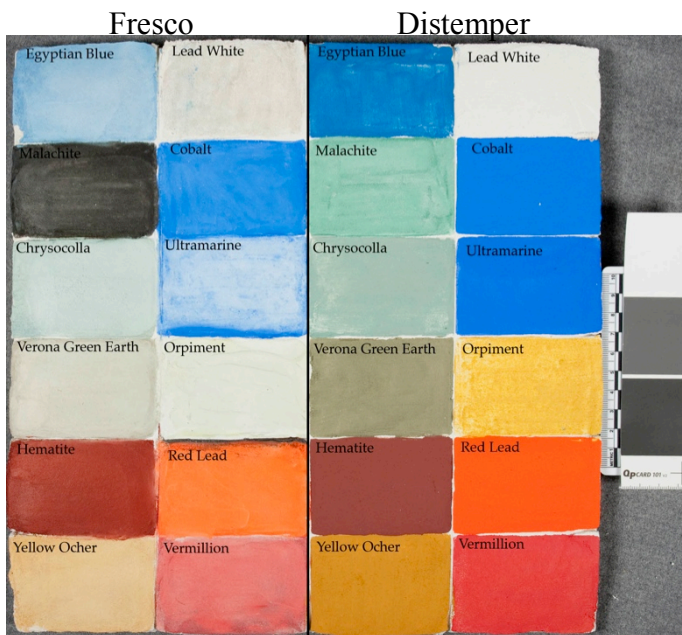


Figure 9: This image shows Tile 1 after it was completed. The 12 squares on the left side were applied using fresco and the 12 squares on the right half were applied a secco using distemper as the binding medium. Note that in the fresco side, the malachite has been affected by the alkalinity of the lime turning black and the overall lighter appearance of the pigments applied in fresco.

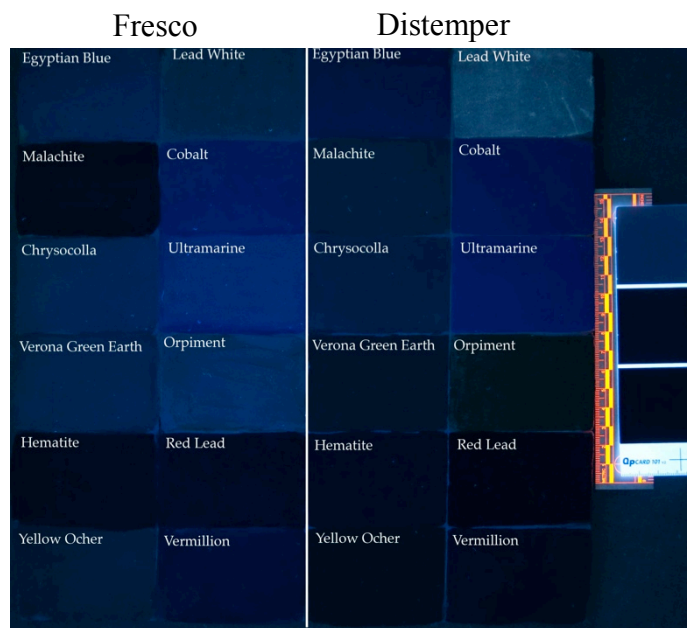


Figure 10: This image shows tile the visible fluoresce of Tile 1 under UV radiation. The image was captured using a Nikon D90 and a long-pass filter to capture only in the visible range.

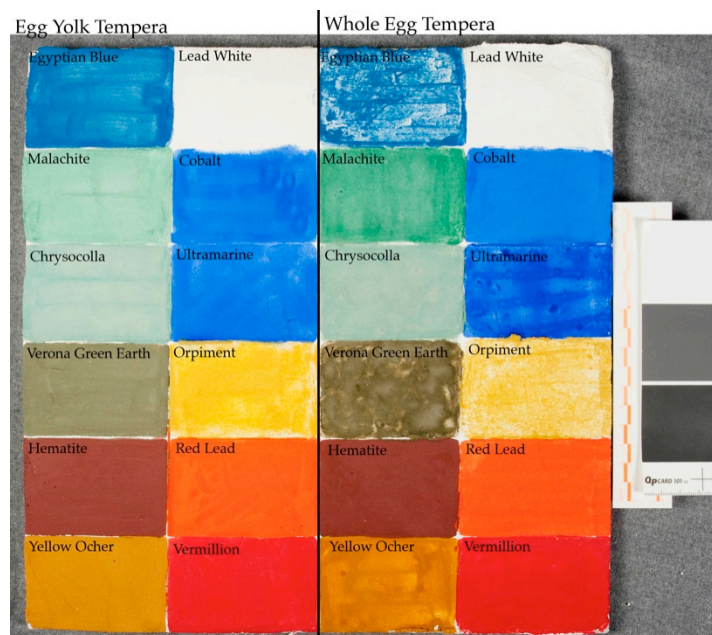


Figure 11: This image shows Tile 2 after it was completed. The 12 squares on the left were applied using egg yolk tempera. The 12 squares on the right were applied using whole egg tempera. Note the patchy appearance of the orpiment, Egyptian blue, and Verona green earth with the whole egg. This resulted from poor affinity of these pigments to the binding media causing poor working properties. The lead white exhibited similar problems for both media as well, but is less apparent in this image.

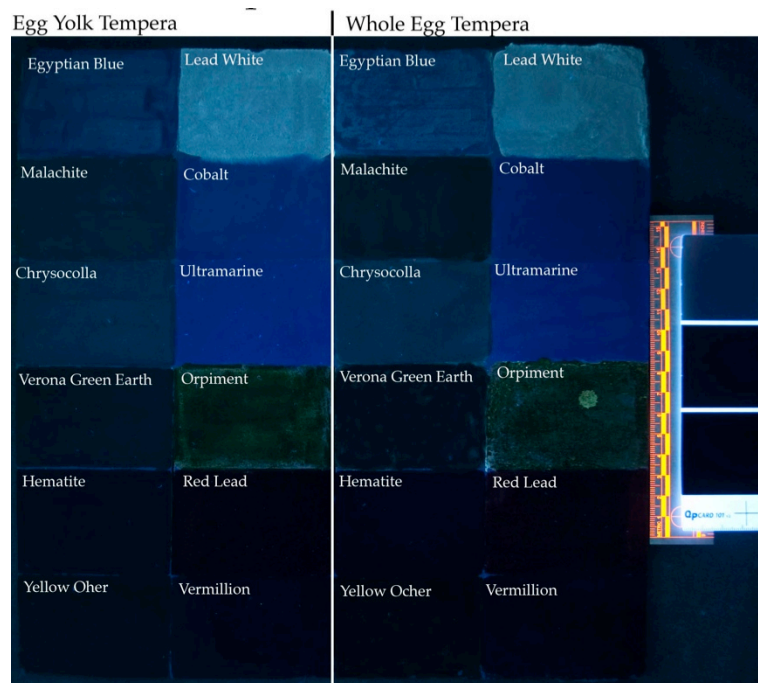


Figure 12: *UV induced visible fluorescence image of Tile 2. The image was captured using a Nikon D90 and a long-pass filter to capture only in the visible range.*

13. Appendix B: Protocol Development

Table 7: FITC Secondary Staining Protocols Tested Using Wet Grinding/ Polishing

Step	Protocol 1a	Protocol 1b	Protocol 1c	Protocol 1d	Protocol 1e
1	25µl block: 10% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 10% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 10% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 10% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 1% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.
2	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 1 hr. at RT	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 6 hr. in fridge (record temp)	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 6 hr. at RT	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for overnight in fridge (record temp)	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 1 hr. at RT
3	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.
4	25µl applied of antibody-FITC conjugate 1:50 in block for 30 min. at RT	25µl applied of antibody-FITC conjugate 1:50 in block for 60 min. in fridge (record T).	25µl applied of antibody-FITC conjugate 1:50 in block for 30 min. at RT	25µl applied of antibody-FITC conjugate 1:50 in block for 60 min. in fridge (record T).	25µl applied of antibody-FITC conjugate 1:50 in block for 30 min. at RT
5	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.
6	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.
7	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent
8	viewed under microscope.	Viewed under microscope.	Viewed under microscope.	Viewed under microscope.	Viewed under microscope.
Results	NR	NR	NR	NR	NR

Table 8: FITC Secondary Staining Protocols Tested with Dry Grinding/Polishing

step	Protocol 1f	Protocol 1g	Protocol 1h	Protocol 1I	Protocol 1J
1	25µl block: 1% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 1% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 1% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 10% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 1% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.
2	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 72 hr. at RT	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 24 hr. at RT	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 6 hr. at RT	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 6 hr. at RT	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 3 hr. at RT
3	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.
4	25µl applied of antibody-FITC conjugate 1:50 in block for 30 min. at RT	25µl applied of antibody-FITC conjugate 1:50 in block for 30 min. at RT	25µl applied of antibody-FITC conjugate 1:50 in block for 30 min. at RT	25µl applied of antibody-FITC conjugate 1:50 in block for 30 min. at RT	25µl applied of antibody-FITC conjugate 1:50 in block for 30 min. at RT
5	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.
6	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.
7	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent
8	Viewed under microscope.	Viewed under microscope.	Viewed under microscope.	Viewed under microscope.	Viewed under microscope.
Results	Positive WeggVU18	Positive WeggVU17	Positive WeggVU15	Positive WeggVU20	Positive WeggVU19

Table 9: Qdot Secondary Staining Protocols Tested Using Wet Grinding/ Polishing

step	Protocol 2a	Protocol 2b	Protocol 2c	Protocol 2d	Protocol 2e
1	25µl block: 10% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 10% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 10% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 10% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 1% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.
2	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 1 hr. at RT	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 6 hr. in fridge (record fridge T).	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 6 hr. at RT	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for overnight in fridge (record fridge T).	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 1 hr. at RT
3	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.
4	25µl applied of antibody-Qdot conjugate 1:100 in block for 30 min. at RT	25µl applied of antibody-Qdot conjugate 1:100 in block for 1 hr. in fridge (record fridge T).	25µl applied of antibody-Qdot conjugate 1:100 in block for 30 min. at RT	25µl applied of antibody-Qdot conjugate 1:100 in block for 1 hr. in fridge (record fridge T).	25µl applied of antibody-Qdot conjugate 1:100 in block for 30 min. at RT
5	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100ul drops PBS at RT, wait 30 seconds, removed drop.
6	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.
7	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent
8	Viewed under microscope.	Viewed under microscope.	Viewed under microscope.	Viewed under microscope.	Viewed under microscope.
Results	NR	NR	NR	NR	NR

Table 10: Qdot Secondary Staining Protocols Tested with Dry Grinding/Polishing

step	Protocol 2f	Protocol 2g	Protocol 3g
1	25µl block: 1% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 1% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.	25µl block: 1% casein in PBS applied to cross section for 10 min at RT. Remove block with adsorbent paper.
2	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 72 hr. at RT	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 6 hr. at RT	25µl ovalbumin antibody diluted 1:200 in blocking solution applied to cross-section incubated for 6 hr. at RT
3	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.
4	25µl applied of antibody-Qdot conjugate 1:100 in block for 30 min. at RT	25µl applied of antibody-Qdot conjugate 1:100 in block for 48 hr at RT	25µl applied of antibody-Qdot conjugate 1:1 in block for 68 hr at RT
5	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.	cross section washed 4x: applied 100µl drops PBS at RT, wait 30 seconds, removed drop.
6	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.	Sample allowed to air dry for 15 min.
7	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent	Covered with coverslip and stoddard's solvent
8	Viewed under microscope.	Viewed under microscope.	Viewed under microscope.
Results	NR	NR w/paint layer: absorption into ground (NSS): WeggVU14	NR

14. Appendix C: List of Materials

Qdot 525 goat F(ab')₂ anti-rabbit IgG conjugate

Product number: Q114441MP

Invitrogen

Eugene Oregon, USA

(541) 465-8300

FITC Anti-rabbit IgG (whole molecule developed in goat) conjugate

Product number: 060M6052

Sigma-aldrich

3050 Spruce st.

St. Louis MO 63103

(314) 771-5765

Anti-chicken egg albumin antibody produced in rabbit

Whole antiserum

Product number: 080M4812

Sigma-aldrich

3050 Spruce st.

St. Louis MO 63103

(314) 771-5765

Phosphate buffered saline (PBS)

Product number:

Sigma-aldrich

3050 Spruce st.

St. Louis MO 63103

(314) 771-5765

Casein

Nido (dry whole milk)

Nestlé USA, Inc.

800 North Brand Blvd.

Glendale, CA 91203

United States

(818) 549 6000

Pigments

Kremer Pigments Inc.,

247 West 29th Street,

New York, NY 10001

(212) 219-2394 or (800) 995 5501

Rabbit skin glue

Talas
330 Morgan Ave
Brooklyn NY 11211
(212) 219-0770

Buehler EpoxiCure two-part epoxy resin
resin 20-8130-032 and hardener 20-8132-008
Buehler
41 Waukegan Road
Lake Bluff, Illinois 60044
(847-295-6500)

Appendix D: Imaging of the Desiccated Qdot Sample

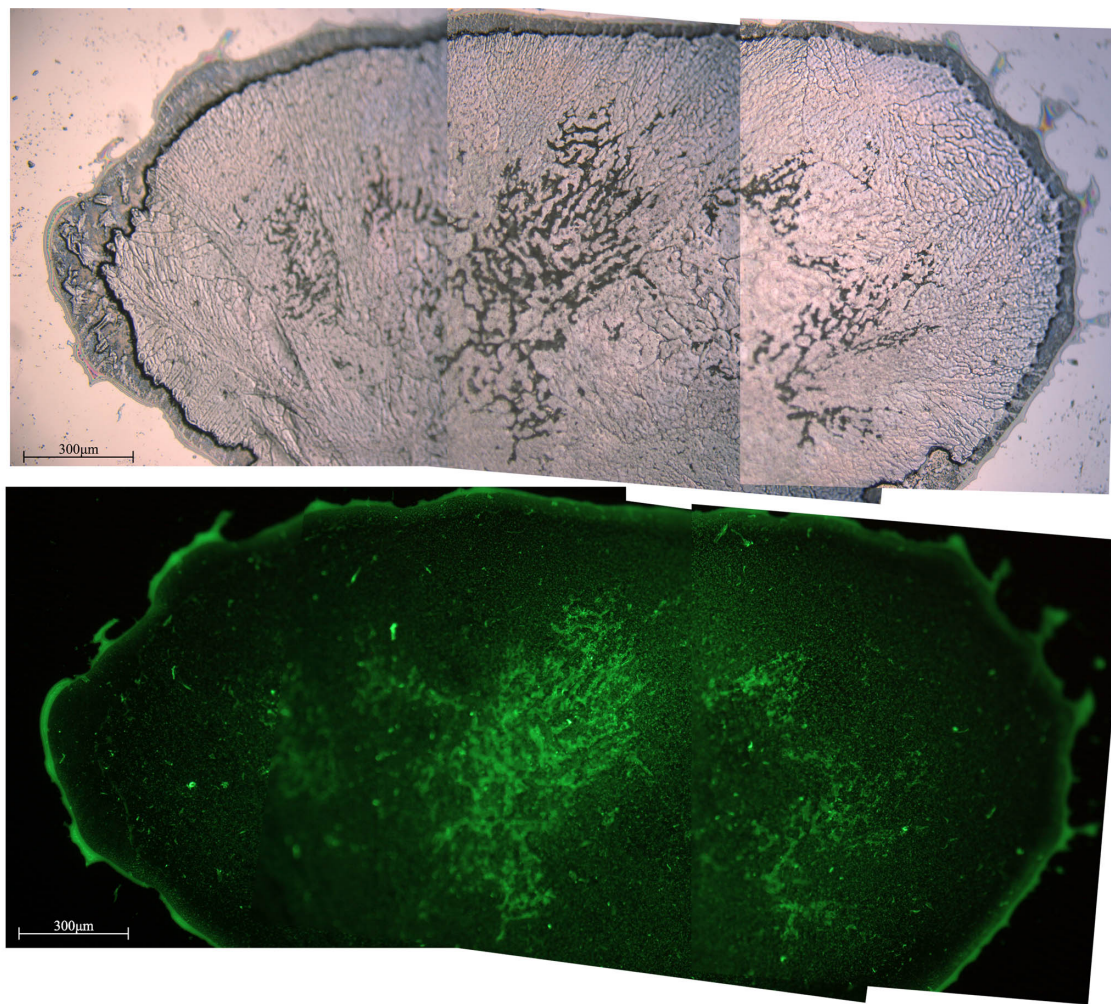


Figure 13: The top digital photomicrograph shows the desiccated Qdot sample under brightfield reflected light and the bottom digital photomicrograph shows the same sample when viewed using the FITC filter cube.

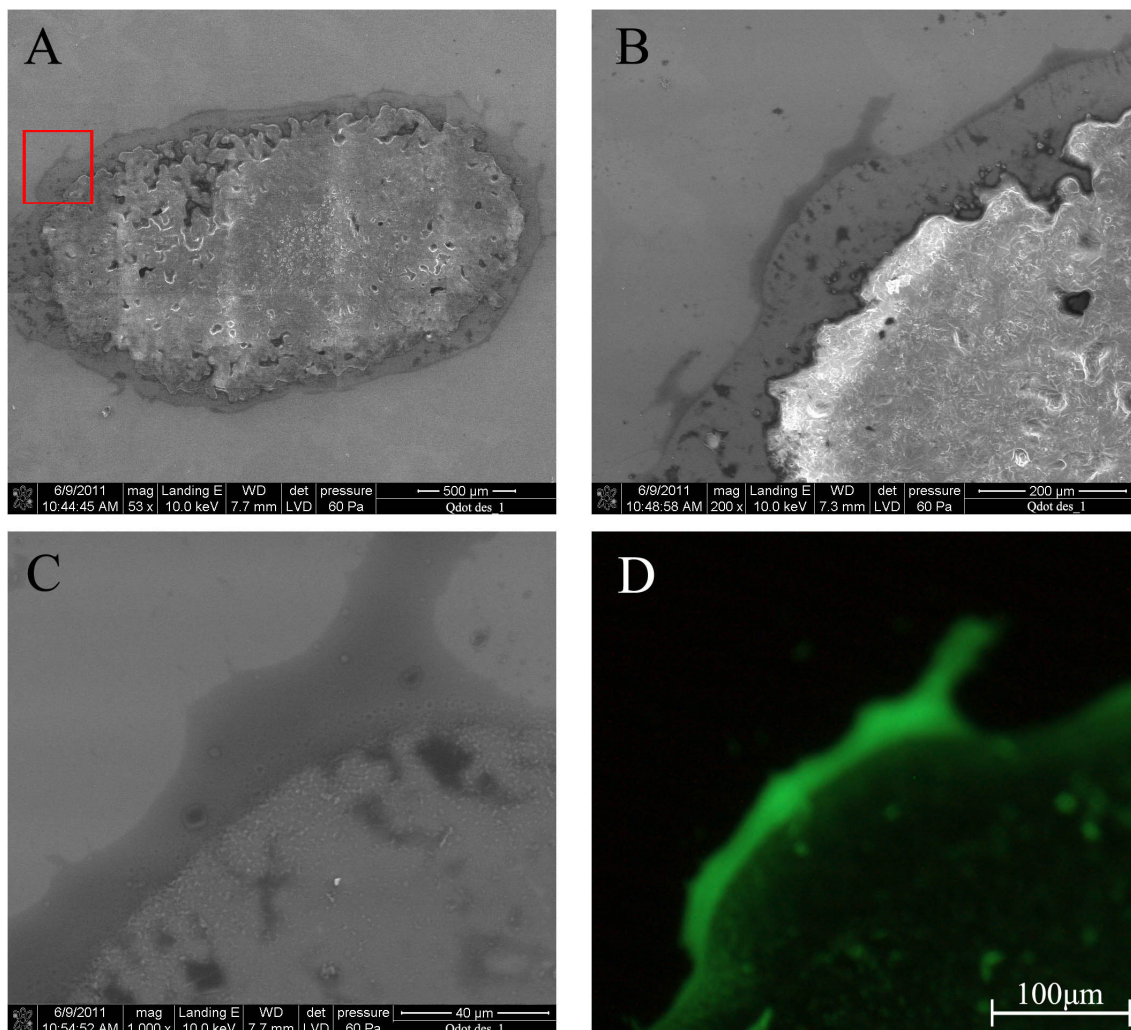


Figure 14: *A shows the SEM montage map of the desiccated sample with the area of focus marked in red. B and C are details taken with the SEM of the area of focus while D shows that same region using IFM.*

12. Bibliography

- Arnold, A, and K Zehnder. 1987. Monitoring Wall Paintings Affected by Soluble Salts. In *The Conservation of Wall Paintings*, edited by S. Cather. Los Angeles: Getty Conservation Institute.
- Arslanoglu, J, S Zaleski, and J Loike. 2011. An Improved Method of Protein Localization in Artworks Through SERS Nanotag-complexed Antibodies. *Analytical and Bioanalytical Chemistry* 399:2997-3010.
- Brajer, N, and N Kalsbeek. 1999. Limewater Absorption and Calcite Crystal Formation on a Limewater-Impregnated Secco. *Studies in Conservation* 44 (3):145-156.
- Cartechini, L, M Vagnini, M Palmieri, L Pitzurra, T Mello, J Mazurek, and G Chiari. 2010. Immunodetection of Proteins in Ancient Paint Media. *Accounts of Chemical Research* 43 (6):867-876.
- Cather, S. 1993. Keynote Address: Complexity and Communication: Principles of In Situ Conservation. Paper read at Conservation of Ancient Sites on the Silk Road: an International Conference on the Conservation of Grotto Sites, at Mogao Grottoes, Dunjuang, The Peoples Republic of China.
- Cather, S, and H Howard. 1986. The Use of Wax and Wax-Resin Preservatives on English Mediaeval Wall Paintings: Rationale and Consequences. In *Case Studies in the Conservation of Stone and Wall Paintings: Preprints of the Contributions to the Bologna Congress, 21-26 1986*, edited by N. Brommelle and P. Smith. London: International Institute for Conservation of Historic Artistic Works.
- Cather, S, D Park, and P Williamson. 1990. Introduction. In *Early Medieval Wall Painting and Painted Sculpture in England*, edited by S. Cather, D. Park and P. Williamson. Oxford: BAR British Series.
- Cennini, Cennino. 1960. *Il Libro dell' Arte*. Translated by D. T. Jr. New York: Dover Publications Inc.
- Chan, WCW, and S Nie. 1998. Quantum Dot Bioconjugates for Ultrasensitive Nonisotopic Detection. *Science* 281 (5385):2016-2018.
- de la Cruz Cañizares, J, MT Doménech Carbó, JV Gimeno Aldelantado, R Mateo Castro, and Reig Bosch, F. 2004. Suppression of Pigment Interference in the Gas Chromatographic Analysis of Proteinaceous Binding Media in Paintings with EDTA. *Journal of Chromatography* 1025:277-285.
- Deerinck, TJ. 2008. The Application of Fluorescent Quantum Dots to Confocal, Multiphoton, and Electron Microscopic Imaging. *Toxicologic Pathology* 36 (1):112-116.

- Dei, L., A Ahle, P Baglioni, D Dini, and E Ferroni. 1998. Green Degradation Products of Azurite in Wall Paintings: Identification and Conservation Treatment. *Studies in Conservation* 43 (2):80-88.
- Dolci, LS, G Sciutto, M Guardigli, M Rizzoli, S Prati, R Mazzeo, and A Roda. 2008. Ultrasensitive Chemiluminescent Immunochemical Identification and Localization of Protein Components in Painting Cross-Sections by Microscope Low-Light Imaging. *Analytical and Bioanalytical Chemistry* 392 (1):29-35.
- FITC1. 2011. Sigma-Aldrich 2011 [cited June 5 2011]. Available from http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=FITC1|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC.
- Gautier, G, and M Perla Colombini. 2007. GC-MS Identification of Proteins in Wall Painting Samples: A Fast Clean-up Procedure to Remove Copper-based Pigment Interferences. *Talanta* 73:95-102.
- Grywacz, CM. 1994. Identification of Proteinaceous Binding Media in Paintings by Amino Acid Analysis Using 9-fluorenylmethyl Chromofomate Derivatization and Reversed-Phase High-Performance Liquid Chromotography. *Journal of Chromatography* 676:177-183.
- Heginbotham, A, V Millay, and M Quick. 2006. The Use of Immunofluorescence Microscopy and Enzyme-Linked Immunosorbent Assay as Complementary Techniques for Protein Identification in Artists' Materials. *Journal of the American Institute for Conservation* 45 (2):89-105.
- Hodgins, G, and R Hedges. 1999. A Systematic Investigation of the Immunological Detection of Collagen-Based Adhesives. Paper read at 6th International Conference on "Non-Destructive Testing and Microanalysis for the Diagnostics and Conservation of the Cultural and Environmental Heritage", at Rome.
- . 2000. Toward the Immunological Detection of Media: The Detection of Artificially Aged Collagen-Based Paints. In *Art et Chimie: La Couleur*, edited by J. Goupy and J. Mohen. Paris: CNRS Éditions.
- Howard, H. 2003. *The Pigments of English Medieval Wall Painting*. London: Archetype Publications Ltd.
- Kakoulli, I. 1994. Philaretos' Akra Tapenoses at Mangori: its technique, condition, and cleaning in the context of late Byzantine Wall Painting in Cyprus, Conservation of Wall Painting Department, Courtauld Institute of Art, London.
- . 2004. Visible Fluorescence Spectral Imaging of Organic Materials on Wall Paintings Using MuSIS HS Technologies: A Case Study of the Tintori Replicas. Los Angeles: Getty Conservation Institute.

- Kleiner, FS, and CJ Mamiya. 2005. *Gardner's Art Through the Ages*. 12 ed. II vols. Vol. I. London: Thomson Wadsworth.
- Koller, M, H Leitner, and H Paschinger. 1990. Reconversion of Altered Lead Pigments in Alpine Mural Paintings. *Studies in Conservation* 35 (1):15-20.
- Larson, LJ, Kyeong-Sook Kim Shin, and JI Zink. 1991. Photoluminescence Spectroscopy of Natural Resins and Organic Binding Media of Paintings. *Journal of the American Institute for Conservation* 30 (1):89-104.
- Massey, R. 1967. *Formulas for Painters*. New York: Watson-Guption Publications.
- Mateo Castro, R, MT Doménech Carbó, V Peris Martínez, JV Gimeno Aldelantado, and Reig Bosch, F. 1997. Study of Binding Media in Works of Art by Gas Chromatographic Analysis of Amino Acids and Fatty Acids Derivatized with Ethyl Chloroformate. *Journal of Chromatography* 778:373-381.
- Meilunas, RJ, JG Bentsen, and A Steinberg. 1990. Analysis of Aged Paint Binders by FTIR Spectroscopy. *Studies in Conservation* 35 (1):33-51.
- Mills, JS, and R White. 1999. *The Organic Chemistry of Museum Objects*. Second ed. San Diego: Butterworth-Heinemann.
- Miyoshi, T, M Ikeya, S Kinoshita, and T Kushida. 1982. Laser-Induced Fluorescence of Oil Colours and Its Application to the Identification of Pigments in Oil Paintings. *Japanese Journal of Applied Physics* 21 (7):1032-1036.
- Mora, P, L Mora, and P Philippot. 1984. *Conservation of Wallpaintings*. London: Butterworths.
- Piqué, F. 2010. The Organic Materials in Wall Paintings Project. *Conservation Perspectives, the GCI Newsletter* 25 (2):12-14.
- R-Pozeilov, YA. 2008. *Digital Photography for Art Conservation*. Los Angeles.
- Ramírez-Barat, B, and S de la Viña. 2001. Characterization of Proteins in Paint Media by Immunofluorescence. A Note on Methodological Aspects. *Studies in Conservation* 46 (4):282-288.
- Rampazzi, L, F Cariati, G Tanda, and M Perla Colombini. 2002. Characteristics of Wall Paintings in the Sos Furrighesos Necropolis. *Journal of Cultural Heritage* 3:237-240.
- Rosewell, R. 2008. *Medieval Wall Paintings*. Woodbridge: The Boydell Press.
- Sawdry, A. 1994. Deterioration of the Romanesque Wall Paintings at Hardham Church West Sussex: A Study of the Influence of Salts and Organic Coatings, Conservation of Wall Painting, University of London, Courtauld Institute of Art, London.

- Silva, R.H. de. 1963. The Problem of the Binding Medium Particularly in Wall Painting. *Archaeometry* 6 (1):56-64.
- Structure of a Qdot Nanocrystal*. 2011. Invitrogen 2011 [cited June 5 2011]. Available from <http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes/Key-Molecular-Probes-Products/Qdot/Technology-Overview.html>.
- Vallance, SL, BW Singer, SM Hitchen, and JH Townsend. 1998. The Development and Initial Application of a Gas Chromatographic Method for the Characterization of Gum Media. *Journal of the American Institute for Conservation* 37 (3):294-311.
- Wolbers, R. 1987. The Use of Direct Reactive Fluorescent Dyes for the Characterization of Binding Media in Cross Sectional Examinations. In *The American Institute for Conservation of Historic and Artistic Works: Preprints of Papers Presented at the 15th Annual Meeting*, edited by A. Brown. Vancouver: The American Institute for Conservation of Historic and Artistic Works.
- . 1988. Aspects of the Examination and Cleaning of Two Portraits by Richard and William Jennys. In *The American Institute for Conservation of Historic and Artistic Works: Preprints of Papers Presented at the 16th Annual Meeting*, edited by S. Z. Rosenberg. New Orleans, Louisiana: The American Institute for Conservation of Historic and Artistic Works.
- Wouters, J, M Van Bos, and K Lamens. 2000. Baroque Stucco Marble Decorations. I. Preparation of Laboratory Replicas and Establishment of Criteria for Analytical Evaluation of Organic Materials. *Studies in Conservation* 45 (2):106-116.