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INTEGRATED HYPERSPECTRAL AND TIME RESOLVED FLUORESCENCE IMAGING COMBINED WITH STATISTICAL DATA ANALYSIS: DIAGNOSTIC INVESTIGATIONS OF WALL PAINTINGS

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ABSTRACT
For the first time we have applied a nanosecond fluorescence lifetime imaging (FLIM) system together with a hyperspectral fluorescence imaging device during a diagnostic investigation of wall paintings. The FLIM apparatus is based on a pulsed laser emitting nanosecond UV pulses at 337 nm for fluorescence excitation and on a nanosecond time-gated image intensifier for acquiring fluorescence emission from the surface. Based on the exponential decay of fluorescence intensity immediately following excitation, FLIM can be used for measuring the fluorescence lifetime map of the surface, thus differentiating between different fluorophores in the field of view. The hyperspectral fluorescence imaging device is based on two filtered lamps emitting at around 365 nm coupled with a tunable liquid crystal filter and a a cooled CCD for collecting fluorescence images at selected different spectral bands of approximately 20 nm FWHM in the visible range. The apparatus can be used to measure the fluorescence spectrum at each pixel in the recorded image.

The integration of the two fluorescence imaging techniques provides both spectral and lifetime information, therefore strengthening the differentiation between fluorescent materials on the surface of a painting. Following acquisition, images can be processed to highlight differences in fluorescence properties (lifetimes and spectra). However a novel approach has been further carried out based on the use of principal component analysis on the acquired images, which can effectively emphasize trends and similarities across the entire image. The portable apparatus has been applied to the monitoring of the condition of the wall paintings in the Baptistery of Colleggiata Church in Castglione Olona. Results will be presented and a final critical discussion of the technique will be given.

INTRODUCTION
Imaging techniques are well appreciated in conservation science. This is due to the fact that they can be used to analyze an artwork not in few selected points, but to visualize larger areas, thus taking account of the compositional heterogeneity of an artwork, which is especially relevant in paintings. Typical imaging techniques applied to the analysis of works of art range from X-ray imaging to thermography, UV-fluorescence and diffuse reflectance in the visible and in the near infrared. Among them, optical imaging techniques are especially attractive due to their simplicity, low cost and universal applicability to complex systems, including wall paintings and stone sculptures.

UV fluorescence is typically used in conservation science for identifying the presence of heterogeneities, which can be ascribed to organic or inorganic fluorescent materials on artistic surfaces. In fact, many artist materials (tempera and oil binders, colorants, lakes, fluorescent modern pigments and varnishes) and restoration compounds (glues, coatings and fixatives) exhibit UV fluorescence [1]. Recently, portable point-like fluorescence spectrometers have been developed and effectively applied to the analysis of artistic surfaces, allowing the identification of specific compounds, which include modern fluorescent pigments [2], natural colorants present in wood and silk textiles [3] and natural varnishes [4]. A strong effort has been further devoted to transform fluorescence spectrometers in imaging devices, especially

important for visualizing the variations in fluorescence in two dimensions [5-8]. Fluorescence imaging is particularly attractive since it may also be used for the analysis of large aeas; further, fluorescence measurements can be easily repeated in time, thus allowing one to monitor possible changes in condition, due to fluctuations in light exposure, humidity, pollution, etc.

In this contest, in our laboratory, after a wide experience with a fluorescence lifetime imaging (FLIM) system applied to the analysis of wall paintings [7] and marble sculptures [8], we decided to develop a new imaging device for recovering fluorescence spectral information. The new apparatus is a hyperspectral fluorescence imaging device based on a liquid crystal tunable filter coupled to a low-noise CCD camera.

The main features of the two imaging devices will be briefly shown, together with a discussion of data analysis. In particular, the results provided by using principal component analysis (PCA) on fluorescence spectral data will be considered. The application of the two devices to the analysis of wall paintings by Masolino in the Baptistery of the Colleggiata Church in Castglione Olona will be finally presented.

SYSTEMS SET-UP

The FLIM apparatus is based on a pulsed laser (LN203C Laser Photonic, Orlando, FL) emitting 1-ns UV pulses at 337 nm and on a nanosecond time gated image intensifier (C9546-01 Hamamatsu Photonics) for acquiring fluorescence emitted by the surface. Based on the exponential decay of fluorescence intensity immediately following excitation, FLIM can be used for measuring the fluorescence lifetime map of the surface, thus differentiating between different fluorophores in the field of view. The apparatus has already been used for the imaging of wall paintings [7] and for the detection of contaminants on marble sculptures [8].

The hyper-spectral fluorescence imaging system is based on two home-made filtered lamps emitting UV light around 365 nm and on a tunable liquid crystal filter (VariSpec, Cambridge Research Instruments) coupled to a cooled CCD (Retiga 2000, QImaging) for collecting fluorescence images at selected different spectral bands in the visible range [9]. The apparatus can be used to record the UV fluorescence spectrum at each point of a surface under analysis.

The integration of the two fluorescence imaging techniques provides both spectral and lifetime information, which are the properties that mainly characterize a fluorescence emission [10]: the fluorescence spectrum of a molecule is primary related to the the structure of the vibrational levels in the ground state of the fluorophore. Different fluorescent compounds are typically characterized by different emission spectra and thus spectral information can be used to identify or at least discriminate different fluorescent materials. In turn, the lifetime of a fluorophore is defined as the average time molecules spend in the excited state before returning to the ground level. The parameter provides a further means of discrimination among fluorophores, since spectrally overlapped signals are often characterized by distinct time behaviors. A further interesting feature is the sensitivity of fluorescence lifetime to the micro- fluorophore environment, which allows one to obtain useful data on the on the binding site of the emitting molecule and on other chemical and physical parameters like pH, polarity of the medium, aggregation state of the fluorophore, etc. Thus, it can be stated that lifetime is effective even for distinguishing changes a fluorophore undergoes when it is surrounded by different chemical environments.

In conclusion, the measurement of both spectral and lifetime properties of an emission strengthen the capability of differentiating between the many fluorescent materials often present on the surface of a painting.

DATA ANALYSIS

Following acquisition, images can be processed to highlight differences in fluorescence properties.

In order to understand the most suitable type of surface analysis, a brief introduction on the use of fluorescence in works of art should be given. Artistic surfaces are complex and heterogeneous objects. A painted layer, for example, is composed of mixtures of inorganic pigmented particles dispersed in a binding medium; colorants can be also present, as well as unknown materials added by restorers. Consequently, the interpretation of the fluorescence signal is not trivial and the identification of the chemical nature of the emitting species, apart from few cases [2-4], usually requires alternative invasive analysis. Thus, data analysis is mainly intended for discriminating between different fluorescent compounds present on the artistic surface.

For what concern time-resolved data, it should be observed that fluorescent systems can be hardly modeled as mono-exponential due to the simultaneous presence of several emitting molecules in the same specimen. Nevertheless, even in the case of a multi-exponential behavior, the kinetics of the emission can be simply modeled as a mono-exponential decay, allowing one to reconstruct what is known as the effective lifetime of the emission [11]. This value can be seen as a good approximation of the average lifetime of the emission; further, it has been shown by different research groups [11,12] that the effective lifetime yields the strongest contrast for the discrimination of different fluorescence compounds and thus is extremely useful for our application. In details, a linear fit is performed on the logarithm of FLIM data, providing the reconstruction of the spatial map of the effective lifetime and of the amplitude of the emission [7]. The first map reveals areas with different chemical composition, while the second gives information on the relative abundance of the fluorescent materials in the field of view. By merging the two maps, a third one, named HSV map, is created on the basis of the HSV model of colors. In this map, the luminance (value) of each pixel is proportional to the fluorescence amplitude, while the hue represents the lifetime, whereas the saturation is kept constant. In this way, the HSV map is easily associated with the functional information provided by the lifetime to the morphology of the analyzed region, given by the fluorescence amplitude [7].

For fluorescence hyper-spectral measurements, the first step in data analysis is the construction of the RGB map of the color of the fluorescence emission; this map provides a quick indication of the properties of the fluorescence emitted in the field of view and is also useful for a comparison of fluorescence spectral measurements to normal UV photography.

A second step in data analysis consists in measuring the physical parameters that characterize a fluorescence spectrum (i.e., the area under the spectral curve, the peak wavelength and the full width at half maximum FWHM), and using these parameters for the reconstruction of the corresponding thematic maps [9]. The first quantity is strictly related to the intensity of the emission, and consequently can provide information on the abundance of the fluorescent compound in the corresponding point of the field of view. The main peak wavelength can provide a rough indication on the nature of the main fluorophore present in the fluorescent compound, taking into account that, when excited by UV light, proteinaceous materials show

a main emission in the blue, whereas lipidic ones gives an emission peaked in the green [9,13]. Finally, the FWHM gives an indication on the presence of secondary fluorescent materials, i.e. the wider the fluorescence band, the more complex is the compound or the larger the number of fluorophores.

In order to enhance differences in spectral features, more refined analyses can be performed. In particular, principal component analysis (PCA) can significantly assist in discriminating between areas characterized by different fluorescent compound [14]. PCA analysis [15] is a common technique for expressing the data in such a way as to highlight their similarities and differences; from a mathematical point of view, PCA is a projection of the spectral information onto a lower dimensional subspace built-up by new orthogonal variables. The new variables are named principal components (PCs), and are chosen in such a way that the first PC describes as much of the covariance among the samples as possible, the second PC as much of the residual covariance and so on. Thematic maps can be depicted by converting the scores of the relevant PC in a grey scale map. Further, a segmented image is typically constructed by segmenting image into sub-areas characterized by the same PC values [16].

MEASUREMENTS ON MASOLINO FRESCOES

The two imaging systems were tested on the analysis of the conservation status of the Renaissance wall paintings by Masolino da Panicale in the first half of the XV century. The paintings are located in the Baptistery of Castiglione Olona, near Varese (Italy), and depict the life of John the Baptist.

Figure 1 – Color image , south wall, depicting the Salomè kneeling before her mother, Herodias with the head of John the Baptist, Masolino di Panicale, Catiglione Olona, 1435.

Ten areas on the painted walls were analyzed with the two fluorescence devices. Generally it was observed that fluorescence lifetime is more sensitive than the fluorescence spectrum for differentiating between areas characterized by different chemical compounds. As already quoted, this observation can be explained by the fact that the lifetime of a fluorophore is further dependent on the micro-environment of the fluorophore. For example, it was noticed (data not shown) that the lifetime of a fluorescent binder mixed to a particular pigment was shortened as a consequence of quenching phenomena induced by pigment particles. In turn,

for what concern the fluorescence hyperspectral device, the information provided by this technique was easier to interpret, since spectral emission can be easily compared to spectra of known fluorescent compounds.

As an example of our analysis, in Figure 1, left panel, a detail of the paintings on the south wall of the Baptistery is given. The area depicts the Salomè kneeling before Herodias with the head of John the Baptist on a platter.

Attention was focused on on fluorescence emissions around Salomè's face (figure 2, left panel). The outcome of analysis, in terms of color of the fluorescence emission is shown in figure 2, right panel. The decorated headdress on Salomè's head is of particular interest in terms of fluorescence emission. Two of the oval balls on the headdress, in fact, are characterized by a red intense emission, whereas a dim blue emission is detectable on the other three.

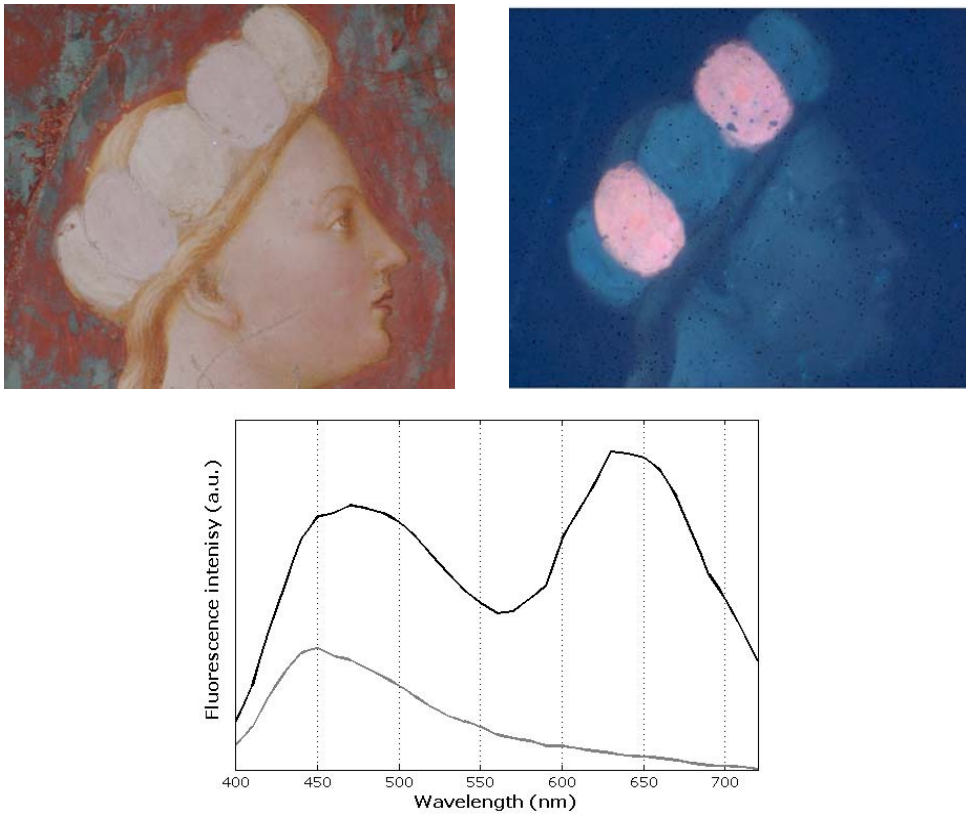

Figure 2: Top-left panel: color image of the area of the area chosen for fluorescence analysis depicting the head of Salomè. Top-right panel: reconstructed RGB map of the color of the fluorescence emission. A red intense emission is observable on two of the oval balls on the headdress of the character.
Bottom panel: fluorescence spectra of the oval balls on the headdress of Salomè: red fluorescent oval balls (black line) and blue fluorescent oval balls (gray line).

The corresponding fluorescence spectra, measured with are imaging spectral device, are also shown (Figure 2, bottom panel). The blue emission in the image is related to a fluorescence spectrum (gray line) with a peak at 450 nm. This emission possibly can be ascribed to the presence of an organic binder used for painting a secco finishing layers or to a protective treatment applied to the fresco surfaces during past restorations. In particular, the emission peaked at 450 nm could be an indication of a proteinaceous material [9]. In turn, the red emission is correlated to a fluorescence spectrum (black line) characterized by two broad peaks, the first centered at 480 nm and the second at 630 nm. The comparison of this

spectrum with spectra of known colorants revealed that the peak in the red is ascribable to the presence of madder lake, probably used by Masolino himself for decorating the headdress.

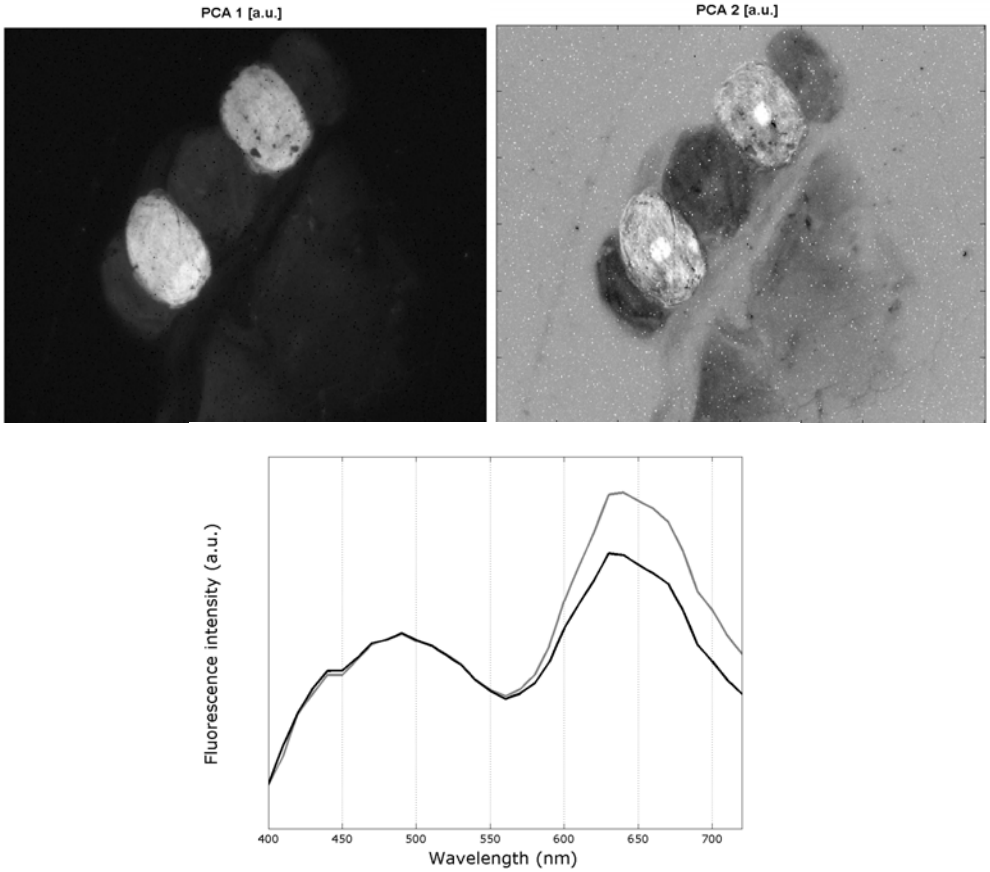

Figure 3 – Top panel: score map of the 1st and 2nd PC resulting from PCA on fluorescence spectral data. Bottom panel: fluorescence spectra of the oval balls on the headdress of Salomè: lateral part of the red fluorescent oval balls (black line) and central part of the red fluorescent oval balls (gray line).

PCA of hyper-spectral fluorescence data performed on the basis of the covariance matrix, revealed further interesting details on the headdress on the head of Salomè (figure 3, top panels). The first component is strictly related to the intensity of emission, whereas the second one highlights areas in the image characterized by different spectral features. In particular, it is possible to observe that the central part of the red fluorescent oval balls of the headdress is strongly highlighted. Considering the corresponding fluorescence spectra, it seems that in these areas there is a greater content of madder lake (figure 3, bottom panel).

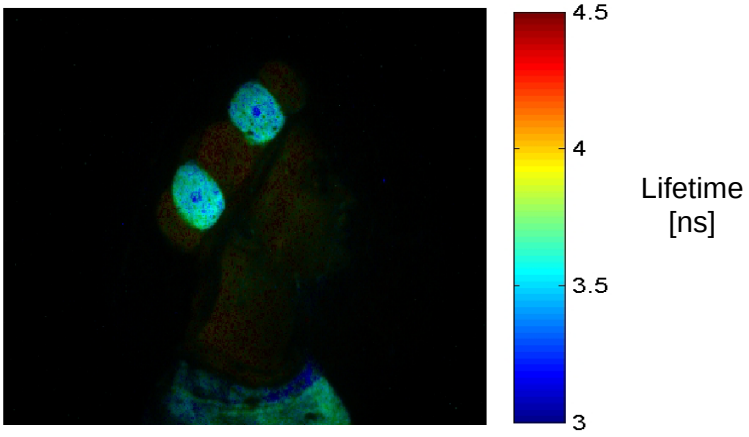

Figure 4 – HSV map resulting from the analysis of time-resolved fluorescence data.

The analysis of the decay of the fluorescence emission, performed with our FLIM set-up, confirmed these differences (figure 4). In particular, the central part of the red fluorescent oval balls is characterized by an effective lifetime near 3.2 ns, whereas a lifetime near 3.7 ns is measured on the lateral part. In turn, the blue fluorescent oval balls are characterized by a longer effective lifetime, near 4.3 ns.

Thanks to information provided by fluorescence spectral data, lifetime information can be better understood. Specifically, it can be stated that madder lake is probably characterized by a very short lifetime, whereas the suspected proteinaceous binder/lighter pigment is characterized by a longer lifetime, near 4.3 ns. When madder lake is mixed with binder/lighter pigment, the lifetime of the emission is the result of the average of the lifetime of madder lake and of the binder/lighter pigment weighted for the relative quantities of the two components in the mixture. Thus, the higher the content of madder lake, the lower is the resulting effective lifetime. Consequently, FLIM analysis confirms that the central part of the red fluorescent balls is characterized by a higher content of madder lake respect to the lateral part of the oval balls.

CONCLUSIONS

The two portable fluorescence imaging devices developed in our laboratory revealed interesting features for the analysis of artistic surfaces. The project is ultimately intended to transform the qualitative approach of UV (Wood lamp) photography, well known to restorers, in a reliable measurement procedure that could also be used to map different fluorescent organic materials present in works of arts.

The measurement of both spectral and lifetime properties of the emission highly reinforce the possibility of differentiating between the many fluorescent materials that can be present on an artistic surface.

In particular, spectral data provides clear indications on the nature of the fluorescent materials present on artistic surfaces, since the spectral shape of the unknown emission can be easily compared to spectra of known fluorescent compounds. Moreover, PCA analysis strengthens capabilities of the hyper-spectral imaging device in differentiating between areas characterized by different fluorescent compounds.

Fluorescence lifetime can significantly corroborate information provided by spectral data. It has been further observed that fluorescence lifetime in certain cases provides a higher discrimination of the fluorescent compounds present in the field of view. This is ultimately due to the inherent sensitivity of the lifetime of a fluorophore to the microenvironment of the fluorophore. Consequently, it can be concluded that the measurement of spectral and lifetime properties of the emission are both essential.

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Back to Top