Safe limits for the application of nonlinear optical microscopies to cultural heritage: a new method for in-situ assessment

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Abstract

Nonlinear optical microscopies (NLOM) are emerging techniques for the monitoring and preservation of cultural heritage, providing morphological, structural and compositional information on a large variety of artistic materials and objects. Being non-invasiveness the main requirement in artwork diagnostics, it becomes increasingly necessary to verify the absence of physicochemical alterations possibly induced by the use of high intensity radiation sources, and thus to find the exposure limits for safe applications. In this work, we propose a new methodology to set the criteria of damage evaluation for near infrared femtosecond pulsed lasers used for examining painted samples by means multi-photon excited fluorescence (MPEF), modality of NLOM. The method is based on the detection of changes in the MPEF signal upon repetitive irradiation on a series of modern acrylic painted samples, complemented by Raman spectroscopy measurements performed before and after irradiation. The obtained results allowed determining a safe average laser power range for the non-invasive determination of thickness in paint layers.

Keywords

Nonlinear optical microscopy, multi-photon excited fluorescence, acrylic paints, non-invasiveness, photo-damage.

1. Introduction

Non-invasive, in-depth optical sectioning of artistic paintings through the most commonly applied optical techniques is often hampered by the presence of opaque materials, such as highly absorbing or scattering pigments. For instance, optical coherence tomography (OCT) [1], a well-established low-coherence interferometric technique, is effective for stratigraphic imaging of materials with high degree of transparency to the radiation used (usually in the near infrared, NIR) such as varnishes. Nevertheless, OCT is not always successful in case of opaque paint layers, besides not providing any chemical information on the analysed

materials [2]. Other imaging techniques allowing for both cross-sectional imaging and chemical characterization, such as confocal fluorescence and Raman microscopy, are also limited to surface analysis in the presence of scattering media [3-5].

In the last years, nonlinear optical microscopy (NLOM) [6-7], in the modalities of multi-photon excited fluorescence (MPEF), second- and third-harmonic generation (SHG, THG), has been introduced in the field of cultural heritage thanks to its proven efficacy in biomedical applications, especially for high-resolution deep-tissue imaging and mapping of subcellular structures [8-11]. MPEF may provide compositional and structural information based on the detection of fluorophores [7], enabling to perform in-depth analysis on a variety of materials. Specifically, it was observed that the imaging penetration depth in biological tissues with multiphoton excitation is significantly higher with respect to confocal imaging, which shows loss of image contrast with increasing depth, due to light scattering [12]. Second- and third- harmonic generations are nonlinear scattering phenomena allowing for the detection of crystalline or highly organized structures without inversion symmetry (SHG) [13], and for the visualization of interfaces, based on local differences in refractive index and dispersion (THG) [6].

The additional benefit of NLOM, as compared to the above-mentioned techniques, is the ability to provide morphological, structural, and chemical information by detecting MPEF, SHG, and THG signals, which are generated simultaneously in the excitation volume of a tightly focused ultrashort pulsed laser. Due to the nonlinear dependence of the processes on the intensity of the excitation source, NLOM interaction is confined within the focal volume of the incident radiation and thus it is intrinsically characterized by high spatial resolution in every direction (typically in the micrometre range). Moreover, the use of NIR radiation to generate nonlinear phenomena allows a deeper penetration with respect to the single-photon excitation at shorter wavelengths (e.g. blue or UV) thanks to the transparency of most pictorial materials to this spectral range. Finally, the possibility to acquire MPEF signals in epi-detection makes this technique suitable for the analysis of a variety of real cases, for which the transmission mode is not feasible - i.e. painted board, wood, canvas, etc.

On these promising bases, NLOM techniques were initially tested for the in-depth analysis of protective layers in paintings and as a support for restoration processes like laser cleaning, which irreversibly modifies morphology and thickness of superficial layers through the selective removal of the outer coatings [14-15]. The combined detection of MPEF and THG signals proved effective for the cross-sectional imaging of varnish layers and for the monitoring of degradation due to aging [16] or laser ablation [17]. Microstructures in wooden artefacts were visualized and characterized by merging SHG and MPEF signals [18], whereas corrosion layers in silver-based objects were identified and quantified by means of MPEF [19]. Recently, pictorial layers were examined with both MPEF [20] and pump-probe microscopy [21-22] for documenting the laser-based removal of acrylic paints [14], and for the micrometric measurement of paint layers' thickness [21,23,24]. MPEF analysis on paint layers suggested that in-depth measurements are feasible, provided that the transparency of the paint layers is sufficiently high at both the excitation wavelength and the emitted fluorescence spectral range.

With the increasing interest in NLOM applied to cultural heritage, it becomes crucial to scientifically prove the safety of the technique. This can be achieved by establishing the range of radiation parameters, which can be applied for exciting nonlinear signals without inducing any deleterious effect on the sample. According to ISO 21254 -2:2001 (revised by ISO 21254-1:2011) [25], laser-induced damage is defined as *any permanent change in the characteristics of the surface or bulk of a specimen that can be observed by an inspection technique*. Therefore, the definition of safe conditions should start by optimizing and developing monitoring strategies according to the specific characteristic of the considered instrument and samples. NLOM laser-induced effects have been extensively characterized in biological tissues [26,27], identifying photobleaching and enhancement of the autofluorescence intensity as markers of photodamage [28,29], but no systematic studies on artistic materials have been performed hitherto. In 2017, Liang et al. [30] examined the degradation induced by nonlinear microscopies in comparison with OCT on painting materials. The samples were irradiated with a Nd:YAG nanosecond-pulsed laser, and photo-damage was examined using a low power Tungsten lamp, generating reflectance spectra in the irradiated spot after each laser pulse. Alterations were found to be strictly related to the different absorption properties of the irradiated materials. Specifically, signs of damage appeared after a few pulses and resulted more common in paint layers than in varnishes, due to pigments' higher absorption. The fluorescence emitted by paints was attributed to a combination of single and multi-photon absorption, in contrast to protective layers, where multi-photon absorption was considered the dominant process. It is worth underlining that in case of coherent nonlinear scattering phenomena, such as SHG and THG, the risk of photo-damage is negligible, since no energy is deposited in the material, which only acts as an energy converter for the incident photons through a single step quantum process. However, the same high intensity beam causing harmonic generation may also be absorbed by the medium through multi-photon absorption, thus eventually leading to photo-damage. Furthermore, considering that both SHG and THG signals need to be excited in a "quasi-resonant" condition, it is almost impossible to excite a multi-harmonic generation process without any non-linear absorption from the sample.

It is well known that the fluorescence emission may be faded by high-intensity and high-repetition pulsed radiation sources causing photo-chemical changes in the molecular structure of fluorophores [31]. It was also observed that fluorescence may be enhanced in presence of nanostructured noble metals, due to the coincidence between the nanostructure resonance wavelength and the fluorophore absorption/emission wavelength - provided that the fluorophore and the metal surface are separated by nanometre distance [32]. Some of the relevant photo-physical mechanisms causing fading are not completely understood, as well as their dependence from the environment [33]. Processes producing reversible changes in the fluorescence emission - e.g. quenching - are due to the reduction of the quantum yield (and consequently of the fluorescence lifetime), and may result from the non-radiative energy transfer from the fluorophore to other acceptor molecules residing physically close (Förster Resonant Energy Transfer, or FRET) [34,35]. In some cases, the fluorescent molecules undergo irreversible chemical modifications with the transition from an excited singlet state to the excited triplet state, causing the permanent loss of fluorescence yield - i.e. photo-bleaching. Due to the structural instability during the excited lifetime, the fluorophore likely interacts with either other molecules or

the local environment (light and molecular oxygen), generating free-radical reactive species. In these cases, it was observed that, while one-photon excited photo-bleaching follows a near-linear relationship with the incident intensity, two-photon excited photo-bleaching is proportional to a higher order dependence on the excitation power [36].

In this study, we define a new methodology for the non-invasive and safe determination of thickness of acrylic paint layers with MPEF, using a NIR fs-pulsed laser. Safe ranges of average laser power were established based on the observed variation of MPEF signal intensity upon repetitive irradiation, which was considered as the signature of possible photochemical alteration. Since both point-wise and image-wise information are often required in cultural heritage diagnostics, we used two different nonlinear setups with different illumination conditions for single-point and imaging acquisitions, respectively (see Table 1). Results obtained with increasing laser powers are compared and discussed, in order to establish safe irradiation thresholds for both approaches. In the imaging approach, to visualize the sign of damage in each small raster-scanned area, MPEF images of larger areas were also acquired before and after each irradiation. Using the same imaging setup, the monitoring was complemented with the acquisition of Raman spectra before and after each MPEF measurement session. Finally, the presence of superficial alteration was verified with optical microscopy (OM).

2. Materials and Methods

2.1 Nonlinear setups

The setup developed at the Instituto de Química Física Rocasolano - Consejo Superior de Investigaciones Científicas (IQFR-CSIC) is a nonlinear optical microscope (Fig. 1) for the point-wise collection of MPEF signals in epi-detection mode. The excitation source is a mode-locked Ti:Sapphire femtosecond laser emitting at 800 nm, with average power of 680 mW, releasing 70 fs pulses at a repetition rate of 80 MHz. A variable neutral density filter (NDC-50C-2M, Thorlabs) was used to control the laser power reaching the sample and the laser beam was focused on the sample by a long working distance objective lens (M Plan Apo HL 50×, Mitutoyo, NA 0.42), with a dichroic beam splitter (FF750-SDi02-25×36, Semrock) with high reflection at 800 nm. Focal plane selection was achieved with motorized translation XYZ stages (Standa 8MVT100-25-1 for XY and Standa 8MTF for Z). The lateral and axial resolutions are 1.5 and 2 μm , respectively, and the spot size (diameter) in the focal plane is 1.5 μm . A LabVIEW interface was used to control both scanning and data acquisition procedures. MPEF signals were collected in the backward direction through the microscope objective lens and a 70/30 beam splitter, in the spectral range 350-625 nm by means of a photomultiplier tube (9783B, ET Enterprises) connected to a lock-in amplifier (SR810 DSP, Stanford Research Systems). A short pass filter (350-625 nm, Thorlabs FGB37S) was placed at the entrance of the photomultiplier to cut off the reflected laser light. The remaining 30% of the MPEF signal was sent through a biconvex lens (focal length 20 cm) to a charged coupled device (CCD, Thorlabs DCC1645C) for online visualization of the sample surface.

Fig 1. Layout of the nonlinear optical microscope at IQFR-CSIC used for MPEF measurements: dichroic beam splitter (DBS), beam splitter (BS), biconvex lens (BCL), bandpass filter (BPF), photomultiplier for MPEF signal (PMT), objective for backwards (O) detection.

The setup developed at the Consiglio Nazionale delle Ricerche - Istituto Nazionale di Ottica (CNR-INO) is a custom-made laser-scanning nonlinear microscope (Fig. 2) [37] for MPEF, SHG, Raman, coherent anti-stokes Raman spectroscopy (CARS) and fluorescence lifetime imaging microscopy (FLIM). Depending on the technique, signal detection is either point-wise or image-wise. The source is an OPO pumped by a doubled Yb-based pulsed laser with 80 MHz repetition rate (Chameleon Discovery, Coherent, Santa Clara, CA, US), with dual output: the primary one at 1040 nm (140 fs) is not used in this study; the secondary one is tuneable across the 680 - 1300 nm range with short pulses (100 fs). The beam was focused on the sample by an air-immersion Plan-Apochromat 10 $\times$ objective lens, (NA 0.45, WD 2.1 mm, Carl Zeiss Microscopy, Jena, Germany) mounted on a piezoelectric scanning system for axial positioning (P-725KHDS PIFOC, Physik Instrumente, Karlsruhe, Germany). Two galvanometric mirrors (Cambridge Technology, Bedford, MA, USA) provide fast beam scanning for imaging on small areas. For bigger acquisitions, mechanical translators (M687-XY, Physik Instrumente, Karlsruhe, Germany) allow for wide range displacement of the sample. Signals were epi-collected with the same objective used for excitation, through a long-pass dichroic filter (FF665-Di02-25x36, Semrock Inc., New York, NY, USA) in order to separate excitation and emission radiations. The detection system is equipped with five kinematic cubes that hold filters and mirrors to send the light signals to the proper detectors. Multiphoton imaging was performed with a photomultiplier tube H7422-40 (Hamamatsu, Hamamatsu City Japan) with sensitivity in the spectral range 360-660 nm, upon proper filtering. Raman spectra were acquired by inserting a short-pass dichroic filter (DMSP805R, Thorlabs Inc., Newton, NJ, USA) and a

flipping mirror before the objective. A Toptica XTRA II (Toptica Photonics AG, Graefelfing, Germany) narrowband laser emitting at 785 nm excited the Raman signal, which was epi-collected and delivered to a Kymera 328i imaging spectrograph (Andor, Belfast, UK), equipped with a 830 grooves/mm diffraction grating.

The scanning system is controlled by a custom-made software developed in LabVIEW 2015 (National Instruments, Austin, TX, US) that also provides the visualization of the acquired images. Raman and fluorescence spectra acquisitions were performed using the software Andor Solis (Andor, Belfast, UK). Images were acquired with a field of view (FOV) of $500 \times 500 \mu\text{m}^2$, including the $100 \times 100 \mu\text{m}^2$ irradiated area ($1 \mu\text{m}$ scanning step in the axial direction), with a resolution of 256×256 pixels. The theoretical radial and axial extent of the PSF, calculated using the formulas reported in by Zipfel et al. [38], correspond to 720 nm and 5 μm , respectively.

Fig. 2. Schematics of the nonlinear optical microscope at CNR-INO used for MPEF measurements: tuneable source telescope lenses (L3-L4); continuous-wave laser telescope lenses (L5-L6); scan lens (L7); tube lens (L8); white light lens (L9); half wave plate (HWP); quarter wave plate (QWP); Glan-Taylor polarizer (GT); flipping mirrors (FM); galvanometric mirrors (GM); camera (C); objective translator (OT); XY-translation stage (TS); white light lamp (L).

The acquisition parameters of the two nonlinear microscopes are summarized in Table 1. Main differences are the pulse duration (70 and 100 fs for IQFR and INO setups, respectively) and the photon dose, namely the number of laser pulses per irradiated point (80×10^6 pulses/point and 200 pulses/point on the sample surface for the IQFR and INO setups, respectively). These latter values were computed considering the repetition rate for the different scanning modalities (point-wise versus area-wise).

Table 1: Comparison of MPEF acquisition parameters of the two NL optical microscopes used in this work.

	IQFR-CSIC	INO-CNR
Laser	Mode-locked Ti:Sa	OPO pumped by Yb-based fs laser
Excitation wavelength	800 nm	800 nm
Repetition rate	80 MHz	80 MHz
Pulse duration	70 fs	100 fs
Laser power	Measured on sample surface	
Objective NA	0.42	0.45
Type of measurement	Pointwise, Axial (z) scan Step on z: 1 μ m	Areal (x, y: 100x100 μ m ²) + Axial (z) scans Step on z: 1 μ m
Detection	Backwards, with PMT, range 360-610 nm	Backwards, with PMT, effective range 360-660 nm
Dose	80x10 ⁶ pulses/point	200 pulses/point

2.2 Optical microscope

A Leica DMRXE upright wide-field microscope system (objective Leica HCX PL FLUOTAR L 20 $\times$, NA 0.45) enabling fluorescence, bright field and dark-field imaging was equipped with a Camera Nikon D3200 (CMOS - APS-C sensor, 24.2 Megapixel resolution, Nikkor AF-S DX 18-55mm f/3.5-5.6 lens) for the acquisition of microscope images.

2.3 Samples

The samples consist of six commercial Extra-fine acrylic paints, Maimeri Brera (IT), which were chosen for their widespread in modern art. They are listed in Table 2 in alphabetic order, with relative acronyms, colour indexes [39] and chemical composition, as declared by the manufacturer. For this study, each paint was laid with a thickness of around 40-50 μ m on glass cover slips (2.5 $\times$ 5 cm², 140 μ m thick).

Table 2. Acrylic tempera paints (Maimeri Brera) with the acronyms used through the text, commercial codes (colour indexes) and chemical composition declared by the manufacturer.

Paint	Acronym	Colour Index	Chemical composition
Cadmium Yellow	CY	PY35 – 77205	Cadmium Sulphide [CdS]
Lemon Yellow	LY	PY175 – 11784	Benzimidazole [C ₇ H ₆ N ₂]
Permanent Blue Light	PBL	PW6 – 77891, PG7 – 74260 PB15:3 – 74160	Titanium Dioxide [TiO ₂], Chlorinated Phthalocyanine [C ₃₂ H ₃ Cl ₁₃ CuN ₈ to C ₃₂ HCl ₁₅ CuN], Copper Phthalocyanine β [C ₃₂ H ₁₆ CuN ₈]
Permanent Green Light	PGL	PY97 – 11767 PW6 – 77891 PG7 – 74260	Titanium Dioxide [TiO ₂], Azo coupling of aniline and acetoacetanilide, Chlorinated Phthalocyanine [C ₃₂ H ₃ Cl ₁₃ CuN ₈ to C ₃₂ HCl ₁₅ CuN]

Primary Red Magenta	PRM	PV19 – 73900	Quinacridone [C ₂₀ H ₁₂ N ₂ O ₂]
Zinc White	ZW	PW4 – 77947	Zinc Oxide [ZnO]

4. Results

MPEF measurements were performed with the IQFR setup by irradiating the samples in different points, on a scanning depth of around 100 μm (1 μm z-step), with different laser powers. Each point was scanned (in the axial z-direction) five consecutive times at a fixed laser power, which was measured on the sample surface. The resulting fluorescence intensity profiles were normalized and plotted together as a function of the depth. A suitable range of power was selected for each paint, based on previous studies [23]. Measurements were performed starting from a given laser power, which was increased or reduced depending on the changes observed in the signal intensity. Specifically, the laser power was decreased and the measurement point changed whenever a variation occurred in the MPEF profile. This procedure was repeated until the fluorescence intensity started to change after repetitive exposures. A reduction of MPEF signal was noticed for the majority of the analysed paints, except for Lemon Yellow, showing a growth in MPEF intensity as the power increased. Noteworthy, the opposite trend was noticed for the other yellow paint, Cadmium Yellow (Fig. 3), thus evidencing that different damage mechanisms are mainly related to the chemical composition of the pigments (see Table 2) and to their interaction with the acrylic binder. Further investigations are envisaged for better understanding these different behaviours.

Fig. 3. MPEF z-profiles of Cadmium Yellow (CY) acrylic paint upon irradiation at laser average powers of 0.4 mW (a), 0.5 mW (b) and 0.6 mW (c), and of Lemon Yellow (LY) paint at 5.5 mW (d), 6.4 mW (e), 7.3 mW (f). At laser power exceeding 0.4 mW and 5.5 mW for CY and LY, respectively, the maximum fluorescence intensity tends to decrease in the case of CY, and to increase in the case of LY, as the number of axial scanning increases, i.e. from Measure 1 to 4.

The INO setup was used to perform MPEF imaging, complemented by Raman spectroscopy and OM. Measurements were performed by irradiating the samples in different regions of interest ($\text{ROI} = 100 \times 100 \mu\text{m}^2$), for five consecutive times with a scanning depth of around $100 \mu\text{m}$ (z-step $1 \mu\text{m}$). Each ROI was scanned at a fixed laser power, and the distance between two successive ROIs was set to 1 mm , in order to facilitate the subsequent identification of the irradiated areas under the optical microscope. To detect precisely the alterations of the fluorescence intensity, we increased the power in steps of 0.25 mW or 0.5 mW between two consecutive ROIs. Furthermore, fluorescence intensity images ($\text{FOV} = 500 \times 500 \mu\text{m}^2$), Raman spectra and OM images were acquired in each ROI before and after each measurement session. Fluorescence intensity images were obtained by irradiating the samples surface within the safe power limits. For each ROI, Raman spectra were compared to the spectrum acquired on a reference area, which was intentionally irradiated at high laser power in order to induce a visible damage on the sample. Such recognizable area also served as fiducial marker for the OM imaging.

As an example, we report the results of the combined application of MPEF, Raman spectroscopy and OM on Permanent Green Light (PGL), which was irradiated with laser power ranging between 2 and 16 mW . In Fig. 4 we show the most representative results acquired on three ROIs, irradiated with 6 mW (a), 7.75 mW (b) and 8.75 mW (c) laser power. At 6 mW the maximum MPEF intensity does not vary with the five z-scans. No sign of alteration is visible in the irradiated ROI in the nonlinear fluorescence intensity images (d), as well as in the OM images (e) acquired before (t_0) and after the five z-scans (t_5). Similarly, Raman spectra (l) acquired after measurements (blue line) and in the non-irradiated area (red line) show no changes in the position of the intensity peaks. The main difference between these two spectra and that acquired in the purposely damaged area (black line) is in the background (see Supplementary Material), which is characterized by higher intensity above 1000 cm^{-1} after five z-scans. Differently, under irradiation at 7.75 mW , the maximum intensity of the MPEF profiles (b) decreases as the number of z-scans increases, possibly due to a photo-bleaching process that reduces the MPEF emission. Under this irradiation power, fluorescence (f) and OM (g) images show a slight change in intensity and hue (which appears darker), respectively. Furthermore, the Raman spectrum (m) acquired after the five z-scans (blue line) approaches that of the damaged area (black line). Finally, a clear sign of damage is detected under irradiation at 8.75 mW , with an evident decrease of intensity in the MPEF profiles (c), as well as in the fluorescence intensity image at t_5 (h). Moreover, the OM image (i) shows the darkening of the green hue after laser scans, and the Raman spectrum of the irradiated area (n) becomes equivalent to that of the fiducial marker.

Fig. 4. Combined analysis on Permanent Green Light acrylic paint: MPEF z-profiles acquired by irradiating the samples with an average laser power of 6 mW (a), 7.75 mW (b) and 8.75 mW (c). Corresponding fluorescence intensity images (d, f, h) and optical microscopy images (e, g, i) acquired before (t0) and after (t5) the five z-scans (Measure 1-5). The irradiated ROI ($100 \times 100 \mu\text{m}^2$) is highlighted by the dashed square. Raman spectra (l, m, n) measured in the purposely damaged area (black line), in a non-irradiated area (red line) and in the irradiated area after the five scans (blue line). In (b, c) a small shift of the MPEF peak is visible, probably due to thermal drift.

The combined application of different techniques for the monitoring of laser-induced effects allowed to define the average damage threshold. Based on the changes in the maximum intensity of the MPEF z-profiles acquired in the same point, we considered any relative change in MPEF signal bigger than 10% as a sign of alteration/damage of the paint. By performing the same measurements with different laser powers in different areas, we defined the upper threshold of damage and a range of laser powers providing safe measuring conditions. The application of other techniques – i.e. MPEF imaging, Raman, OM – supports and confirms the reliability of the proposed monitoring method. As an example, we report the results on Permanent Blue Light and Zinc White (Fig. 5) paints. In PBL after irradiation at 8.5 mW, the alteration was highlighted by the increase of either the maximum signal intensity in the z-profiles (a) or the background in the Raman spectrum (g), as well as in the fluorescence intensity image (c) where a light square is clearly visible. However, no alteration was observed in the OM image (d). On the other hand, the damage threshold of the Zinc White

Fig. 5. Comparison between the results obtained on Permanent Blue Light (PBL) and Zinc White (ZW) acrylic paints, irradiated with a laser power of 8.5 mW and 41.5 mW, respectively. MPEF z-profiles measured after each z-scan (a, b), fluorescence intensity images (c, e) and optical microscope images (d, f) acquired after the five z-scans. The irradiated ROI ($100 \times 100 \mu\text{m}^2$) is highlighted by the dashed square. Raman spectra (g, h) measured in the purposely damaged area (black line), in a non-irradiated area (red line) and in the irradiated area after the five scans (blue line) for the PBL (g) and ZW (h) samples.

For a more detailed characterization of the fluorescence behaviour of the paints, as well as for determining the damage threshold, we increased the number of power levels and we plotted the values of the maximum fluorescence intensity of the five MPEF profiles acquired in each area as a function of laser power. It was found that the damage threshold significantly varies depending on the chemical composition of the paints. As an example, the results obtained on Permanent Green Light and Zinc White are reported in Fig. 6 a-b and Fig. 6 c-d, where the maximum intensity is plotted as a function of laser power in the range 2-15 mW and 0-80 mW, for PGL and ZW respectively. The different colours correspond to subsequent irradiation iterations. In both cases, we observed a rather wide range of laser powers in which the maximum intensity of the five consecutive measurements remains unchanged for all the irradiated areas: within such range, the data can be fitted to a quadratic function, thus revealing a second-order nonlinear dependence of the signal, possibly related to a second-order nonlinear process – i.e. two-photon absorption or SHG. Above this range, the five values of

Fig 6. MPEF intensity changes as a function of average laser power for Permanent Green Light and Zinc White acrylic paints: plots of the maximum intensity values acquired at the first (black dots), second (red dots), third (green dots), fourth (blue dots), fifth (magenta dots) irradiation scans at each laser power in the range 2-15 mW for PGL (a) and 0-80 mW for ZW (c); the dashed rectangle highlights the range of powers that is magnified in (b) and (d); the damage threshold (light-red rectangle) is observed between 7.5 - 8 mW for PGL and 39.5 - 41.5 mW for ZW.

Finally, we observed that the range of laser power threshold identified for the acrylic paints considered in this study differed considerably depending on the setup used. Specifically, we measured a range of thresholds between 0.5 and 10 mW with the IQFR setup and a range between 10 and 45 mW with the INO setup. This discrepancy is attributed to the different illumination conditions, namely to the number of laser pulses per irradiated point. This latter parameter is therefore crucial for defining the threshold of damage for a specific material: a higher photon dose reduces the threshold of damage, as well as the laser power and the exposure time. Once defined the safe limits for the application of MPEF, cross sectional measurements can be performed in safe conditions, in good comparison with other non-invasive optical techniques, like OCT. Finally, it is

worth to underline that the proposed methodology could be applied for monitoring the damage of materials other than paints, such as varnishes, wood, parchments, and textiles.

5. Conclusions

We proposed a new methodology to establish safe measurement conditions for the application of multi-photon excitation fluorescence on painted artworks. The monitoring of laser-induced alterations was performed with a new procedure enabling the detection of photo-chemical changes inside the focal volume of the laser beam. The application of MPEF on a series of commercial acrylic paints highlighted the dependence of the emitted fluorescence intensity on materials composition and illumination conditions. Specifically, fading or enhancement of fluorescence was observed in paints characterized by similar colour but different chemical composition. Additionally, the comparison between the results obtained with two different setups revealed that the photon dose used for irradiation is a crucial parameter: the higher the dose, the lower the damage threshold – i.e. the laser power at which the fluorescence intensity starts to decrease after consecutive irradiations. Working below these limits allows for the cross-sectional imaging of paint layers by MPEF without risks of damage, while still gathering enough nonlinear optical signals at a suitable signal-to-noise ratio. This methodology for the assessment of safe MPEF measurement conditions in painting substrates is in line with procedures used in biology, where analysis of changes in intensity of fluorescence of dyes and autofluorescence are monitored. Phototoxic effects, which may precede changes of fluorescence in biological systems, could be generated also in sensitive heritage substrates, thus calling for extended research on this issue. Further analysis on different painting materials are in progress to better understand the mechanisms of damage, the relationship between the fluorescence behaviour and the chemical composition of pigments and binders, as well as the influence of laser illumination conditions on the threshold of damage.

Conflicts of Interest

There are no conflicts to declare.

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Supplementary Material

This section provides a detailed description of the method used for the analysis of the spectroscopic data.

We performed an accurate analysis of Raman spectra in order to separate the fluorescence and the Raman components of each spectrum and highlight variations in background and in Raman spectral shape.

Raman spectra were processed to remove the fluorescence signal through an automated iterative routine (Vancouver Raman Algorithm) [1]. More in detail, the spectral contribution of fluorescence was fitted to a 5th order polynomial function and subtracted from each acquired spectrum. (Fig. S1)

Fig. S1. Raman spectral analysis. a) black line: Raman spectrum of the fifth scan of PGL area irradiated with 6 mW of laser power; red line: 5th order polynomial function fit. b) Raman spectrum resulting from the subtraction of the fluorescence background.

Furthermore, we proceeded to compare the shape of the Raman spectra measured in the fiducial marked area (black line) with those acquired in the non-irradiated area (red line) and in the irradiated area after the five scans. In particular, referring to the example here shown in Fig. S2 on PGL sample, the plotted spectra correspond to those above-mentioned with the other three spectra corresponding to those acquired in the same sample using laser powers of 6 mW (blue line), 7.75 mW (green line) and 8.75 mW (magenta line).

This comparison evidences a decrease in the intensity of Raman peaks, moving from the bottom to the top of the Fig. S2, showing that the first two spectra, respectively corresponding to the non-irradiated (red line) and to the irradiated area with a safe laser power (blue line), have the same intensity and shape, while the signal intensity decreases with increasing laser power.

This automated iterating routine has been applied to all the Raman spectra acquired. Since the shape of Raman spectra does not change and their intensity and fluorescence background decrease while increasing the laser power, both contributes are shown in Fig. 4 and 5 in the paper.

Fig. S2. Comparison between Raman spectra acquired from PGL sample. Black line: Raman spectrum of the fiducial marker area; red line: Raman spectrum of the non-irradiated area; green, blue and magenta lines: Raman spectra of areas irradiated with laser power of 6 mW, 7.75 mW and 8.75 mW respectively.

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