Two photon confocal microscopy is an emerging technique in the field of laser scanning microscopy which has an inherent capability for optical sectioning in 3D. We have used this two photon confocal scanning microscopy as a nondestructive tool to study onset of corrosion of metallic substrates beneath the paint layer in-situ without removing or peeling off the paint layer. The result shows that the onset of corrosion can be studied by a combination of reflection and two-photon fluorescence modes of detection in confocal microscopy. We have used the newly developed localized spectrometer (multi-photon or confocal) to study the micro-environmental changes inside the polymeric paint coatings. We have also explored a new technique called "Optical Coherence Tomography" (OCT) for imaging through the paint coatings. In the process, we have developed a Confocal enhanced OCT (CEOST) for imaging through highly scattering media. CEOST was found to have a better S/N ratio while imaging through highly scattering coatings compared to two photon/confocal or normal OCT.
Introduction:

Two-photon confocal microscopy is an emerging technique used for three dimensional optical sectioning, mainly used in biological samples. In two-photon confocal microscopy, one uses near IR wavelength laser beam to excite a two-photon fluorophore which exhibits up-converted fluorescence emission in the visible wavelength region. Two-photon confocal laser scanning microscopy (2PCLSM) has several advantages over its single-photon counterpart. One of the obvious advantages arises from the quadratic dependence of the two-photon induced fluorescence intensity on the excitation intensity. Under proper excitation power, this property limits the fluorescence emission to the vicinity of the focal point, thus providing excellent depth resolution even without using a confocal aperture. The penetration depth of the UV or even visible excitation light can be poor in many organic materials where the linear attenuation and scattering are high. This normally limits the depth in a material that one can probe using one photon confocal laser scanning microscopy (1PCLSM). On the other hand, the near IR light has a much greater penetration in polymeric materials.

In two photon confocal microscopy, due to the large penetration depth of the excitation light (IR range), one can easily investigate the surface, the bulk, and any underlying fractures or defects of any polymer or paint coatings. With some of the new fluorophores developed at the Air Force Laboratory which change their fluorescent properties with changes in the environmental conditions, confocal microscopy would be a powerful technique to investigate the behavior of a multi-layer paint coating’s structural deformation under environmental changes like temperature changes or exposure to corrosive vapors. All studies reported here were done on polymeric paint coatings with thickness of the order of 2-5 scattering mean free path (MFP).
The main limitation of two-photon microscopy, is the fact that when the scattering is really high (maximum thickness of 5 MFP) with high pigmentation of the paint coatings the confocal principle cannot be used. The penetration in highly scattering media can be improved by adding optical coherence gating along with the multi-photon or confocal gating. So we have tried confocal enhanced optical coherence gating (which is useful up to a thickness of ~30 MFP) for imaging under highly scattering paint coatings. OCT (Optical Coherence Tomography) is another new optical imaging technique that allows high-resolution cross-sectional imaging of turbid sample. OCT is analogous to ultrasound imaging except that light waves rather than acoustic waves are used. In OCT, the interference property of light reflected from a sample provides information from the reflective boundaries and backscattering sites in the sample. Interference will occur only when the path length of the reflected light and reference light of OCT nearly match. By changing the reference path length, reflected signal from different depths of the sample can be made to interfere. Coherent signal intensity represents the backscattering or reflectance of the site.

We have also added localized Multiphoton/single photon spectroscopy capability to our confocal setup to detect the minute chemical changes occurring inside the polymeric coatings. This can be a valuable tool for the detection of any kind of spectral shifts in the material before the real onset of visible changes in the material. Thus coupling a spectrograph with TPLSM to make our own two-photon localized spectrofluorometer, we were able to acquire the two-photon excited localized fluorescence spectra.

**Two photon Confocal microscopy of paints and substrates:**

In two photon confocal microscopy, due to the large penetration depth of the excitation light (IR range), one can easily investigate the surface, the bulk, and any underlying fractures or defects of any polymer or paint coatings. We believe that with the new fluorophores with very high two photon absorption crosssection (almost two order more than the commercially available dyes) developed at the Air Force Laboratory, confocal microscopy would be a powerful technique to investigate the
behavior of a multilayer coating or paint, structural deformation under external stress and their thermal degradation and to induce and probe surface modifications.

**Multichannel confocal microscopy for study of Microstructure, Interface:**

By doping multiple layers of paint with different dyes and configuring the confocal microscope for redirecting different emission wavelengths to different photodetectors, one can probe the properties of different paint layers distinctively. For this study, the aluminium substrates were coated with three layers of epoxy paint (Hobbyepoxy enamel). The top and bottom layers were doped with two new chromophores, 2,5-bisbenzothiazole 3,4-didecycloxy thiophene (BBTDOT) and 4-[N-(2-hydroxy ethyl)-N-(methyl) amino phenyl]-4'-(6-hydroxyhexyl sulfonyl)stilbene (APSS), both of which absorb at 800 nm by two photon absorption, but fluoresce at 460 nm and 520 nm respectively. The intermediate layer was left undoped. These fluorophores were dissolved in paint at a concentration of 1% wt./wt. Fig.1 shows the vertical crosssection image of multilayer paint.

![Layer Diagram](image)

**Fig.1** Two-Photon Two Color Image of Three Layers of Paint on an Aluminum Substrate.
The images presented here were obtained with excitation from a mode-locked Ti:Sapphire laser oscillator (we have used NJA-4 from Clark-MXR Inc., our own home built laser pumped by Argon laser or Tsunami from SpectraPhysics) tuned to a center wavelength of ~800nm and producing a train of ~90 fs pulses, at a frequency of ~80-90 MHz. The average power used for imaging was around 15mW.

Using the setup described above, it was possible to image through the entire thickness of all three layers (120µm thick low pigmented paint which was equivalent to ~2 MFP). Fig.1 shows a three-dimensional image of multiple layer paint coating on an aluminum substrate. The top and bottom layers have been assigned false colors by a computer, based on the signal strength between the two detecting channels, for better visual discrimination. Such images can yield information about the layer thickness, bonding between layers, and diffusion across layers (note the gradual changes in fluorescence intensity between the adjacent layers).

![Image of multiple layer paint coating](image)

**Fig. 2**
By using multi-channel detection of two-photon fluorescence and reflection signals, it is possible to study the interfaces of multi-layer paint coatings as well as the substrate properties under the coating (Fig.1). In this case the confocal microscope was configured for observing the fluorescent emission in one channel and the reflected light in second channel. In Fig.1, the vertical cross section of a three layer coating on an uneven metal substrate with different pigment concentration is shown. The topmost layer was doped with a two-photon dye (AF-240) and the combination of fluorescence (shown as red in figure) and reflectance (shown as green in figure) clearly shows the multi-layer structure of paint coatings. Even the difference in concentration of the pigment in different layers can be visualized in the reflection mode confocal microscopy.

Fig. 3

In Fig.3, a 50 μm thick paint layer on an uneven aluminum substrate is imaged in reflection mode confocal microscopy. In this case also, the paint layer and the bottom aluminum substrate are clearly distinguishable. Similarly the reflection mode confocal and two-photon fluorescence images of the paint coating is able to show clearly the grain structure in the paint coating. Even small defects in the paint layer can be seen in fluorescence mode images.
The optical sectioning using the two photon confocal microscopy can yield the surface profile of the substrate. This can be used as a nondestructive testing tool to study the corrosion or other degradation of the metal surface below the paint coatings.

The samples used in this study consisted of single layer of epoxy paint (Polymer paint obtained from Dept. of chemical coatings, Irvine, California) on aluminium substrate. The paint layers were doped with a two photon absorbing dye (AF 240, obtained from Wright Laboratory, U. S. Air.Force), which have high two photon absorption crosssection at 800 nm and fluoresce at ~470 nm. The aluminium substrates were artificially corroded with strong acids before coating them with the paint layer.

**Fig. 5** Three dimensional reconstruction of an artificially corroded metallic substrate and paint coating imaged through the dye doped paint

Fig.5 shows the surface profiles of the artificially corroded aluminum substrate. In this negative contrast images, as one probes deeper through the polymer films, the fluorescence intensity decreases as the aluminum substrate does not fluoresce. When the focal point reaches the bottom of the deepest ridge the signal level drops to zero. In Fig.5 the boundary between the corroded region and the uncorroded region is clearly visible as the paint doped with dye has penetrated into the corroded region and give fluorescence.
Figure 6 shows the image obtained shows the image of the bottom aluminum substrate where one can monitor both the inner layers of the polymeric paint in fluorescence mode (6(a)) as well as the surface profile of the substrate in reflection (6(b)) mode.

(a)  

(b)

Fig. 6 Multichannel confocal microscopy (fluorescence mode (a) and reflection mode (b)) for studying the onset of corrosion of metallic surface beneath the paint layer:

Multichannel confocal microscopy for studying the effect of environmental conditions on the paint coatings:

Polymeric paints and coatings on metallic surface tend to degrade with time due to environmental effects (heat, moisture etc.). It is desirable to develop a method or technique by which one evaluates the degradation of paint layers without removing or peeling off the paint layers. We conducted studies for detecting the onset of corrosion of metallic substrates by using a dye which changes its properties depending on the environmental conditions. Acidic environment is a major cause for the corrosion of metals even when a protective paint coating is there. To demonstrate our technique, we used a 60 μm thick paint film coated on a metallic substrate, exposed to different levels of acidic environment. The paint film was pre-doped with a very low percentage of (0.5 % by weight) two-photon dye AF-240. AF-240 is very sensitive to the acidic environment and they get photo-bleached at a very low level of acidic
exposure. By monitoring the photo-bleaching of the dye using TPCLSM, it was possible to monitor the penetration of acidic vapor through the protective paint coating to the metallic substrate.

Fig. 7 (A) Fluorescence mode (Dye is bleached by acid)  
Fig. 7 (B) Reflection mode (Non-uniformity and change in micro-structure in paint layer is visible)

The fig.7 shows the fluorescence and reflection mode images of a paint coating after one hour exposure to acidic environment, imaged at 40μm below the surface. Here the images clearly indicate the penetration of acid vapors deep into the polymeric paint coating. In reflection mode confocal image we can see the bulging due to the damage in the paint coating. This can be better studied with the technique of localized spectrometry.

We have also tried to study the effect of temperature on paint coatings. Fig. 8 shows the effect of heating on 60μm thick paint film. The paint film was heated up to 100°C for 1 hour. The fluorescence image clearly shows the development of cracks.

Fig. 8 3D image created from a TPCLSM image of a paint film after heat treatment  
Fig. 9 3D image created from a TPCLSM image of a paint film after applying pressure
Similarly Fig. 9 shows the 3D image of another 50μm thick paint coating after applying high pressure on the coating. In both cases the changes occurring inside the film was clearly visible in 3D recreation of the TPCLSM images.

**Confocal Enhanced Optical Coherence Tomography:**

![Confocal Enhanced Optical Coherence Tomography Diagram]

**Fig. 9** Schematic diagram of OCT setup.

Figure 9 shows a schematic of the OCT system used in our experiments. A compact SLD (model C86064E, EG&G Inc.) which can output 8 mw at 850 nm with 20 nm spectral bandwidth, is used in the system. The full-width-half-maximum (FWHM) optical coherence length of the SLD is 32 μm in air. The longitudinal resolution is half of the coherence length because light travels round trip in the sample. The distribution ratio of linear polarization light from SLD input into two arms can be controlled by means of rotation of a half wave plate (HWP) to achieve maximum coherent signal. The first polarizing beam splitter (PS1) separates two orthogonally polarized components to two arms. The vertically polarized beam goes to the reference arm, the other to the sample arm. The horizontally polarized light first passes through the second polarizing beam splitter (PS2), and then the back-scattered light is reflected by PS2 because the horizontally polarized light becomes vertically polarized light after travelling through the quart wave plate (QWP) twice. Finally polarization of the two beams becomes the same
and they combine at 50% splitting mirror (SM) to get the interference. The interference signal is modulated by a 90 kHz electro-optic phase modulator in the reference arm. The signal is detected by a silicon photo-receiver (PR, Model 2031, New Focus Inc.) with a large area photodetector and electronic amplifier. Finally the signal is demodulated by a digital lock-in amplifier and collected by a computer.

Fig. 10

Fig.10 (a) and (b) shows the OCT images of paint coating and the bottom substrate. For this the paint film was prepared at a pigment:thinner ratio of 1:1 by weight. At this high loading of the pigment, confocal microscopy was unable to penetrate deeper into the paint coating. But as shown in the fig.8, the OCT was able to image the corroded area inside a 50 μm thick paint film. Fig.8 (a) shows the image of the paint surface and (b) shows the corroded metal surface below the paint coating. From this it is clear that the coherence gating used in the OCT is able to distinguish the ballistic light (light carrying information about the reflecting surface) from the scattered light in a much better way compared to confocal gating.

By combining the resolution of confocal microscope and the ability of Coherence gating to separate ballistic light from the scattered light, we have made a Confocal enhanced Optical Coherence microscope (CEOCT). For this a confocal aperture was added in front of the detector in OCT setup. We were able to image a
metallic grid embedded inside ~100 μm thick polymeric paint coating (~ 10 MFP).

Figure 11(A) shows the conventional OCT image while 11 (b) shows the same sample imaged using a CEOCT setup. It is clear that the addition of confocal aperture to the OCT did improve the image quality significantly, while imaging through a highly scattering media like pigmented paint coating.

![Image](image_url)  

(A)

![Image](image_url)  

(B)

**Fig. 11 OCT (A) and CEOCT (B) images of a metallic grid embedded inside a polymeric paint film.**

**Confocal Localized spectrometer:**

We have developed our own system of Two-photon Laser Scanning Micro-spectrofluorometer (TPLSMF) for the purpose of multi-color spectral imaging. With one-photon or two-photon confocal laser scanning microscopy, images are acquired through broad band emission filters, which cannot distinguish between small changes in the spectral profiles of the sample. To get a complete picture of the sample, their
emission spectra also have to be monitored. In order to acquire the spectral images, we have developed a confocal/Multiphoton localized spectrometer, which has the spatial resolution comparable to the confocal/Multiphoton microscope as well as a spectral resolution of ~1nm. TPLSM, TPLSMF has inherent localization ability and much less photodamage compared with one-photon system. Because excitation is limited to the focus point, TPLSMF can provide spectral resolution of the diffraction-limited spot size without any pinhole. This feature makes it very promising in studying the dynamic changes inside the living cells. In contrast, in one-photon confocal microspectrofluorometry, researchers have to reduce the aperture to get localized spectra, which is in fact a tradeoff between signal levels and spatial resolution. Therefore, it is hard to reach the theoretical spatial resolution of using one-photon confocal microspectrofluorometer.

Spectrograph consists of a fiber (1 mm, multi-mode) coupled monochromator (Holoscope from Keiser Inc.), equipped with a cooled CCD (Princeton Instruments) as detector. The Setup for Two-photon Laser Scanning Microspectrofluorometry (TPLSMF) is shown as Figure 12.

Fig. 12 Schematic diagram of the confocal/Multiphoton localized spectrometer and micro-transmission spectrometer. This fiber coupled localized spectrometer was built upon the existing confocal microscope
The fluorescence acquired under the microscope was directed to a fiber by a dichoric mirror without passing any filters inside the scanhead. An absorption filter in the IR range was used inside the spectrograph to cut off the excitation lines from laser.

Fig. 13 shows an image and spectra taken using this versatile confocal microscope and localized spectrometer of a polymeric paint film (at a depth of ~50 μm from surface, i.e., ~2 times the MFP of the paint film.). The change in fluorescence as well as the spectrally resolved imaging clearly shows the penetration of acidic...

Fig. 13 Polymeric paint coatings doped with two-photon chromophore AF-240, was made on aluminum substrates jept in acidic environment. Two-Photon confocal spectrally resolved images and localized spectra taken at two different points are shown here. Spectra are taken from an area of the size ~ 10 μm X 10μm.
environment into the film. This is a potential tool to detect the onset of corrosion due to acidic or other environmental changes.

Conclusion:

Our study demonstrates that confocal microscopy can be used as a tool for nondestructive evaluation of multilayer coatings and inspections of any corrosion on the underlying surface without removing or peeling off the paint layer. We were able to non-destructively study the onset of damages in paint coatings using confocal microscopy (two-photon fluorescence and reflection mode). We have also shown that the new technique of OCT as a potential candidate for non-destructive evaluation of paint coatings. So with a combination of confocal/Multiphoton gated imaging, coherence gated imaging and the study of the change in spectral profile using a confocal/Multiphoton localized spectrometer, one can study the effect of change in environmental conditions on a pigmented polymeric paint coating, or the onset of corrosion of a metallic substrate through the coating.