

## SHORT TECHNICAL NOTE

# Destruction of polymer growth substrates for cell cultures in two-photon microscopy

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### Summary

The choice of the growth substrate for cell cultures used in fluorescence microscopy is guided by several factors including the type of cells studied and the type of microscopy used. Usually, cells can be cultured on either polymer or glass substrates. One type of polymer, termed Aclar, presents several attractive features: the adhesive properties are better than those of glass, the optical properties are comparable to those of glass, it is biochemically inert, unbreakable, flexible and has a high surface tension, convenient for seeding cells on the cover slip. However, here we show that when imaging with two-photon microscopy, which is based on a femtosecond pulsed laser source, local damage of the Aclar substrate occurs, starting at an average intensity of  $10^5 \text{ W cm}^{-2}$  at the focal point and for exposure times insufficient to cause cell damage. This leads to the appearance of gas bubbles on cultures plated on Aclar cover slips, which perturb the imaging. By contrast, this phenomenon does not occur on borosilicate cover slips, probably because of their different physical (thermal conductivity, absorbance, melting point) and material homogeneity properties. Thus, for cell culture applications using pulsed lasers with high intensities, the use of glass is preferable to Aclar. The results also reveal that substrates can be more susceptible to thermal damage than the cells themselves.

### Introduction

The choice of growth substrates for cell cultures is of critical importance for cellular imaging studies (Kleinfeld, 1988). One

significant challenge in establishing cell cultures is that most cells require attachment to grow and/or proliferate. For many cell types, a charged hydrophilic surface is necessary to ensure a good adhesion and growth, thus glass (which is naturally negatively charged) or treated plastic (Permanox, polystyrene) are well-suited. However, certain types of cells, such as neurones, do not adhere sufficiently to these substrates. In that case, glass or plastic surface must be treated to improve their adhesive properties: an adequate surface chemistry for neurone attachment and growth is either obtained by adding poly-D-lysine on the surface or by chemically modifying the surface by adding amines (Letourneau, 1975; Kleinfeld *et al.*, 1988; Zheng *et al.*, 1994; Banker, 1998).

The choice of growth substrate also depends upon the biological application. Generally, for applications such as fluorescence microscopy, a cover glass is preferred to a plastic cover slip because glass provides superior optical properties, is generally less autofluorescent in the UV/blue range and is more resistant to solvents. Furthermore, certain types of plastic have birefringent properties that can degrade image quality, especially for differential interference contrast.

However, it is known that the application of Aclar fluoropolymer films (Honeywell Inc., Pottsville, PA) provides several advantages compared to glass and considerably simplifies the preparation of cultured cells for all types of microscopy, including light microscopy, fluorescence microscopy, scanning electron microscopy and transmission electron microscopy (Kingsley & Cole, 1988). These films are thermoplastic made from fluorinated-chlorinated resin (chlorotrifluoroethylene). They have similar optical properties to glass (no birefringence, high transmission properties in the visible and near infra-red), are as thin as cover glass, flexible, unbreakable, less expensive, have higher adhesive properties for neurones and a higher surface tension

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than glass. Aclar can be easily manipulated and cut to prepare custom-made sizes of cover slips. For high resolution imaging of cultured living cells, using for instance 63× or 100× oil immersion objectives on an inverted microscope, it is necessary to modify Petri dishes by boring a hole in the centre and gluing a cover slip with sylgard to cover the hole. To perform immunocytochemistry on cells grown on these cover slips, one can easily peel off the flexible Aclar cover slips in order to mount on slides. For applications using electron microscopy, Aclar cover slips are more appropriate than glass, because the material can be cut easily with a microtome. It has several advantages including flexibility and good adhesive properties and does not suffer from the limitations of other commonly used polymer substrates. Thus, in several respects, Aclar cover slips are a preferable substrate to glass for cell culture combined with multiple microscopy applications.

Two-photon laser scanning fluorescence microscopy has become one of the latest powerful techniques for in-depth 3D imaging of living cells. It has high spatial and temporal resolutions and the region of photodamage and photobleaching is limited to the focal volume (Konig, 2000). However, while imaging neurones grown on Aclar cover slips with two-photon microscopy, we observed that above a certain pulsed laser energy threshold, gas bubbles appeared, precluding further imaging. By contrast, this phenomenon never appeared when cultures were made on a glass substrate. We determined that these bubbles came from the local absorption of the pulsed laser energy by the Aclar substrate. This heating phenomenon should be taken into consideration in the design of polymer substitutes for glass substrates for nonlinear imaging approaches.

## Materials and methods

### Neurone cultures

Neonatal hippocampal neurones were grown at high density (~2000 cells mm<sup>-2</sup>) in serum-free media based on a protocol developed by Brewer *et al.* (1993) and then modified by Hudmon *et al.* (2005). Dissociated cells were plated by adding three small (~50 µL) drops of cell suspension in the centre of the cover slips. Once the cells had attached (~10–15 min later), the cells were washed once with growth media and then 0.5 mL fresh media was added. The culture was maintained at 37 °C and aerated with 5% CO<sub>2</sub>. Three-week-old neurone cultures were transfected with green fluorescence protein cDNA using lipofectamine 2000 (Invitrogen Canada Inc., Burlington, Canada), according to the manufacturer's protocol, except that the lipid-DNA mixture was incubated for only 5 h. The cells were kept for 12–36 h prior to imaging. The neurones were cultured on 120-µm-thick cover slips (Aclar 22C or Aclar 33C) or on circular 150-µm-thick cover glass (Fisherbrand – microscope cover glass 12-545-86 25CIR-1D, Fisher Scientific Canada, Nepean, Canada). Aclar and glass cover slips were thoroughly cleaned in ethanol, water and 10%

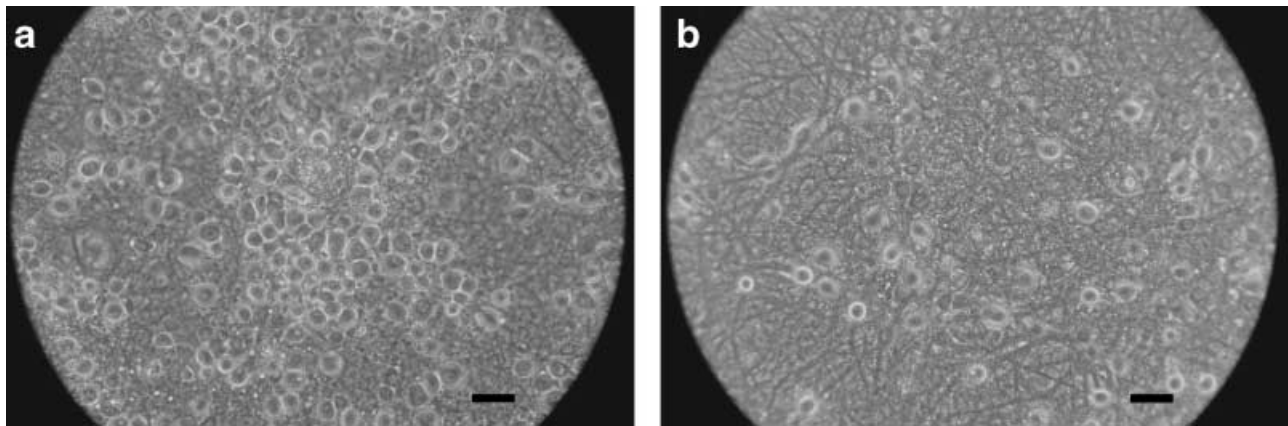
nitric acid to ensure the removal of surface impurities. Poly D-lysine (250 µg mL<sup>-1</sup>, BD Biosciences Canada, Mississauga, Canada) was used to coat the cover slips. It was dissolved in 100 mM borate buffer at pH 8.5 and the surface being treated was covered completely by this solution for 3 h. During observation, cultures were maintained in a physiological medium (142 mM NaCl, 10 mM HEPES, 2.5 mM KCl, 10 mM glucose, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>).

### Two-photon imaging

The two-photon microscope system used in the experiments consisted of a mode-locked Ti: sapphire laser (Coherent MIRA 900B, Coherent Inc., Santa Clara, CA) pumped with an Ar-ion laser (Coherent INNOVA 310C) coupled with a Zeiss confocal laser-scanning microscope (LSM 510, Carl Zeiss Canada Ltd., Toronto, Canada). The Ti: sapphire laser was operated at a repetition rate of 76 MHz with an average output power of 1 W, a pulse width of 128 fs and a wavelength of 820 nm. The initial pulse duration was characterized using a commercial autocorrelator Carpe (APE/Inrad Inc., Northvale, NJ); the spectrum and central wavelength was measured by a laser spectrum analyser (IST-Rees, Imaging & Sensing Technology Corporation, Horseheads, NY). An acousto-optical modulator was used to control the laser power at the sample plane. The mean laser power was measured after the microscope objective in air by a Gentec powermeter (model SOLO P/E, Gentec, Québec, Canada). The power out of the objective lens was varied between 1 and 20 mW. The excitation light was focused by a 63×/0.8 water immersion objective (Achromplan, Zeiss). The average intensity at the focal point was estimated by dividing the measured average power in air after the objective by the area of the measured point spread function in water. The point spread function was measured with fluorescent microspheres of 500 nm radius (Molecular Probes, Eugene, OR) excited by the Ti: sapphire laser. The radius of the point spread function was 0.7 µm. The energy fluxes measurements in air deviated from the real energy fluxes (a water immersion lens was used) because dispersion and absorption are different in water and air. The ratio of energy fluxes estimated in air and in water, taking into account the different focal distance in 'glass-air' and 'glass-water' media, is probably one order of magnitude (Born & Wolf, 1999).

The linear transmission spectra (spectral range from 200 to 1000 nm) of Aclar films and cover glass were measured using a spectrometer (Spectrometer UV-VIS-NIR, CARY 500, Varian Canada Inc., Mississauga, Canada). To test the birefringence properties of these films, they were placed between two crossed polarizers and illuminated by a linear polarized laser. The transmitted laser power was measured with the previous powermeter.

The scanning and image acquisition were controlled with the Zeiss LSM software. Single neurones grown either on Aclar or glass cover slips were imaged using a stack of scans



**Fig. 1.** Hippocampal neurone cultures grown on different growth substrates. Phase contrast images of neurones cultured on (a) an Aclar cover slip and (b) cover glass. Note the higher density of cells on Aclar compared to glass. Scale bar = 50  $\mu\text{m}$ .

of 20  $\mu\text{m}$  depth (10  $\mu\text{m}$  above and below the centre of the cell in the  $z$ -axis) with a 2.24  $\mu\text{s}$  pixel time, a 1.9 s scan time and 512  $\times$  512 resolution; the total acquisition time was 30 s. The distance between each scanning plane of the stack was 2  $\mu\text{m}$ , thus 11 slices were recorded per stack.

## Results

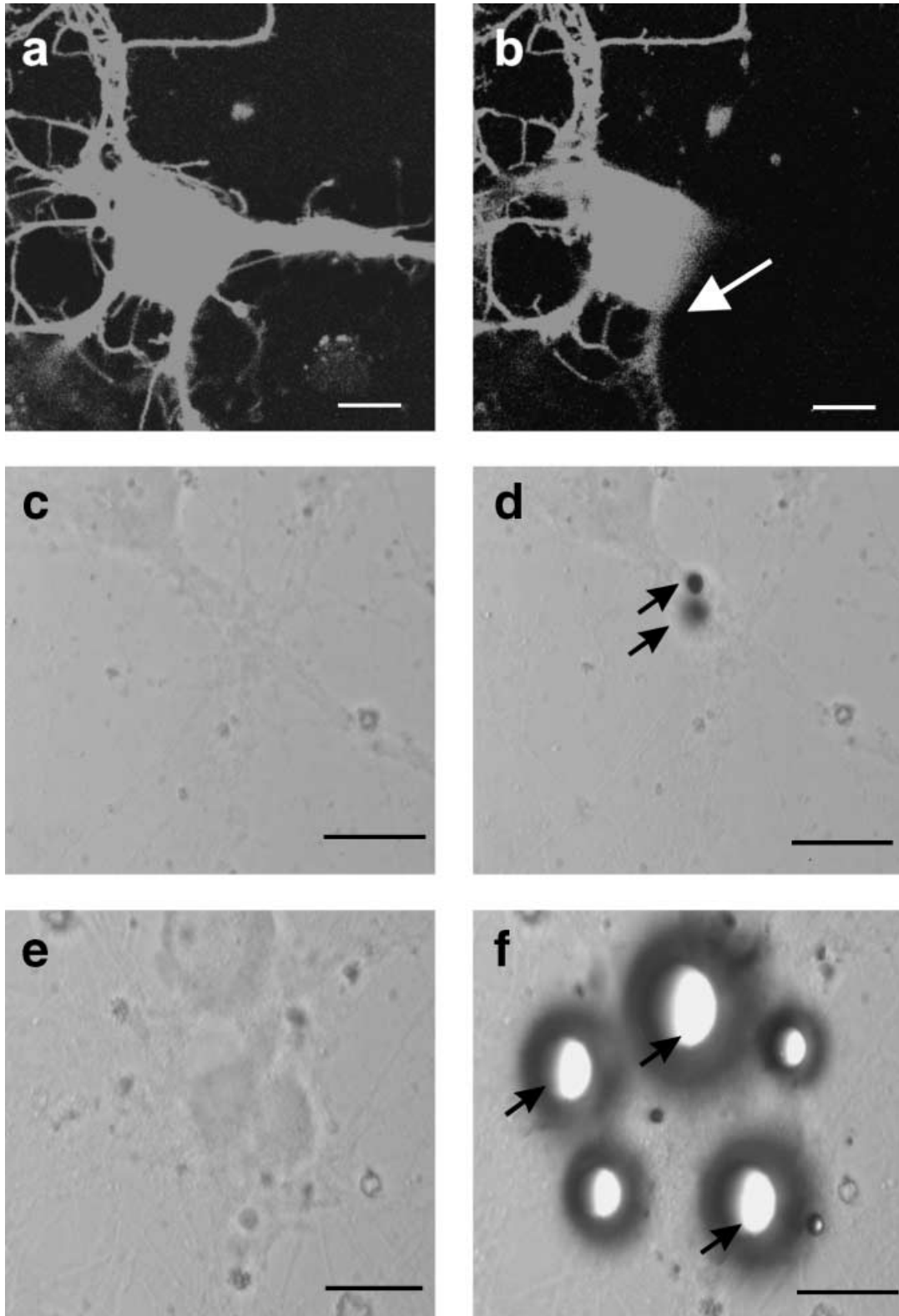
We set out to perform two-photon imaging on cultured hippocampal neurones transfected with green fluorescence protein. We plated neurones on either Aclar film or borosilicate cover glass. Figure 1 shows examples of hippocampal cultures grown on both substrates. We observed that the neurones were at a higher density on the Aclar substrate mainly because the surface tension is higher for Aclar than for glass, causing cells to remain more concentrated in the centre of the Aclar cover slip (Fig. 1a). On cover glass, cells tended to spread out from the centre leading to a lower density of cells on the substrate (Fig. 1b). This feature of Aclar might be advantageous for specific applications such as producing high density of cells in a small area. Other advantages of Aclar film compared to glass are that it can be cut easily without shattering, is unbreakable and considerably less expensive.

During two-photon imaging of cells grown on Aclar cover slips, we noticed that above a mean laser power threshold of 2 mW (measured after the objective), some bubbles appeared on the cover slips (Fig. 2f), even though the exposure time (11 slices recorded, 1.9 s scan time per slice) was usually insufficient to cause morphological changes to neurones. Figure 2(d,f) shows that bubble size grows with increasing laser power, eventually preventing proper imaging of the cells. Figure 2(a,b) illustrates the distortion of a neurone whilst imaging in two-photon mode. Bubbles had already appeared during the acquisition of the first stack of images. The number of bubbles increased with laser power (Fig. 2d,f).

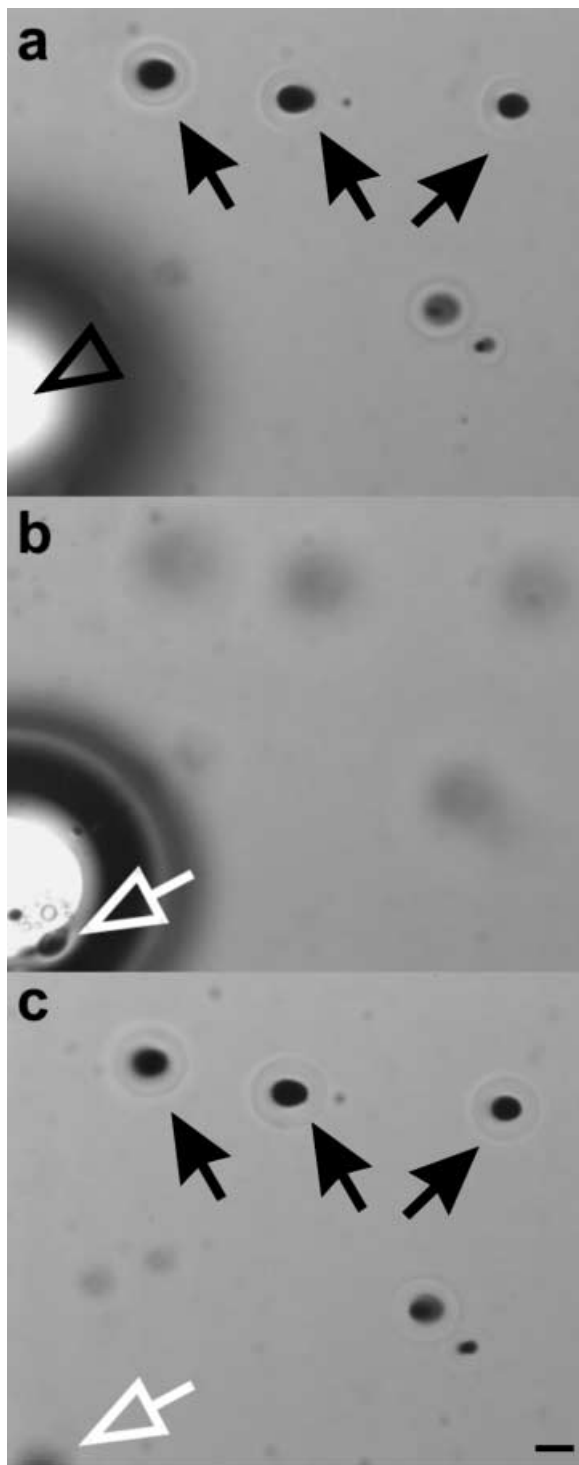
With neurones cultured on cover glass, no bubbles were observed even at the highest laser power available (40 mW after the objective, equivalent to energy per pulse of 0.5 nJ). These results suggested that the origin of the bubbles was an interaction between the laser and the Aclar film, as no bubbles appeared on the cover glass.

To determine the origin of these bubbles, we carried out a series of tests. We first examined whether the occurrences of bubbles were due to an interaction with the culture media or the cells. The bubbles also occurred with other types of cultures, such as Human Embryonic Kidney cells (HEK 293). We replaced the culture medium (neurobasal A with HEPES) with distilled water and observed no effect on the incidence of bubbles on Aclar cover slips. We next irradiated both types of cover slips coated with poly-D-lysine but without cells. Bubbles appeared on the Aclar cover slips but not on the cover glass, suggesting that the appearance of bubbles was due to interaction of the pulsed laser with the Aclar material itself. We next carried out a series of experiments to characterize the laser interaction with uncoated Aclar and glass cover slips. To reproduce the neurone 3D imaging conditions (stack of 20  $\mu\text{m}$  in thickness), the different substrates were scanned with a stack of 11 scans, 2  $\mu\text{m}$  apart (10  $\mu\text{m}$  above and below the substrate/media interface) whilst varying the laser power.

Morphological changes and bubbles on plastic substrate occurred starting at a 2 mW average laser power. Figure 3(a) illustrates a bubble appearing on the left side. In Fig. 3(b), some structural changes in the plastic were visible through the bubble at a different focal depth. This suggested that the origin of these bubbles may be due to structural changes in the Aclar. Such damage may constitute absorption sites of laser energy, creating local heat production and the occurrence of bubbles. To test this, cover slips were washed with water and ethanol and examined under phase contrast microscopy. Thorough washing did not alter the



**Fig. 2.** Neurone images grown on an Aclar cover slip—appearance of bubbles in two-photon microscopy. (a, b) Appearance of bubbles during imaging of a neurone with a laser power of 8 mW (stack of 11 scans 2  $\mu\text{m}$  apart). (b) The distortion of the neurone is indicated by the white arrow. (c, e) Phase contrast images of neurone substrate before two-photon imaging. (d, f) Phase contrast images after 11 scans (2  $\mu\text{m}$  apart) in two-photon mode at (d) 4 W and (f) 12 W. Black arrows point to bubbles. Scale bars = 10  $\mu\text{m}$ .



**Fig. 3.** Characterization of the interaction of the pulse laser with an Aclar cover slip (average power of 25 mW) and cover glass. Phase contrast photomicrographs. (a) A gas bubble is visible on the left side (arrowhead). Few structural changes are visible (black arrows). Note the rings around these structural changes suggesting the existence of regions with a different refractive index. (b) A different focal depth reveals that a bubble appeared on a damage site in plastic (outline arrow). (c) After washing the cover slip, the bubbles disappeared, but damage sites in Aclar remained (black and outline arrows).

appearance of the structural damage in Aclar (Fig. 3c), but the bubbles disappeared, suggesting that the latter consisted of gas.

Structural damage to the Aclar was confirmed by scanning electron microscopy (Fig. 4). The morphological changes were spherical in shape and appeared as randomly distributed protrusions (Fig. 4a,b). By contrast to Aclar, neither bubbles nor visible morphological changes on glass substrate were induced by the laser (Fig. 4c).

We then determined the laser intensity threshold at which the protrusions occurred in pulsed mode. We scanned cover slips as stacks of optical sections, 2  $\mu\text{m}$  apart, for 30 s. These scans were repeated 20 times at different positions on the cover slip. Figure 5(a) shows the protrusion appearance probability, which was defined by the frequency of occurrence of protrusions over 20 scans. The threshold for appearance of protrusions was identical for both thermoplastic materials (Aclar 22C and 33C) and corresponded to a mean power after the objective of 2 mW (equal to energy per pulse of 0.025 nJ and an average intensity of  $10^5 \text{ W cm}^{-2}$ ). Beyond this threshold, the size of the protrusions was greater than 1  $\mu\text{m}$  (Fig. 5b) and increased linearly with power. On average, the total number of protrusions was 5-fold greater on Aclar 33C than on Aclar 22C and increased linearly with laser power. No protrusion appeared on the cover glass at powers up to 35 mW.

The observed differences between the substrates could be due to their optical properties. We thus examined their birefringence properties, determined the refraction indices and measured linear transmission spectra of these materials. All substrates showed no birefringence properties at all. The refraction indices were: cover glass  $-1.522$ ; Aclar 22C  $-1.653$ ; Aclar 33C  $-1.652$ . Figure 6 shows the linear transmission spectra of Aclar films and cover glass. In the visible and in the near infrared ranges the transmission of both Aclar substrates was 2% higher than that of glass. Between 200 and 300 nm, glass was absorbing whereas Aclar transmitted the light as observed by Kingsley *et al.* (1988). At a wavelength of 273 nm, Aclar 22C and Aclar 33C had a transmission of 88% and 72%, respectively. For the spectral range 300–350 nm, the transmission of Aclar 22C was 4% higher than that of glass. Thus, in terms of optical properties, Aclar represents a good substitute for glass for cell cultures and fluorescence microscopy.

Finally, we tested the absorption process in continuous mode and determined that irradiation of the cover slip surface with the Ti: sapphire laser in continuous mode (at the same average intensity at the focal point  $10^5 \text{ W cm}^{-2}$  as in the mode-locked regime) also induced damage to the Aclar surface.

## Discussion

The results revealed that the origin of the bubbles on Aclar cover slips following laser scanning excitation with a pulsed laser was an interaction between the laser beam and the polymer

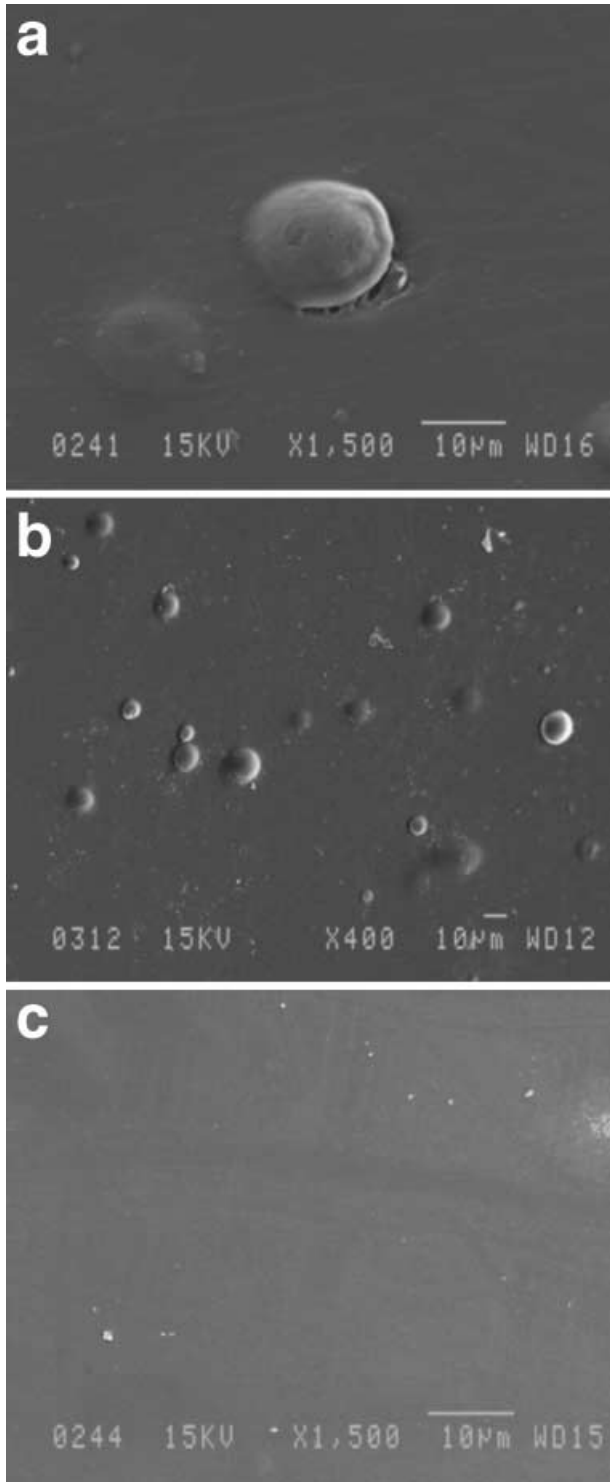


Fig. 4. Scanning electron micrographs of samples irradiated with pulse laser (average power of 25 mW). (a, b) Aclar; the damage sites on Aclar appear to be protrusions. (c) Glass; no protrusion was observed on glass.

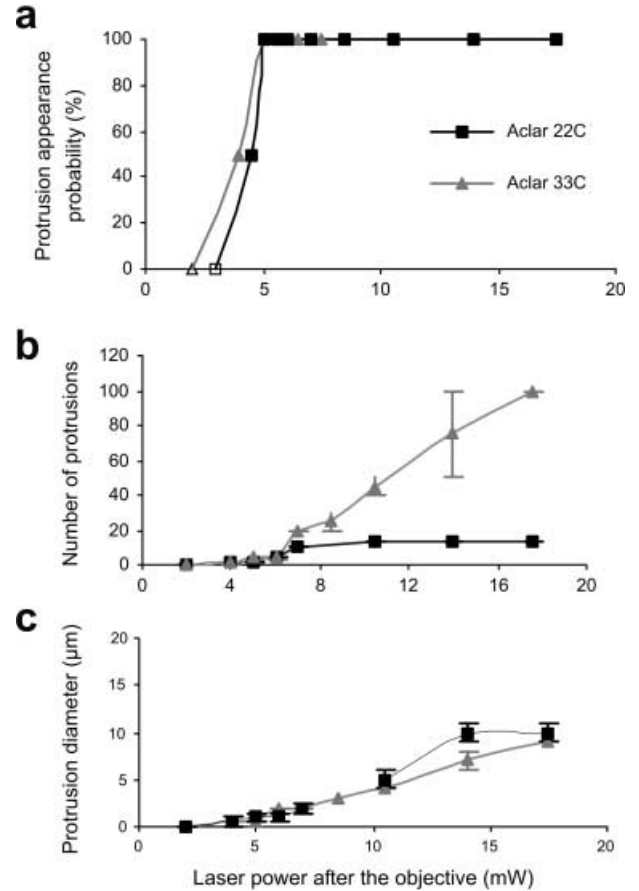


Fig. 5. Effect of laser power on occurrence of protrusions on Aclar cover slips. Aclar cover slips were scanned in two-photon mode (10 stacks, 2 µm apart). (a) Protrusion appearance probability versus laser power after the objective. (b) Protrusion number versus laser power after the objective. (c) Protrusion diameter versus laser power after the objective.

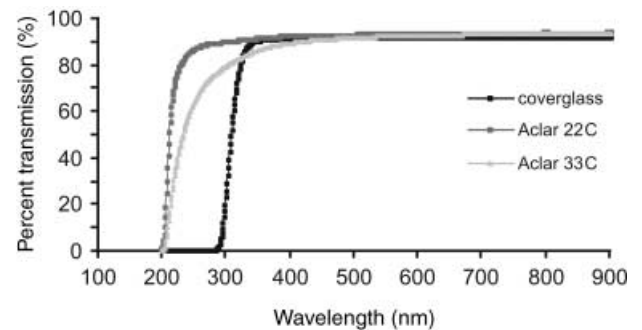


Fig. 6. Transmission spectra of Aclar and glass.

growth substrate. Above an average laser power threshold (after the objective) of 2 mW (corresponding to an average intensity at the focal point of  $10^5 \text{ W cm}^{-2}$  and a peak intensity of  $2 \cdot 10^{10} \text{ W cm}^{-2}$ ), some structural changes appeared on the Aclar surface in random locations. This laser power value is

within a typical range of power used in two-photon microscopy for live cells in culture (Konig, 2000). The gas bubbles seem to arise from these structural changes. Some of the laser energy must therefore have been absorbed by the Aclar. Nevertheless, when imaging cells at or above this threshold intensity, cell damage does not appear systematically. The difference arises from the loss of energy due to absorption and diffusion properties of the biological tissue and from the fact that the laser focus is not always restricted to the substrate surface during cell imaging.

Typically, the imaging in scanning mode corresponds to a multishot interaction. As the time between pulses (13 ns) is smaller than the diffusion time for the energy to go beyond the focal volume (20  $\mu\text{s}$ , calculated knowing the thermal conductivity and heat diffusivity  $10^{-3} \text{ W cm}^{-2}$  (Dyer, 2003)), the laser energy can be deposited in the substrate more rapidly than it can leave the focal volume by thermal diffusion. In this case, the accumulation of heat and the creation of defects from shot to shot occurring in the interaction volume can allow the melting point of the polymer (206 °C) to be reached (Ladieu *et al.*, 2002; Ashkenasi *et al.*, 2003). Consistent with this, the size of protrusions increased with laser power and their size was larger than the diameter of the focal volume (1  $\mu\text{m}^3$ ) suggesting that damage is linked to thermal diffusion. Furthermore, a series of rings surrounding the damage sites was observed. These rings probably represent changes in the refractive index. They may be created by the changes in physical and chemical properties of the polymer because of heat diffusion.

By contrast, borosilicate glass has a 5-fold greater thermal conductivity (16–20  $\times 10^4 \text{ cal}\cdot\text{cm cm}^{-2}\cdot\text{s}^{-1}\cdot\text{°C}$  for glass vs. 5.3–4.7  $\times 10^4 \text{ cal}\cdot\text{cm cm}^{-2}\cdot\text{s}^{-1}\cdot\text{°C}$  for Aclar) and a 10-fold higher melting point than Aclar (1400–1600 °C for borosilicate vs. 190–206 °C for Aclar) (Carslaw & Jaeger, 1959; Weast, 1989–90). Thus, glass not only has a higher melting point, it also has better heat dissipation characteristics, explaining the absence of damage to the glass. In addition, previous studies on borosilicate glass indicate that the optical breakdown for this material is of the order of  $10^{13} \text{ W cm}^{-2}$  (Schaffer *et al.*, 2001a,b, 2003). In our experiments, the laser intensity at the focal volume (between  $10^{10}$  and  $10^{11} \text{ W cm}^{-2}$ ) was insufficient to create cracks in or melt the glass.

One hypothesis to explain the convex shape of the structural damage (protrusions) in Aclar is that the damage was a photo-mechanical process. As the heating time was shorter than the characteristic time of mechanical relaxation, substrate heating may have taken place at nearly constant volume, leading to a rise in pressure in the irradiated volume. At the Aclar surface, the pressure gradient leads to forces driving the acceleration of the top layers in the direction normal to the surface (Zhigilei, 1997). Local heating can also explain the presence of gas bubbles at the level of damage sites, probably because the rise in temperature caused desolubilization of oxygen or local vaporization of water.

The swelling behaviour on Aclar may also be explained by the fragmentation of the polymer molecular chain and the recombination of molecules giving rise to gas formation (chlorine or fluorine) as observed previously during laser ablation studies on other polymers such as Polymethyl methacrylate (PMMA), Polytetrafluoroethylene (PTFE) (Himmelbauer *et al.*, 1996; Baudach *et al.*, 2000; Serafetinides *et al.*, 2001; Yingling, 2001).

Whatever the hypothesis concerning the origin of the structural changes on Aclar, the laser energy must have been absorbed by the Aclar. The distribution of damage spots on the Aclar surface was random. Aclar film contains 16 randomly distributed chemicals, according to the manufacturer's chemical data sheet, and these could absorb the laser energy. The presence of inhomogeneities introduced during manufacturing processes, and strains imposed while making the films suggests that there may be microsites of higher absorption (Neidlinger, 1987; Dusek & Duskova, 2000).

There was a 5-fold difference between the number of structural changes in Aclar 33C and Aclar 22C. The experiment with the continuous laser at the same average intensity as in the pulsed mode shows that the absorption process is partially linear. However, in pulsed mode, multiphoton absorption processes may occur. Figure 6 shows that, at a wavelength  $\lambda = 273 \text{ nm}$  (corresponding to the energy of three photons at a wavelength  $\lambda = 820 \text{ nm}$ ), Aclar films have significantly different transmission values (88% for Aclar 22C vs. 72% for Aclar 33C). This absorption difference (more than twice as large for Aclar 33C than Aclar 22C) may explain the higher propensity for damage with Aclar 33C. Another indication of the possible multiphoton absorption process is the nonlinear dependence of the curve for Aclar 33C.

In conclusion, although Aclar presents several attractive features (biochemically inert, unbreakable, flexible, high surface tension and comparable optical properties as glass), its physical and chemical properties (chemical composition, thermal conductivity and melting point) may preclude its usability for high power laser imaging applications. The latter parameters should therefore be taken into consideration in the design of polymer substitutes for glass substrates for nonlinear imaging approaches.

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