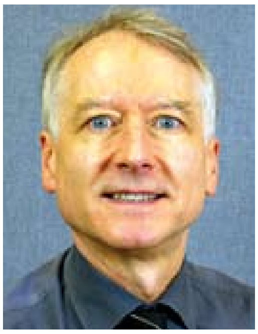
Apple-green birefringence?



Professor Alexander J Howie

Editor's introduction: Amyloid disease, or amyloidosis, is a rare condition where abnormal proteins accumulate in a patient's organs and tissues. There are several types that cause different clinical syndromes. The only way to make a definitive diagnosis is to take a biopsy and stain the tissue sample with Congo red, a diazo dye first synthesized in 1883. Under polarised light it shines not red but a greenish hue termed, classically, "apple-green birefringence". Even a microbiologist like me remembers the phrase from my student days. But I had never seen it demonstrated until I reviewed the images to accompany this article. "Apple-green" does seem to me to describe the colour nicely – but it turns out it is much more complex than that. Why does the colour happen at all? What's going on? In this in-depth article Alexander Howie and Douglas Brewer explain it all.

Every medical student is taught that amyloid stained by Congo red shows apple-green birefringence in polarised light. Like many well-known 'facts', this is not quite right.

Figure 1 shows amyloid stained by Congo red, examined between crossed polariser and analyser. Amyloid appears bright and coloured against the dark background. This is the appearance usually seen in practice, and is typical of many published figures said to show apple-green birefringence. Is there more than one colour? Which colour is apple-green?

When the slide is rotated, the colours exchange positions (Figure 2). When the polariser or analyser is rotated, virtually any colour can be produced (Figures 3 and 4). How is all this explained?

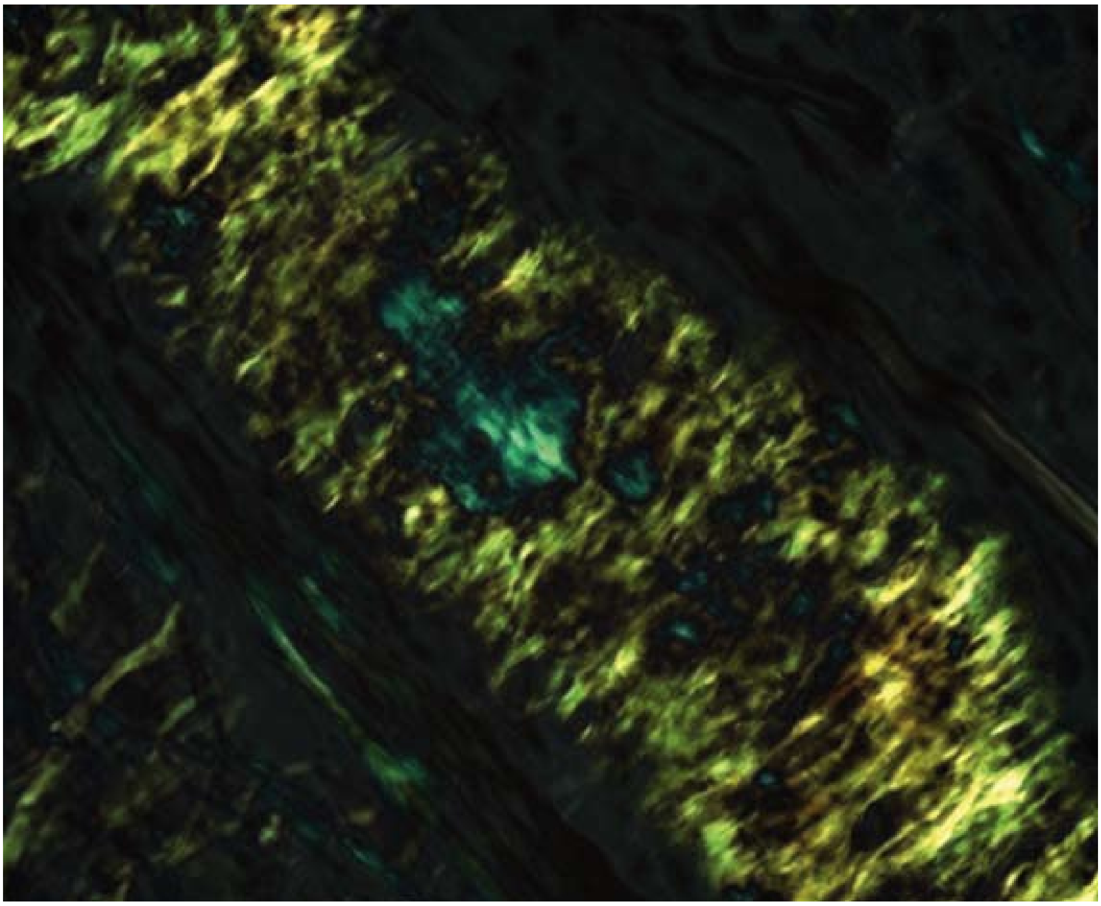


Figure 1
Congo red-stained amyloid between crossed polariser and analyser

How birefringence explains the brightness

Light travels more slowly through a transparent material than through air. The refractive index is the ratio of the velocity of light in air to that in the material. The higher the refractive index, the slower the transmission. Some materials transmit light polarised in one plane at a different velocity from light polarised at right angles. These have two extremes of refractive index, and are birefringent.

Birefringent materials such as starch particles appear bright when examined between crossed polariser and analyser (Figure 5). A polarised light wave splits into two vectors in these materials, one passing through the axis with the higher refractive index, or slow axis, and the other through the fast axis.

When the vectors reach air again, they recombine into one wave, but one vector has been retarded behind the other. Instead of vibrating only in one plane, which is linear polarisation, the tip of the combined wave now traces out an ellipse. This elliptical polarisation means that some light is in the plane of the crossed analyser, and can be transmitted, to make the materials appear bright against the dark background (Figure 6).

This only occurs if the slow and fast axes are not parallel and perpendicular to the polarised light, when waves cannot give vectors in the two axes and no birefringent effects are possible. This explains the 'Maltese cross' appearance of some materials, when one axis runs radially and the other circularly.

Congo red molecules are orientated on amyloid fibrils. This makes the dye birefringent and allows it to produce elliptically polarised light.

How birefringence partly explains the colours

Between crossed polariser and analyser, materials such as starch that appear bright white, without any colour, have the same birefringence at every wavelength of light. To appear coloured in these conditions, a material transmits wavelengths unequally. Congo red attached to amyloid appears coloured. One explanation is that its birefringence varies with wavelength.

Congo red in unpolarised light has peak absorption of wavelengths intermediate between blue and green, and appears red. To appear green, a material absorbs light at both ends of the spectrum, in the violet and red. Absorption of violet alone gives yellow, and absorption of red gives blue. Green is a mixture of blue and yellow. How can Congo red, with its peak absorption in the blue/green, absorb in the violet and red, to give green?

The answer is that the refractive index of a light-absorbing material is not constant at every wavelength, but changes dramatically around an absorption peak. The index sinks to a minimum on the shortwave side, and jumps to a maximum on the longwave side. This is anomalous dispersion of the refractive index (Figure 7).

Congo red molecules only absorb light polarised parallel to their orientation on amyloid fibrils. Consequently, the long axis of Congo red-stained amyloid shows anomalous dispersion, while the axis at right angles has an almost constant refractive index. The birefringence is the difference between the two refractive indices and varies with wavelength, reaching its greatest values around the absorption peak, in the blue/green. This should give most elliptical polarisation and so most transmission of light in the blue/green.

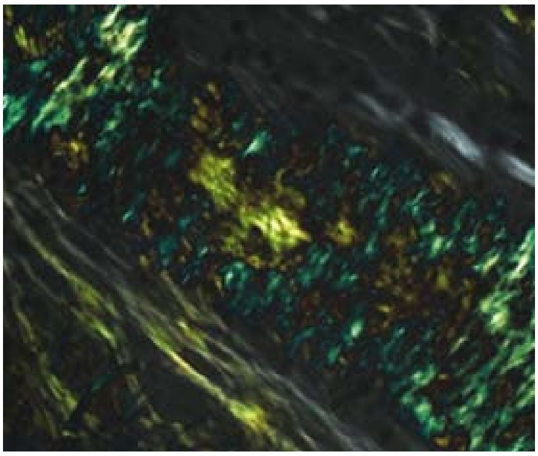


Figure 2
The section in Figure 1 rotated 90°, with the image presented in the same orientation

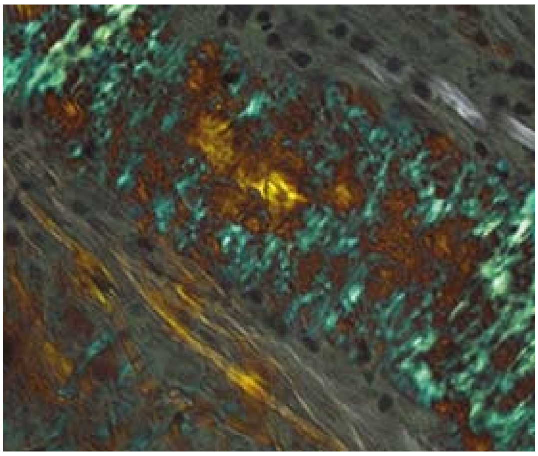


Figure 3
The section with polariser and analyser slightly uncrossed

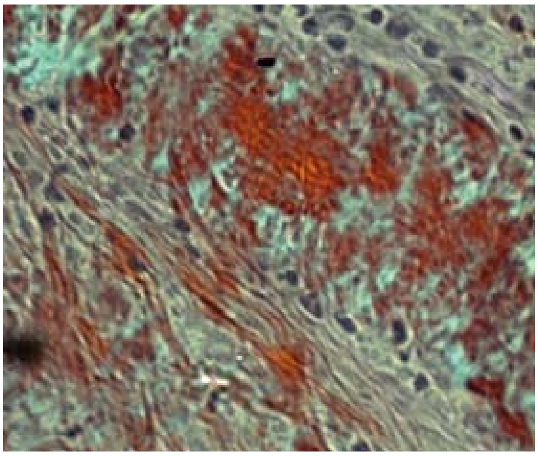


Figure 4
The section with further uncrossing of polariser and analyser

How birefringence is not the whole answer

A paradox is that Congo red has its maximum absorption in the blue/green, which should reduce transmission of those wavelengths, and yet has its maximum birefringence in the blue/green, which should give maximum transmission. In reality, these two processes do not cancel each other, because they are not mirror images. When they combine, the spectrum passed through a crossed analyser is the net outcome, with least transmission in the violet and red. This gives green in ideal conditions.

Figure 5
A starch granule between crossed polariser and analyser, with a Maltese cross appearance

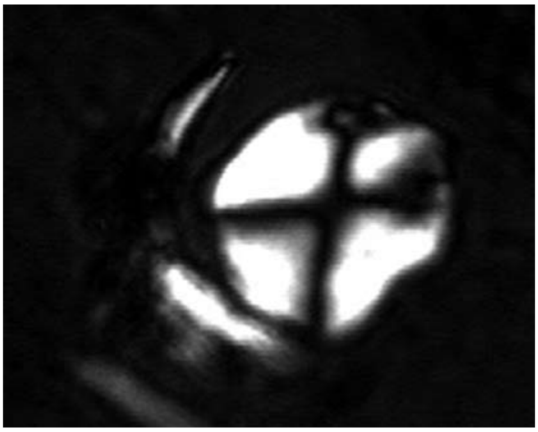


Figure 6
A birefringent object with its axes at 45° between crossed polariser and analyser, interacting with linearly polarised light to produce elliptically polarised light

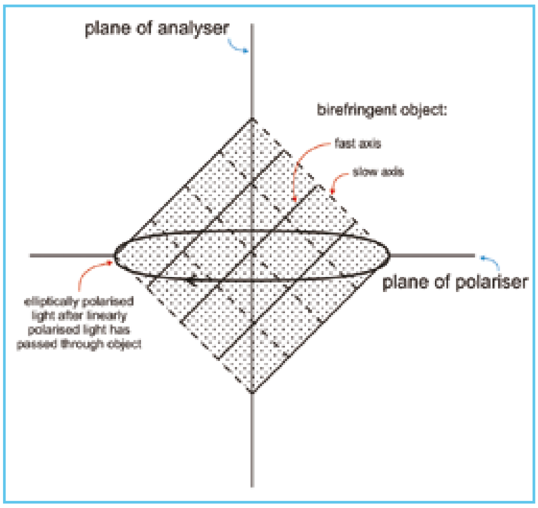
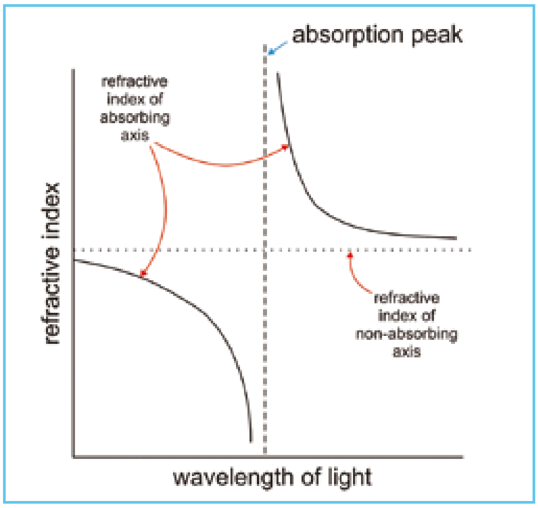


Figure 7
Anomalous dispersion of the refractive index around an absorption peak



How pure green is not usually seen

In practice, pathologists hardly ever see a pure, single green colour. The reason depends on anomalous dispersion. On the shortwave side of the absorption peak of Congo red, the refractive index of the absorbing axis, which is parallel to amyloid fibrils, is lower than that of the non-absorbing axis at right angles. This is called negative birefringence, when the higher refractive index, or slow axis, is at right angles to the long axis of a material. On the longwave side, the birefringence becomes positive, because the slow axis is now parallel to amyloid fibrils.

Negative and positive birefringence both give elliptically polarised light, but the direction of rotation of the ellipses is opposite. Combined with effects of absorption, negative birefringence transmits blue and positive transmits yellow. Together these appear green.

Any extra birefringence in the light path can introduce elliptical polarisation, which may cancel elliptical polarisation produced by Congo red, converting it back to linearly polarised light that cannot pass the analyser. This occurs if the ellipses have opposite directions of rotation and if they affect light of the same wavelength. This is called compensation.

Virtually all routine microscopes have additional, unwanted birefringence in components such as objective lenses, and extraneous birefringence could be introduced by glass slides or coverslips. This so-called strain birefringence may partly or completely eliminate either negative birefringence, reducing or removing blue to give yellow/green or yellow, or positive birefringence, giving blue/green or blue, depending on the relative orientation of the different factors. This explains Figure 1. When the section is rotated by 90°, strain birefringence may produce opposite effects, explaining Figure 2.

Pure green may be seen on a routine microscope sometimes by chance, and more reliably if a piece of apparatus called a compensator is introduced (Figure 8). This has a birefringent plate that can be rotated to give polarised light of variable ellipticity and direction of rotation, and can be adjusted to compensate strain birefringence and restore the original ellipticity (Figure 9). This can also mimic or exaggerate strain birefringence (Figure 10).

In everyday practice, there is no need to get either a compensator or a proper, strain-free polarising microscope, unless demonstration of a pure green colour is considered essential.

How other colours are produced

As the polariser or analyser is rotated from the crossed position, birefringent effects progressively decline. At the same time, light is directly transmit-

Figure 8
A Brace-Köhler compensator. Rotation of the graduated dial turns the birefringent plate in the arm, which produces polarised light of variable ellipticity

ted through the polariser, specimen and analyser, and the background lightens.

As the plane of polarisation of either polariser or analyser approaches the absorbing axis of orientated Congo red, more blue/green light is absorbed, and the colour approaches the red seen in unpolarised light. If the colour between crossed polariser and analyser is pure green, this changes progressively to yellow, orange and then red. If the plane of polarisation goes the opposite way and approaches the non-absorbing axis, colour is progressively lost.

When there is strain birefringence, this can modify the colours to give almost any colour, as can easily be seen in practice.

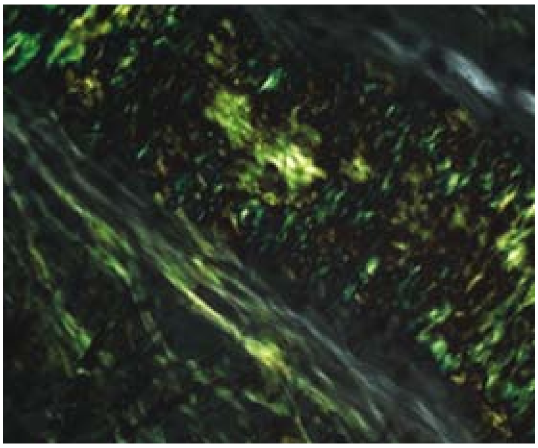


Figure 9
The section in Figure 1 with a compensator in the light path, rotated to give a uniform green

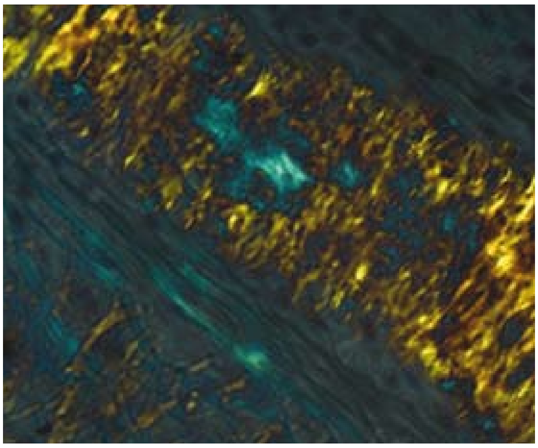


Figure 10
The section in Figure 1 with a compensator in the light path, rotated to give even more yellow and blue

Occasionally, the term ‘apple-green dichroism’ is used. This is not correct. ‘Dichroism’ means the change in appearance of orientated Congo red from red to colourless, depending on the plane of polarisation. This is shown with only a polariser or only an analyser, not both.

What is a better expression?

Frequently, Congo red-stained amyloid is said to show apple-green birefringence in polarised light, although there is rarely a pure green colour. Even if there is, birefringence, meaning there are two extremes of refractive index, cannot be green. Also, birefringence alone does not explain the colour and particular conditions of polarised light are required, with the specimen between crossed polariser and analyser.

Colours transmitted by an analyser under these conditions are called anomalous. Congo red-stained amyloid is more accurately said to show anomalous colours between crossed polariser and analyser. This phrase is just about the same length as the usual one, but this is hardly likely to make medical students or anyone else give up ‘apple-green birefringence in polarised light’.

Alexander J Howie
Professor of Renal Pathology
University College London

Douglas B Brewer
Emeritus Professor of Morbid Anatomy
University of Birmingham

Acknowledgment
We are grateful to Jackie Lewin for the diagrams.

Reference

1. Howie AJ, Brewer DB, Howell D, Jones AP. Physical basis of colors seen in Congo red-stained amyloid in polarized light. *Lab Invest* 2008;88:232–242.