

A REVIEW OF FLUORESCEIN ANGIOGRAPHY, INDOCYANINE GREEN ANGIOGRAPHY, AND OTHER FLUOROPHORES IN OPHTHALMOLOGY

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Purpose: To review fluorophores used in ophthalmology and summarize properties relevant to imaging, diagnosis, and safety.

Methods: Discussion of excitation, emission, and other essential properties of specific fluorophores.

Results: Sodium fluorescein, a fluorophore, is used for angiography and remains the cornerstone for retinal vascular imaging. Indocyanine green is also a fluorophore that absorbs light at longer wavelengths, enabling improved choroidal visualization and aiding in the diagnosis of various vascular, inflammatory, and neoplastic diseases. It is also used to stain the internal limiting membrane during vitreoretinal surgery. Other fluorophores, such as rose bengal, are used topically and can be used in the treatment of corneal fungal infections. Riboflavin has uses in the cornea, and curcumin has potential uses because of its binding affinity to amyloid.

Conclusion: In addition to fluorescein and indocyanine green, there are expanding uses for additional fluorophores, with further investigation needed for new imaging and treatment techniques.

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The term “cyanescence” has been incorrectly used to describe images generated with indocyanine green fluorescence angiography using infrared imaging. As this usage has appeared in both lectures and the literature, correction of the misnomer may enhance

clarity and promote precision. Here, we review existing fluorophores and recent new fluorophores that are used in ophthalmology to encourage adoption of accurate and consistent terminology.

Light is electromagnetic radiation that can be treated as a wave or a series of photons. The visible spectrum ranges between approximately 400 nm violet light to around 700 nm red light. Wavelengths above 710 nm to around 1,000 nm constitute near infrared light.¹ Wavelength is inversely related to frequency. The frequency of light is directly proportional to its energy. A shorter wavelength is thus associated with a higher frequency and therefore higher energy. In addition, the scatter of light is inversely proportional to the fourth power of the wavelength. Light of a shorter wavelength is scattered significantly more than light of a longer wavelength. For example, blue light of 480 nm is scattered about 7 times more than a near-infrared wavelength of 780 nm.

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Luminescence is the emission of light by a substance after it has been excited by higher-energy light. The emitted light has lower energy than the absorbed light and can be observed as fluorescence or phosphorescence. Fluorescence is a type of luminescence that occurs when specific molecules return to their original ground state after being excited to a higher energy state by absorbing light. The emitted light is of a longer wavelength (lower energy) than the absorbed light because there is a loss of energy from heat during the short lifetime of the excited state. Molecules that fluoresce are called fluorophores. The fluorescence quantum yield of a molecule, which varies among fluorophores, is the ratio of photons emitted to those absorbed. A perfect quantum yield would be 1, whereas a molecule that does not fluoresce when exposed to energy has a quantum yield of 0. Fluorescence can also occur outside the visible spectrum, and cameras can be used to record fluorescence in nonvisible regions of the electromagnetic spectrum.

Fluorescence lifetime imaging microscopy is a technique that measures the time a fluorophore remains in its excited state before emitting a photon and returning to its ground state.² When explicitly applied to ophthalmology, this technique is referred to as fluorescence lifetime imaging ophthalmoscopy. Fluorescence lifetime imaging ophthalmoscopy enables the in vivo measurement of fluorescence lifetimes of endogenous fluorophores within the retina.³

Fluorescein in Ophthalmology

There are myriad chemicals that are fluorophores, including rhodamine, curcumin, green fluorescent protein, luciferin, and fluorescein. Of these, fluorescein is the best known to ophthalmologists. It absorbs light of approximately 490 nm (blue light) and emits fluorescent light of approximately 514 nm (green light), with a very high quantum yield of up to 0.95 under optimal conditions.⁴ Because it emits light in the visible light range, it is ideal for evaluating retinal vessels. However, the choroidal circulation is not well visualized because of the scatter or absorption by the lens, vitreous, retina, and retinal pigment epithelium. In addition, fluorescein has a low molecular weight (MW) of 376 g/mol. Once injected intravenously, approximately 80% binds to albumin; the rest is unbound. Because of its low MW, free fluorescein diffuses out of vessels and into surrounding tissues easily when there is a breakdown of the blood-retinal barrier.

The introduction of fluorescein angiography in ophthalmology is usually ascribed to two medical students at the University of Indiana Medical School, Harold Novotny and David Alvis.⁵⁻⁷ Their article really

was a tour de force because it laid out many of the concepts of fluorescein angiography that are still in use today. Interestingly, the manuscript was not accepted by the *American Journal of Ophthalmology* and was therefore published in *Circulation*, a journal of the American Heart Association.⁷ Although the camera used in their work was slow and took photographs every 12 seconds, the authors were able to demonstrate fluorescein angiography in a normal fundus and in the eyes of patients with hypertension and diabetes. Notably, the work was preceded by studies in cats by Chao, Flocks, and Miller from Stanford University, who initially injected trypan blue and subsequently described fluorescein angiography (using a cobalt blue emission filter) in 1958 and 1959.^{8,9} After publication of the work of Novotny and Alvis, Maumane, Gass, and others began to use and popularize the technique of fluorescein angiography in ophthalmology.^{10,11}

Sodium fluorescein can also be used topically. For example, it can stain the cornea surface where the epithelial barrier has been disrupted, and it can reveal aqueous leakage from wounds, which is seen as dilution of the overlying fluorescein in the Seidel test.¹²

Sodium fluorescein is typically only used during pregnancy and during breastfeeding when it is necessary because it is classified as U.S. Food and Drug Administration Pregnancy Category C (animal studies have shown an adverse effect on the fetus). However, there are no well-controlled studies that have demonstrated complications in humans.¹³ Especially in the era of optical coherence tomography angiography, the need for fluorescein angiography during or after pregnancy is probably only rarely necessary. Although allergy to fluorescein is rare, it can happen and can be quite severe (including anaphylaxis). Therefore, as with all substances being injected, it is important to obtain a history of any prior reactions and to be prepared to handle a severe reaction.¹⁴

Because fluorescein is absorbed from the gastrointestinal tract, fluorescein can be given orally. This is typically done for patients who cannot tolerate intravenous fluorescein, such as children and some adults. When administered orally, late circulation of fluorescein in the fundus is captured. As the kidneys excrete sodium fluorescein, particular care must be taken in patients with renal insufficiency, as the sodium load can cause hypertension and fluid retention.¹⁵

Indocyanine Green and Indocyanine Green Angiography

Since 1971, Flower and associates had worked on the development and use of another fluorophore in ophthalmology, indocyanine green (ICG).^{16,17}

Interestingly, its use in ophthalmology was initially resisted because of questions about the value it added above the then widely used fluorescein. In addition, when used early in its development, primitive and expensive early technology resulted in images that were of low quality. Since then, however, ICG angiography has become recognized as an important method to image the deep choroidal vessels.

Indocyanine green has a MW of 775 g/mol, which is about double that of sodium fluorescein. It has a much lower quantum yield of between 2% and 13% depending on its environment.¹⁸ In addition, it strongly binds to plasma proteins. Because of the higher MW and its plasma protein binding, it diffuses less easily than fluorescein. Indocyanine green fluoresces by absorbing light with a maximum of 780 nm, and its fluorescence emission is maximal around 810 nm. The choroid is better visualized at these longer wavelengths because of less scatter and absorption by the retina; conversely, the longer wavelength does not allow for the exact resolution as with fluorescein. Like fluorescein sodium, ICG is also considered a Pregnancy Class C drug.¹⁹

The fluorescence of ICG is especially useful for visualizing pathologic condition in the choroid. Increased ICG fluorescence is described as “hyperfluorescence,” and decreased ICG fluorescence (relative to what would be expected for a standard control) is defined as “hypofluorescence.” There are some that have used the improper terms “hypercyanescence” and “hypocyanescence.” These terms are incorrect because the phenomenon that is being evaluated is fluorescence.²⁰

In cases of inflammatory diseases such as sarcoidosis and birdshot retinochoroidopathy, the choroidal lesions show hypofluorescence because there are inflammatory infiltrates that do not stain or leak.²¹ Choroidal hemangiomas show uptake of ICG in the large vessels²²; ICG accumulates in tumors in the choroid²³ and in systemic tumors.²⁴ In central serous chorioretinopathy, there is staining of the reactive choroidal vessels.²⁵ In pseudoxanthoma elasticum, the angioid streaks can be stained by ICG fluorescence.²⁶

More recently, we and others have shown that ICG staining is a promising biomarker of transthyretin amyloid. The time to binding is long, so imaging needs to extend to approximately 15 minutes post-administration.²⁷ We have also shown that the amount of staining is related to the extent of systemic involvement.²⁸ Indocyanine green staining seems to diminish after systemic treatment as well.

Because of its propensity to accumulate in tumors, ICG has also been used for a photothermal treatment of choroidal or retinal tumors.²⁹ It is estimated that 88% of the absorbed infrared 780 nm light is converted to thermal energy and photoreactive species.³⁰

In an in vitro study, tumors that were irradiated with ICG and near-infrared laser increased the temperature by 12% to 15% higher than laser treatment without added ICG.³¹

Indocyanine green also stains the internal limiting membrane when applied to the retinal surface. This property is used in the surgical treatment of macular holes and other retinal pathologies.^{32,33} The photothermal effect of shining light on ICG may cause retinotoxicity in rare cases.³⁴

Indocyanine green undergoes hepatic metabolism. Therefore, in patients with mild renal insufficiency, the use of ICG is not an absolute contraindication. Sodium iodide is added to stabilize the ICG. Most cases of “iodine” allergy are to the contrast agent that is attached to the iodine rather than to the iodine itself. In addition, seafood allergy is often to the proteins of the seafood and not the iodine, so actual allergies to ICG and iodine are very rare.³⁵

Other Fluorescent Chemicals Used in Ophthalmology

Rose Bengal

Rose bengal is a molecule with a MW of 974 g/mol. It absorbs light in the visible yellow range, with a maximum at 559 nm, and emits it at a maximum of 571 nm. In ophthalmology, it has been used topically to stain degenerating epithelial cells and to identify tear film abnormalities.³⁶ Rose bengal can both fluoresce and be activated to a photoreactive species.³⁷ This latter property has been recently exploited in the treatment of corneal infections, especially by *acanthamoeba*.³⁸

Riboflavin

Riboflavin is a water-soluble vitamin with a MW of 376 g/mol. It is a fluorescent compound and has maximum absorption peaks in the ultraviolet and violet ranges of 390 nm and 438 nm. The emission peak is at 532 nm, so a significant amount of the absorbed light is used to produce reactive free radicals. If the riboflavin is placed on the cornea and ultraviolet light is applied, these reactive species can then cross-link the collagen in the cornea.³⁹ The cross-linking increases the elastic modulus of the cornea, slowing or stopping the progression of the keratoconus.

Other Possible Future Ophthalmic Fluorophores

Curcumin is a compound with a MW of 368.46 g/mol, isolated from turmeric. The absorption maximum

is around 420 nm in the violet range. The maximum fluorescence emission peak is at 500 nm, and the quantum yield is a low 0.17. It seems to bind amyloid species, and congeners are being developed to bind and fluoresce more effectively.⁴⁰ Alzheimer disease involves the deposition of amyloid in the central nervous system, and some treatments are in development.⁴¹ Techniques, including positron emission tomography, are used to detect the deposition of amyloid in tissues such as the brain.^{42,43} There is some evidence that amyloid might also deposit in the retina.⁴³ Being able to visualize the amyloid with a low-cost imaging technique may be a great help in early diagnosis and evaluating the treatment of Alzheimer disease.

Conclusion

In summary, fluorescent molecules have key roles in ophthalmology because they allow for visualization, diagnosis, and in some cases treatment of ophthalmic disease. Fluorescein remains the gold standard for retinal vascular imaging but is complemented by ICG's properties, which enable visualization of the choroid. In addition, riboflavin has therapeutic use in corneal cross-linking. Other agents, such as rose bengal, play a role in *Acanthamoeba* treatment, and curcumin's binding properties with amyloid could be helpful in the future as technologies expand to visualize the retina in patients with Alzheimer disease. Accurate and consistent terminology improves clarity and enhances communication in both research and clinical practice.

Key words: fluorescein angiography, fluorescence, fluorophore, indocyanine green angiography.

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