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Introducing Euro-Glo, a rare earth metal chelate with numerous applications for the fluorescent localization of myelin and amyloid plaques in brain tissue sections

Larry Schmued*, James Raymick

National Center for Toxicological Research, Food and Drug Administration, 3900 NCTR Rd Jefferson AR, 72079, United States

HIGHLIGHTS

- Euro-Glo is a novel fluorochrome consisting of an organic chelate of the rare earth metal Europium.
- Euro-Glo allows for fluorescent localization of myelinated fiber bundles, individual fibers and toxicant induced myelinopathies.
- Euro-Glo staining also labels amyloid plaques, with the surround appearing red and the core appearing blue in color.
- Comparing Euro-Glo staining with a number of well characterized histochemical tracers suggests that it may have an affinity for glycolipids.
- Euro-Glo is the first rare earth metal chelate to be applied histologically for the study of the brain.

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ABSTRACT

The vast majority of fluorochromes are organic in nature and none of the few existing chelates have been applied as histological tracers for localizing brain anatomy and pathology.
New method: In this study we have developed and characterized a Europium chelate with the ability to fluorescently label normal and pathological myelin in control and toxicant-exposed rats, as well as the amyloid plaques in aged AD/Tg mice.
Results: This study demonstrates how Euro-Glo can be used for the detailed labeling of both normal myelination in the control rat as well as myelin pathology in the kainic acid exposed rat. In addition, this study demonstrates how E-G will label the shell of amyloid plaques in an AD/Tg mouse model of Alzheimer's disease a red color, while the plaque core appears blue in color. The observed E-G staining pattern is compared with that of well characterized tracers specific for the localization of myelin (Black-Gold II), degenerating neurons (Fluoro-Jade C), A-beta aggregates (Amylo-Glo) and glycolipids (PAS).
Comparisons with existing methods: This study represents the first time a rare earth metal (REM) chelate has been used as a histochemical tracer in the brain. This novel tracer, Euro-Glo (E-G), exhibits numerous advantages over conventional organic fluorophores including high intensity emission, high resistance to fading, compatibility with multiple labeling protocols, high Stoke's shift value and an absence of bleed-through of the signal through other filters.
Conclusions: Euro-Glo represents the first fluorescent metal chelate to be used as a histochemical tracer, specifically to localize normal and pathological myelin as well as amyloid plaques.

1. Introduction

1.1. Background

For over 150 years neuroanatomists have used organic or metallic stains to selectively stain components of the nervous system in great detail. Subsequently, a plethora of specialized histochemical methods have evolved. The use of fluorescent tracers has become increasingly popular for several reasons. One reason for this is that much fewer dye molecules are needed to be detected with fluorescent excitation, as compared to bright field absorption. Another advantage of fluorescent tracers is their suitability for multiple labeling studies, by virtue of the fact that different tracers can be selectively or simultaneously visualized by employing different excitation/emission filters. Although the majority of fluorescent histochemical tracers are simple organic compounds, there has

* Corresponding author.
E-mail addresses: larry.schmued@fda.hhs.gov (L. Schmued), james.raymick@fda.hhs.gov (J. Raymick).

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been a growing interest in applying chelates of certain REMs for both imaging and analysis of biochemicals, cells or tissues.

The actual physics underlying the properties of REM, or "Lanthanide", chelates is quite complex and has been reviewed in depth elsewhere (Bünzli, 2010). Many of the useful properties of these compounds can be attributed to differences in the number of electrons in the 4th orbital. Some of the more useful properties this configuration confers include the fluorescent and paramagnetic properties of certain REM compounds. Just as the nature of the particular REM used in the tracer preparation is critical to its function, so is the associated organic molecule. This organic portion of the compound must serve multiple functions including forming a soluble REM complex, sensitizing the metal's fluorescence and targeting a specific anatomical structure of interest. Previously reported biological applications tend to fall into two categories, in vitro bio-assays and live cell imaging.

1.2. Biological applications of REM chelates

The earliest biological application of REM chelates dates back to 1969 when a Europium complex was used to stain *E. coli* bacterial smears (Scaff et al., 1969). Interest in this field was rekindled a decade later when it was shown that certain REMs, such as trivalent Eu, Sm, Tb, and Dy, could be complexed with certain chelators, such as polyaminocarboxylates and beta diketonates, to make useful fluorescent tracers for use in time-resolved luminescent microscopy (Soini and Hemmilä, 1979). Subsequently, there has been a proliferation of new REM chelates and associated bio assays to be used with in vitro assays and cellular imaging studies. More recent reports of in vitro assay applications include their use in assays for peroxidase (Lin et al., 2006; Liu et al., 2012), carbonate (Smith et al., 2012a, 2012b), DNA (Nishioka et al., 2006), proteins (Evangelista et al., 1988), steroids (Mikola et al., 1993), glycoproteins (Lee et al., 1998), antigens (Diamandis, 1988), pH levels (Zhang et al., 2011) and DNA hybridization assays (Dickson et al., 1995). REM chelates have also been used to image live cells, either in tissue culture or in situ. Tissue culture applications include imaging mitochondria (Butler et al., 2015; Smith et al., 2012a, 2012b; Divya et al., 2013), nitric oxide (Liu et al., 2012), liposomes (Mignet and Scherman, 2010), intracellular pH (Zhang et al., 2011) and ribosomes, lysosomes and nucleoli (Montgomery et al., 2008).

1.3. Potential significance of REM based studies

There are several reasons for the increased interest in REM based tracers, primarily due to their unique fluorescent and paramagnetic properties. Some Lanthanides are intensely fluorescent, especially Europium and Terbium chelates, and are also quite stable and resistant to fading. These tracers are typically excited by ultraviolet light, making them suitable for multiple labeling studies in conjunction with commonly used green or red fluorochromes that require blue or green light excitation respectively. A major advantage of REM based tracers is their very sharp excitation and emission peaks. Another major advantage REM based tracers have over conventional organic fluorochromes is their exceptionally large Stoke's shift value. These two latter properties give REM based tracers a major advantage in that there is virtually no "bleed-through" of the tracer when visualizing other fluorescent tracers in multiple labeling studies. Another potential advantage of REM based tracers is their relative long post-illumination luminescence (Lin, 2012). This allows an image to be captured post-illumination, resulting in negligible background fluorescence, since it is not significantly luminescent. This is known as time resolved microscopy. Another advantage of using REM chelates is their relatively low toxicity, making them suitable for use in in vivo studies. Even the most soluble forms of the most reactive Lanthanide, Europium, has an oral rat LD_{50} value of 5 g/kg (Merck Index, 2006), reflecting an extremely low acute toxicity profile. Another potential advantage of REM based tracers is the fact that some are strongly paramagnetic, thus allowing a Gadolinium chelate, gadopentate dimeglumine, to be used clinically as a MRI contrast agent (Haustein et al., 1993). In light of all of the aforementioned advantageous attributes of REM based tracers, it is somewhat surprising that there are no examples in the literature of this class of tracer being used histologically to examine tissue sections. There is also no prior application of such tracers to the study of the nervous system, aside from one study of cultured PC-12 cells (Liu et al., 2012). Therefore, the present study constitutes the first example of REMs being used for the selective labeling of fixed brain tissue sections.

1.4. Models of brain pathologies

The tracer labeling pattern seen in wild type rodent brains was compared to that seen with genetic and toxicant models of certain brain pathologies. Specifically, double transgenic mice with mutations in the gamma secretase and amyloid precursor protein exhibited conspicuous amyloid plaques throughout the forebrain by 1 year of age. Myelin pathology was modeled by the administration of kainic acid. Kainic acid is a neurotoxin that causes damage to both nerve cells and myelin and was therefore used in this study to evaluate the usefulness of Euro-Glo for detecting myelin pathology. Kainic acid is a glutamate agonist that lesions primarily the neocortex, alocortex and the thalamus (Hopkins et al., 2000) via an excitotoxic mechanism.

2. Materials and methods

2.1. Materials

Euro-Glo (Europium tri [1,10-phenanthroline-5-amine]), Amylo-Glo, Black-Gold II and Fluoro-Turquoise were obtained from Histo-Chem Inc. (Jefferson AR). Antibodies to GFAP were obtained from Dako, (Carpentaria CA). Secondary biotinylated antibodies and streptavidin-FITC conjugates were obtained from Jackson Immunoresearch (West Grove PA). Kainic acid, periodic acid, Schiff's reagent, sodium thiosulfate and DAPI were all obtained from Sigma (St. Louis MO).

2.2. Animals

All animals were used in accordance with NCTR/FDA/NIH institutional animal care and use guidelines. Ten adult C57 Sprague Dawley rats were 3–6 months of age when sacrificed. Five of these rats were given kainic acid (10 mg/kg/i.p.) and subsequently sacrificed 2 days later. Ten AD/Tg mice (Jackson Labs. Bar Harbor ME) were sacrificed at 12 months of age. This B6C3-Tg (APPswe,PSEN1dE9)85Dbo/J strain of mice express mutations in both the sequence of the APP protein and the gamma secretase enzyme, resulting in a significant amyloid plaque buildup by 1 year of age.

2.3. Neurotoxicant exposure

A subset of the rats were given the neurotoxin, kainic acid (KA). KA was administered via a single i.p. injection of 10 mg/kg and the animal was sacrificed 1–2 days later.

2.4. Tissue processing

Following euthanasia with Euthasol, animals were perfused via the ascending aorta with 10% formalin in 0.1 M neutral phosphate buffer. The brains were removed and post-fixed overnight in the same fixative solution plus 20% sucrose for cryoprotection. The brains were then cut at a thickness of 25 μm on a freezing-sliding

microtome. The brain tissue sections were either collected in wells containing 0.1 M neutral phosphate buffer for short term storage in the refrigerator, or in wells containing antifreeze (ethylene glycol: glycerin: distilled water: 0.1 M neutral phosphate buffer 1:1:1:1) for long term storage in the freezer. After two 3 min rinses in distilled water, the tissue sections were mounted onto gelatin coated slides from distilled water and allowed to dry on a slide warmer at 55 ° C for at least 30 min.

2.5. E-G staining procedure

Slide mounted tissue sections were first rehydrated by immersion in distilled water for 5 min. The slides were then transferred to a 0.025% solution of Euro-Glo dissolved in a distilled water vehicle for 1–4 days at room temperature. The progression of the stain may be microscopically monitored daily until optimal staining is achieved. Once fully stained, the slides were then given a 3 min rinse in distilled water and then transferred to an ammonium borate solution for 1 min. Following a distilled water rinse, the progression of the differentiation can be monitored microscopically and the slide returned to the ammonium borate solution if the background remains too high. However, differentiation times in excess of 6 min are rarely needed and longer times can potentially bleach the specific labeling. To avoid fading under UV examination, the tissue should be well rinsed as indicated below. The ammonium borate differentiating solution was made by dissolving 4 g of boric acid and 10 ml of concentrated (40%) ammonium hydroxide in 90 ml of distilled water. Should the slides become over differentiated, they may be re-stained in the Euro-Glo solution. Once differentiated, the slides were rinsed through three 5 min distilled water rinses. Insufficient rinsing of differentiating solution can result in fading under examination. Slides were then cover-slipped with an aqueous non-fluorescent mounting media, Ever-Glo, (Histo-Chem AR) consisting of polyvinyl alcohol, glycerin and borate buffer. Polar mounting media such as DPX and Permount are not suitable for coverslipping E-G stained tissue. Other aqueous mounting media such as Prolong-Gold (Thermo Fisher, CA) were found suitable for short term examination but allowed the section to fade from the edges inward within a few days.

2.6. Supplemental staining procedures

2.6.1. Amylo-Glo

Amylo-Glo fluorescent labeling of amyloid plaques was accomplished using the previously described method (Schmued et al., 2012). In brief, this involved immersing slide mounted tissue sections in 70% ethanol for 5 min, DW for 3 min and then for 10 min in a staining solution of Amylo-Glo prepared by adding 1 ml of the stock solution to 99 ml of saline. When examined under UV light, Amylo-Glo labeled amyloid plaques appeared bright yellow in color.

2.6.2. Periodic Acid Schiff (PAS)

Periodic Acid Schiff (PAS) reagents were used to label complex glycoproteins. The staining method of Culling (1974) was followed. This involved 10–20 min immersion in 1% periodic acid resulting in the oxidation of adjacent hydroxyl groups of sialic acid sugars to di-aldehydes. Subsequent immersion of the tissue in the colorless Schiff's reagent for 10–30 min labeled the polysaccharides a red color.

2.6.3. Fluoro-Turquoise

Fluoro-Turquoise was conjugated to gelatin using the previously described protocol and then delivered intravascularly at the time of animal sacrifice (Sarkar et al., 2013). Basically, the following FT-gel in situ labeling paradigm was used: After euthanasia, animals were perfused intravascularly with saline, followed by 4% formaldehyde in neutral 0.1 M phosphate buffer and then followed by another saline rinse. Subsequently, 100 ml of a warm (55 °C) solution of 2% Fluoro-Turquoise gel was perfused via the ascending aorta. Brains were then removed from the cranium and post fixed overnight in the same formaldehyde solution plus 20% sucrose while kept at 5 °C. Brains were sectioned on a freezing sliding microtome at a thickness of 25 μm. Sections were examined under UV light excitation.

2.6.4. Black-Gold II

Black-Gold II labeling of myelin was achieved by following the method described in the original article (Schmued et al., 2008). In brief, formalin-fixed, frozen-cut and slide mounted tissue sections were rehydrated briefly in distilled water. They were then transferred to a 0.3% solution of Black-Gold II dissolved in 0.9% sodium chloride and kept at a temperature of 60 °C for 15–20 min. The sections were then rinsed briefly in distilled water, fixed for 3 min in 2% sodium thiosulfate and then rinsed through three 5 min rinses in tap water.

2.6.5. Fluoro-Jade C

Fluoro-Jade C staining of amyloid plaques was achieved as described in the original article (Schmued et al., 2005). Briefly, slide mounted tissue sections were rehydrated and then incubated in a 0.06% potassium permanganate solution in distilled water for 10 min. Following a brief rinse, sections were incubated in a 0.0001% solution of FJ-C, dissolved in 0.1% acetic acid, for 15 min. The slides were then rinsed in distilled water, air dehydrated, cleared by brief immersion in xylene and coverslipped with DPX mounting media. Sections were examined under blue light excitation.

2.6.6. DAPI fluorescent Nissl

DAPI fluorescent Nissl counterstaining was achieved by immersing tissue that had already been stained with E-G, as described above, in a solution of 0.0001% DAPI dissolved in distilled water vehicle for 3–5 min. Tissue sections were then briefly rinsed in distilled water. Alternatively, a DAPI counterstain can be achieved by using certain commercial aqueous mounting media that contain this fluorochrome. Sections are visualized under UV light excitation.

2.6.7. Anti-GFAP immunofluorescent

Anti-GFAP immunofluorescent staining was achieved by on-the-slide staining procedures in which the various solutions are applied directly to the tissue sections which are kept in a humidity chamber. For multiple labeling with E-G, slides were first pre-stained with E-G as described above. 10% horse serum in 0.9% saline is applied directly onto the tissue sections for 20 min and then rinsed with 2 changes of saline. The primary polyclonal rabbit anti-GFAP antibody (Dako, CA) was used at a 1:500–1:1000 solution in saline and was allowed to stay on the section overnight at room temperature. The slides were then rinsed with 2 changes of saline and then incubated in the biotinylated donkey anti-rabbit secondary antibody (Jackson Immunoresearch, PA) at a dilution of 1:200 for 2 h. Following two saline rinses, the sections are incubated in an avidin-FITC conjugate solution in saline at a dilution of 1:250 for 1 h. Sections were then rinsed twice with saline and then coverslipped with the same mounting media as described above for the E-G stained slides.

3. Results

3.1. Myelin staining in normal brains

Wild type untreated rat and mouse brain sections stained with Euro-Glo resulted in the bright salmon red colored staining of

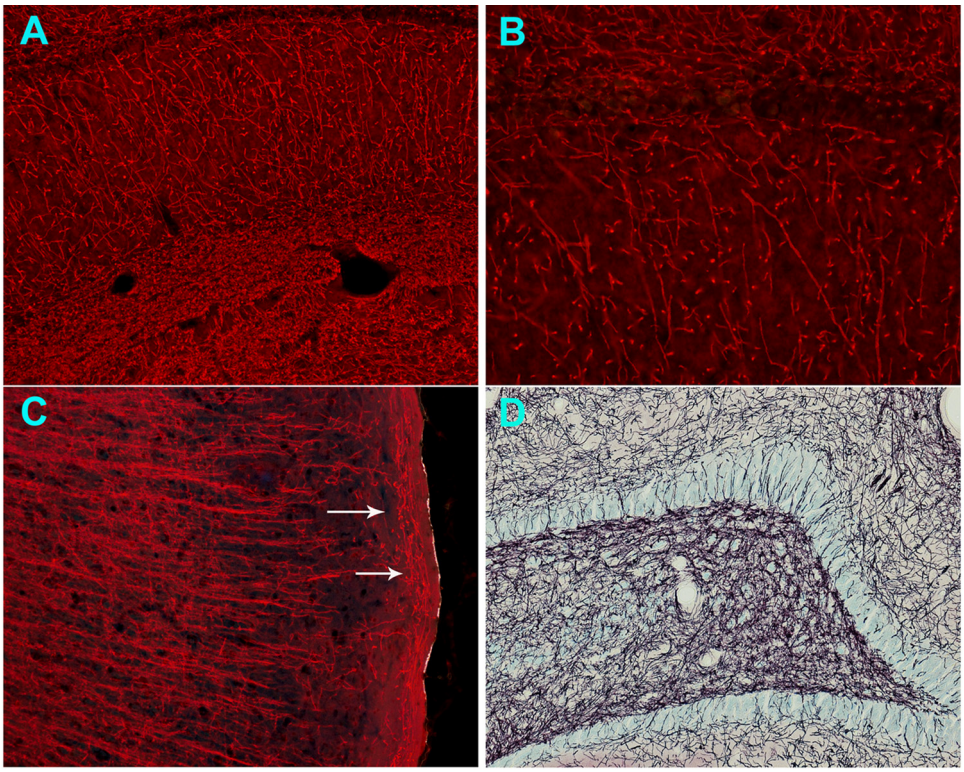

Fig. 1. Demonstrates E-G labeling of myelin within the rodent forebrain. Specifically, (A) survey view of hippocampus; (B) high mag view of hippocampal myelination within the dentate gyrus; (C) myelin staining within the superficial cortex of both the radial fibers within layers II and III plus the fine parallel fibers of layer I (arrows); (D) Black-Gold II labeling of hippocampal myelination reveals a comparable labeling pattern between it and E-G.

all white matter. This includes both the large myelinated fascicles (Fig. 1A low mag) as well as the fine individual myelinated fibers (Fig. 1B high mag) of the hippocampus. Examination of brain regions containing the finest myelinated fibers, including the parallel fibers of layer I cortex (Fig. 1C) exhibited labeling of high resolution and contrast. In addition, cell bodies exhibited a relatively pale green fluorescence. Staining of adjacent tissue sections with the bright field myelin stain (Fig. 1D) Black-Gold II supports the notion that E-G labels all myelin since both tracers labeled comparable myelinated structures.

3.2. Amyloid plaque staining in aged AD/Tg mice

In addition to labeling the normal myelin, as previously described, E-G also labels amyloid plaques within the brains of aged AD/Tg animals. Extensive extracellular plaque labeling was found especially throughout the cortex and the hippocampus of these animals. Plaque morphologies included those previously described including diffuse, condensed and dense-core varieties. For the purpose of this study the dense core plaques were analyzed in detail, because they exhibit the greatest morphological diversity. Examination of plaques at low magnification reveals a relatively homogeneous flocculent appearing labeling of the entire plaque region surrounding the core, which itself appears blue-green in color (Fig. 2A). Examination at higher magnifications reveals a variable number of round vesicular-like structures within the plaque itself (Fig. 2B). Although truncated myelinated fibers can be seen adjacent to amyloid plaques, there were no conspicuously labeled myelin fragments contained within the plaques.

3.3. Black-Gold II staining of amyloid plaques

As previously reported (Schmued et al., 2013), B-G II will stain individual myelinated fibers up to the point at which they come in contact with the most peripheral aspect of the plaque (Fig. 2C). Although the plaques tend to acquire a pale lavender color, the intact or fragmented black fibers associated with myelination are never seen.

3.4. Amylo-Glo labeling of amyloid plaques

Amylo-Glo labels much of the plaques including the core as well as the more radially oriented fibular neurites (Fig. 2D). In comparison, the E-G does not appear to label the neurites and the plaque cores appear as a different color.

3.5. Fluoro-Jade C labeling of amyloid plaques

Fluoro-Jade C histochemistry resulted in the labeling of dense cores, neuritic filaments and occasional vesicular-like structures (Fig. 2E). Aside from the vesicular-like structures, there was little similarity between the plaque staining patterns of FJ-C versus E-G.

3.6. PAS staining of amyloid plaques

PAS histochemistry labeled the entire plaque in a relatively homogenous fashion similar to that seen with Euro-Glo (Fig. 2F). One difference, however, was that the PAS stained plaques included labeling of the plaque cores, as opposed to the E-G labeling, in which the larger cores appear blue-green in color. The PAS technique also stained the large myelinated tracts, like E-G, although it did not stain the fine individual myelinated fibers.

3.7. Myelin staining of toxicant exposed brain tissue sections

Rats that were exposed to kainic acid exhibited myelopathies in multiple brain regions, especially the hippocampus, neocortex, piriform cortex and thalamus, which was consistent with previous

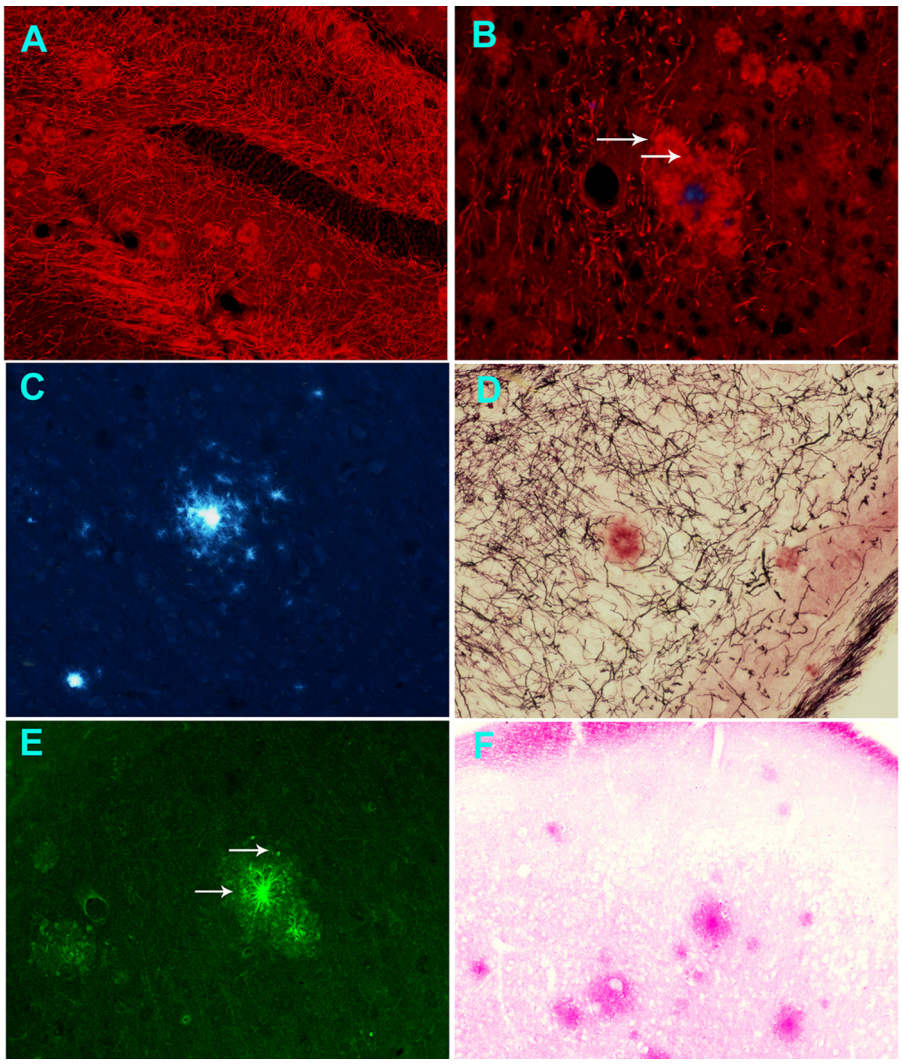

Fig. 2. Illustrates the staining of amyloid plaques with E-G as well as other specific histochemical markers shown for comparison. Examples of E-G staining of plaques are seen at survey magnification in the hippocampus (A) and at high magnification in the perirhinal cortex (B) revealing roughly circular flocculent staining as well as the presence of some vesical-like structures (arrows). Plaques were stained with A-G (C), which generally co-localizes with anti-A-beta immunohistochemistry, labeling both the plaque cores and the more distal radial fibrils. Comparing amyloid plaque staining with the myelin specific tracer, Black-Gold II (D), it is apparent that the nearly black myelinated fibers reach the most distal borders of the plaques, but are not actually incorporated into the plaques. Fluoro-Jade C staining of amyloid plaques (E) shows the staining of their cores, neurites and occasional vesicular appearing structures (arrows). Use of PAS histochemistry (F) shows, in addition to the labeling of the large myelinated anterior olfactory tract at top (image inverted), both the cores and peripheries of the amyloid plaques were labeled.

descriptions of this toxicant mediated pathology (Hopkins et al., 2000). The nature of the morphological changes of the observed myelin structures included edematous varicosities, fragmentation and demyelination (Fig. 3A). There were no changes to the fluorescent intensity of normal vs lesioned white matter, aside from total demyelination, in which case no labeling was seen.

3.8. DAPI cellular counter staining

Combined labeling with DAPI allowed for the simultaneous visualization of all cellular nuclei and endoplasmic reticulum (Nissl bodies) (Fig. 3B). The observed pattern of staining is consistent with that previously reported and allows for easy visualization of the nature and number of cells in relation to E-G labeled myelinated axons or amyloid plaques.

3.9. Fluoro-Turquoise gel vascular labeling

Combined labeling with Fluoro-Turquoise gel delivered intravascularly at the time of sacrifice combined with E-G staining allows for visualization of brain vasculature in relation to myelin or amyloid plaques (Fig. 3C) as seen here in the rat cortex.

3.10. Anti-GFAP astrocyte labeling

Combined labeling with GFAP immunofluorescent methods, as seen here (Fig. 3D) in the dentate gyrus of the hippocampus, simultaneously demonstrates the presence of hypertrophied astrocytes (green) and myelin pathology (red) in the hippocampus of a kainic acid exposed rat.

4. Discussion

4.1. Unique and advantageous properties

As discussed in the Background section, certain REM chelates including Europium exhibit numerous advantages over conventional organic fluorescent dyes when employed in a variety of in vitro assays. These advantages include intense fluorescence, very large Stoke's shift values, very sharp excitation and emission

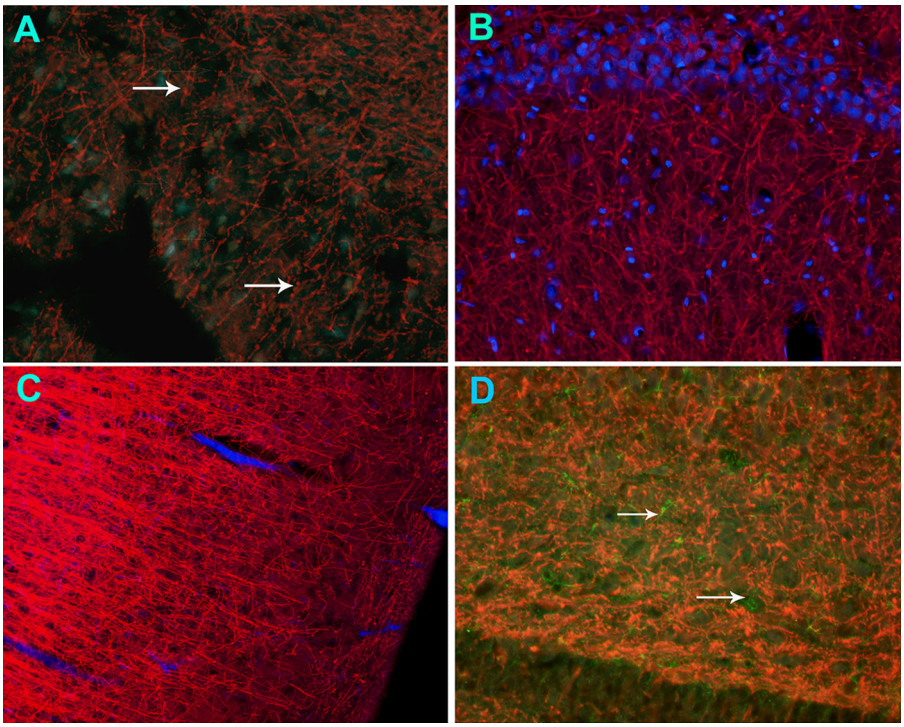

Fig. 3. Figure 3 shows how E-G can be used to both localize toxicant induced pathologies, as well as co-localizing with other fluorescent probes of contrasting color. E-G staining of pyriform cortex from a kainic acid exposed rat (A) reveals myelin pathologies including conspicuous edematous varicosities (arrows). Following E-G staining with DAPI staining (B) allows for co-localization of myelin and cell nuclei simultaneously with UV light excitation. Post mortem perfusion of the vascular system of a rat with Fluoro-Turquoise labeled gelatin combined with E-G staining (C) results in the simultaneous localization of blood vessels of the cortex and the myelinated fibers using UV light excitation. A tissue section from a kainic acid exposed rat first stained with E-G and then processed for anti-GFAP immunofluorescence (D) reveals hypertrophied astrocytes (arrows) relative to changes in myelination using combined UV and blue light excitation.

peaks, long post-excitation luminance times and high resistance to fading. In light of these numerous attributes, it is somewhat surprising that prior to the present work, no studies have employed REM chelates as fluorescent histochemical tracers. The Stoke's shift value refers to the distance between excitation and emission wavelengths. A large Stoke's shift value is especially beneficial when used in multiple labeling studies since it ensures that there will be no "bleed-through" when visualizing complementary fluorescent tracers. It also facilitates the unprecedented simultaneous localization of two different colored fluorophores using just one excitation wavelength. Thus the blue emission of DAPI or Fluoro-Turquoise can be visualized simultaneously with the red emitting E-G with only UV excitation. The sharp excitation and emission peak profiles translate to lower background levels of auto fluorescence, when viewing the distribution of the tracer microscopically. The exact emission and excitation profiles for this tracer have not yet been resolved, mainly because soluble aqueous solutions exhibit negligible fluorescence and only become highly fluorescent upon precipitation as a basic salt, or after binding to either myelin or amyloid plaques in fixed brain tissue sections. However, empirical use of the tracer would suggest that the observed red colored emission likely corresponds to the reported sharp emission peak of 611 nm for the Europium doped Yttrium oxide phosphor (Yen et al., 2007). The primary excitation peak reported for this Europium phosphor is 253 nm, which would presumably be too short of a wavelength to visualize with Hg vapor illumination, although secondary peaks between 364 and 395 nm could be excited using ultraviolet light. The compound's intense fluorescence confers several advantages over conventional fluorochromes including short exposure times and high sensitivity, which result in the ability to detect very few tracer molecules. Another advantage of REM chelates is their high stability, both when examined under UV light illumination and following extended archival storage. This advantageous property is, however, dependent on the use of certain mounting media. All solvent based mounting media, such as Permount or DPX are counter-indicated, presumably because they extract the lipids from the myelin. Some of the aqueous based anti-fade mounting media were found to be better than others as previously indicated.

4.2. Histochemical affinities

Euro-Glo exhibits an affinity for myelinated fibers and, therefore, will label both large fascicles as well as fine individual myelinated fibers in both normal and damaged brains. When compared with conventional bright field myelin stains, like Black-Gold II, both exhibit the same pattern of labeling in control as well as neurotoxin exposed tissue. The level of sensitivity and resolution of the two tracers seems to be comparable and both methods label the fine parallel fibers of layer I cortex, typically the most difficult myelinated fibers in the brain to stain. Although the physical properties of inorganic Europium salts are well documented, much less is known about the affinities of the Europium chelates for specific biological targets. So although the exact endogenous biochemical(s) that E-G binds to is not known, it is possible to make some inferences on what these biochemical targets might be. For example, it is known that exposing the tissue sections to non-polar solvents prior to immersion in the E-G solution eliminates all staining (data not shown). This would suggest that E-G targets certain lipid containing molecules that are susceptible to solvent extraction. The observation that formalin fixation of the tissue does not prevent the solvent mediated inhibition of staining suggests that the endogenous E-G ligand does not have a proteinatious component, since most proteins are immobilized by formalin fixation. Further insights on the possible identity of E-G's endogenous targets may be inferred from comparing its staining with the staining achieved using certain specialized histochemical techniques, including the following:

Table 1
Comparison of E-G staining of myelin and plaques with other well characterized histochemical markers.

Tracer Affinity ⇒ Anatomical Structures⇓	E-G	BG II Myelin	A-G Amyloid Aggregates	PAS Glycoproteins- Glycolipids	FJ-C Degenerating Neurons
Large Myelinated Fasicles	X	X			
Individual Myelinated Fibers	X	X			
Myelin Pathology	X	X			
Degenerating Neurons and Neurites					X
Plaque Cores	X Blue		X	X	X
Plaque-Surround Radial Rays			X		X
Plaque-Surround Flocculent Material	X Red			X	
Plaque-Surround Vesicle-Like	X	X		X	X

4.2.1. Amylo-Glo

Amylo-Glo, like A-beta immunohistochemistry, both label essentially the same components of the plaques including dense cores and radial fibular and diffuse A-beta aggregates. This is in contrast to Euro-Glo which labels the entire plaque in the brains of AD/Tg animals. This difference in plaque labeling suggests that E-G stains a biochemical component of the plaque other than the aggregated A-beta.

4.2.2. Black-Gold II

Black-Gold II, which is specific for myelin in fixed tissue sections failed to reveal any intact, or fragmented, myelin within the plaques. This would suggest that E-G is not simply co-localizing with myelin contained within the plaques.

4.2.3. Fluoro-Jade C

Fluoro-Jade C, which is specific for localizing degenerating neurons, also labels plaque cores, neurites and occasional vesicle-like structures. Since this differs significantly from the E-G staining, it would suggest that E-G does not label the neuritic component of amyloid plaques.

4.2.4. The PAS stain

The PAS stain, which has an affinity for compounds containing sialylated glycosides was found to stain both the large myelinated fascicles in control rats and the amyloid plaques in AD/Tg mice. Therefore, keeping in mind the aforementioned conditions and limitations, it is possible to propose a class of biomolecules whose properties conform with these restrictions. Specifically, such a candidate molecule should contain both lipids and complex glycosides but not proteins. An abundant class of compounds in the brain that fits this description is the gangliosides. These molecules contain fatty acids, glycerol and complex polysaccharides high in galactose and *n*-acetylneuraminic acid (Lehniger, 1970). Gangliosides are membrane components that are especially concentrated in neurons and oligodendrocytes, which would be consistent with the E-G staining of myelin and amyloid plaques. Although a primary component of amyloid plaques is polymerized A-beta fibrils, it has been suggested that gangliosides serve as the site of plaque nucleation (Matsuzaki et al., 2010). Additionally, the presence of the common ganglioside, GMD1a, has been localized immunohistochemically in amyloid plaques from human brain tissue (Nishinaka et al., 1993).

4.3. Comparison of E-G staining with other histochemical markers

From a methodological perspective, one obvious difference between the E-G staining vs. the other tracers is that the E-G staining requires more time. This suggests that, as with immunocytochemistry, numerous relatively weak chemical bonds are formed between the dye and its target tissue. Examination of Table 1 reveals both similarities and differences in the plaque staining pattern as revealed by diverse histochemical markers. Of these well characterized markers, the PAS stain seems to correlate the most closely with the E-G labeling. Specifically, both stains labeled large myelinated fibers as well as amyloid plaques in their entirety, although the PAS stain does not also label fine myelinated fibers and stains both plaque cores and surrounds monochromatically. The polychromatic staining of the plaques with E-G is quite an unusual phenomenon which is never seen with conventional organic fluorophores. Although the significance of this polychromatic staining is unknown, it is conceivable that the red color indicates Europium in its trivalent state, while the blue color could be indicative of Europium in its divalent state. If true, this would suggest that the plaque cores contain a yet unidentified reducing agent.

4.4. Potential follow-up studies

The present findings would seem to present the opportunity for a number of related follow-up studies. Although we have speculated on the possible endogenous ligands for E-G, it will be important to resolve this matter definitively. Another potentially interesting follow-up study could be investigating the potential uses of E-G with time resolved fluorometry. Potential advantages of this form of microscopy include very high contrast images since Europium chelates will luminescence long (over 1 ms) after background auto-fluorescence ceases to be detectable. Possible future studies could also look at the structure-activity relationship of other REM chelates. For example, the usefulness of other fluorescent REMs metals, such as Terbium, could be investigated to see if a related green fluorescent chelate could be produced. Similarly, other organic chelators with different functional groups could be investigated to potentially target other brain structures. For example a chelator possessing multiple carboxyl groups, like the Fluoro-Jade dyes might also be suitable for labeling degenerating neurons. Another potential application worth investigating would be the tracer's potential usefulness as a MRI tracer/contrast agent. Chelates of Gadolinium are presently used clinically as MRI contrast enhancers, having the highest magnetic susceptibility of all the REMs. However, co-localization of these compounds via fluorescent microscopy is not feasible due to the negligible fluorescent emission of Gadolinium compounds. In contrast, Europium and Terbium chelates are both highly fluorescent and significantly paramagnetic. In terms of relative magnetic susceptibility, Europium has 9.3% and Terbium has 54.2% of Gadolinium's magnetic susceptibility, suggesting they may also be applicable to MRI imaging.

5. Conclusion

In conclusion, this study introduces a novel Europium chelate, Euro-Glo, which represents the first such REM complex to be applied for the fluorescent histochemical localization of specific structures within the brain. These findings confirm the hypothe-

sis that fluorescent rare earth metal chelates can be used for the histochemical localization of specific structures within the brain. This tracer has been shown to have an affinity for both myelin and amyloid plaques. It is therefore also capable of detecting pathological myelin following exposure to neurotoxins, as well as labeling amyloid plaques in AD/Tg mice. As a REM chelate, E-G exhibits numerous advantages over conventional organic fluorochromes including sharp excitation and emission peaks, a very large Stoke's shift value, intense emission, high stability (permanence) and long post-illumination luminescence times. Histologically, these attributes translate to a very bright stain with low background and negligible bleed-through when combined with other fluorochromes in multiple labeling studies.

Disclaimers

The mention of trade names or commercial products does not constitute endorsement. This work does not necessarily reflect the views of the entire FDA.

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References

Butler, S.J., Delbianco, M., Lamarque, L., McMahon, B.K., Neil, E.R., Pal, R., Parker, D., Walton, J.W., Zwier, J.M., 2015. EuroTracker® dyes: design, synthesis, structure and photophysical properties of very bright europium complexes and their use in bioassays and cellular optical imaging. Dalton Trans. 44 (March (11)), 4791–4803, http://dx.doi.org/10.1039/c4dt02785j.

Bünzli, J.C., 2010. Lanthanide luminescence for biomedical analyses and imaging. Chem. Rev. 110 (May (5)), 2729–2755, http://dx.doi.org/10.1021/cr900362e.

Culling, C., 1974. Histological and Histopathological techniques. Butterworth Ltd., pp. 262–269.

Diamandis, E.P., 1988. Immunoassays with time-resolved fluorescence spectroscopy: principles and applications. Clin. Biochem. 21, 139–150, be.

Dickson, E.F., Pollak, A., Diamandis, E.P., 1995. Ultrasensitive bioanalytical assays using time-resolved fluorescence detection. Pharmacol. Ther. 66 (May (2)), 207–235.

Divya, V., Sankar, V., Raghu, K.G., Reddy, M.L., 2013. A mitochondria-specific visible-light sensitized europium β-diketonate complex with red emission. Dalton Trans. 42 (September (34)), 12317–12323, http://dx.doi.org/10.1039/c3dt51117k, Epub 2013 Jul 15.

Evangelista, R.A., Pollak, A., Allore, B., Templeton, E.F., Morton, R.C., Diamandis, E.P., 1988. A new europium chelate for protein labeling and time-resolved fluorometric applications. Clin. Biochem. 21 (June (3)), 173–178.

Haustein, J., Laniado, M., Niendorf, H.P., Louton, T., Beck, W., Planitzer, J., Schöffel, M., Reiser, M., Kaiser, W., Schörner, W., et al., 1993. Triple-dose versus standard-dose gadopentetate dimeglumine: a randomized study in 199 patients. Radiology 186 (March (3)), 855–860.

Hopkins, K.J., Wang, G., Schmued, L.C., 2000. Temporal progression of kainic acid induced neuronal and myelin degeneration in the rat forebrain. Brain Res. 864 (May (1)), 69–80.

Lee, Y.C., Kawasaki, N., Lee, R.T., Suzuki, N., 1998. Quantum-dye labeled proteins for glycobiology: a viable nonradioactive alternative tracer. Glycobiology 8 (September (9)), 849–856.

Lehniger, A., 1970. Biochemistry. Worth Publishers, pp. 200–201.

Lin, Z., Wu, M., Wolfbeis, O.S., Schäferling, M., 2006. A novel method for time-resolved fluorometric determination and imaging of the activity of peroxidase, and its application to an enzyme-linked immunosorbent assay. Chemistry 12 (March (10)), 2730–2738.

Liu, M., Ye, Z., Wang, G., Yuan, J., 2012. Development of a novel europium (III) complex-based luminescence probe for time-resolved luminescence imaging of the nitric acid production in neuron cells. Talanta 99, 951–958.

Matsuzaki, K., Kato, K., Yanagisawa, K., 2010. A-beta polymerization through interaction with membrane gangliosides. Biochem. Biophys. Acta 1801, 868–877.

O'Neil, M. (Ed.), 2006, fourteenth edition, p. 3905.

Mignet, N., Scherman, D., 2010. Liposome biodistribution via europium complexes. Methods Mol. Biol. 606, 509–518, http://dx.doi.org/10.1007/978-1-60761-447-0_34.

Mikola, H., Sundell, A.C., Hänninen, E., 1993. Labeling of estradiol and testosterone alkyloxime derivatives with a europium chelate for time-resolved fluoroimmunoassays. Steroids 58 (July (7)), 330–334.

Montgomery, C.P., New, E.J., Parker, D., Peacock, R.D., 2008. Enantioselective regulation of a metal complex in reversible binding to serum albumin: dynamic helicity inversion signaled by circularly polarized luminescence. Chem. Commun. (Camb.) 36 (September (28)), 4261–4263, http://dx.doi.org/10.1039/b810978h, Epub 2008 Sep 10.

Nishinaka, T., Iwata, D., Shimada, S., Kosaka, K., Suzuki, Y., 1993. Anti-ganglioside GD1a monoclonal antibody recognizes senile plaques in brains of patients with Alzheimer-type dementia. Neurosci. Res. 17, 171–176.

Nishioka, T., Yuan, J., Yamamoto, Y., Sumitomo, K., Wang, Z., Hashino, K., Hosoya, C., Ikawa, K., Wang, G., Matsumoto, K., 2006. New luminescent europium (III) chelates for DNA labeling. Inorg. Chem. 45 (May (10)), 4088–4096.

Sarkar, S., Raymick, J., Paule, M.G., Schmued, L., 2013. In situ demonstration of Fluoro-Turquoise conjugated gelatin for visualizing brain vasculature and endothelial cells and their characterization in normal and kainic acid exposed animals. J. Neurosci. Methods 219 (October (2)), 276–284, http://dx.doi.org/10.1016/j.jneumeth.2013.07.020, Epub 2013 Aug 14.

Scaff Jr., W.L., Dyer, D.L., Mori, K.J., 1969. Fluorescent europium chelate sta. Bacteriology 98 (April (1)), 246–248.

Schmued, L.C., Stowers, C.C., Scallet, A.C., Xu, L., Fluoro-Jade, C., 2005. Results in ultra high resolution and contrast labeling of degenerating neurons. Brain Res. 1035 (July (1)), 24–31.

Schmued, L., Bowyer, J., Cozart, M., Heard, D., Binienda, Z., Paule, M., 2008. Introducing Black-Gold II, a highly soluble gold phosphate complex with several unique advantages for the histochemical localization of myelin. Brain Res. 1229 (September (10)), 210–217, http://dx.doi.org/10.1016/j.brainres.2008.06.129, Epub 2008 Jul 15.

Schmued, L., Raymick, J., Tolleson, W., Sarkar, S., Zhang, Y.H., Bell-Cohn, A., 2012. Introducing Amylo-Glo, a novel fluorescent amyloid specific histochemical tracer especially suited for multiple labeling and large scale quantification studies. J. Neurosci. Methods 209 (July (1)), 120–126, http://dx.doi.org/10.1016/j.jneumeth.2012.05.019, Epub 2012 Jun 15.

Schmued, L.C., Raymick, J., Paule, M.G., Dumas, M., Sarkar, S., 2013. Characterization of myelin pathology in the hippocampal complex of a transgenic mouse model of Alzheimer's disease. Curr. Alzheimer Res. 10 (January (1)), 30–37, PMID. 23157338.

Smith, D.G., Pal, R., Parker, D., 2012a. Measuring equilibrium bicarbonate concentrations directly in cellular mitochondria and in human serum using europium/terbium emission intensity ratios. Chemistry 18 (September (37)), 11604–11613, http://dx.doi.org/10.1002/chem.201201738, Epub 2012 Aug 2.

Smith, D.G., Pal, R., Parker, D., 2012b. Measuring equilibrium bicarbonate concentrations directly in cellular mitochondria and in human serum using europium/terbium emission intensity ratios. Chemistry 18 (September (37)), 11604–11613, http://dx.doi.org/10.1002/chem.201201738, Epub 2012 Aug 2.

Soini, E., Hemmilä, I., 1979. Fluoroimmunoassay: present status and key problems. Clin. Chem. 25 (March (3)), 353–361.

Yen, Y., Shionoya, S., Yamamoto, H., 2007. Phosphor Handbook, 2nd edition. CRC Press, p. 197.

Zhang, X., Jiao, Y., Jing, X., Wu, H., He, G., Duan, C., 2011. pH-sensitive fluorescent sensors based on europium (III) complexes. Dalton Trans. 40 (March (11)), 2522–2527, http://dx.doi.org/10.1039/c0dt01325k, Epub 2011 Feb 4.