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Natalie Pankova,[a,b,c] Xu Zhao,[c] Huiyuan Liang,[c] David Sung Hyeon Baek,[a,c] Hai Wang,[c] and Shelley Boyd[a,b,c,d,e,*]

[a]University of Toronto, Department of Laboratory Medicine and Pathobiology, 1 King's College Circle, Toronto, Ontario M5S 1A8, Canada
[b]University of Toronto, Department of Ophthalmology and Vision Sciences, 340 College Street, Toronto, Ontario M5T 3A9, Canada
[c]St Michael's Hospital, Keenan Research Centre for Biomedical Science, 209 Victoria Street, Toronto, Ontario M5B 1W8, Canada
[d]St. Michael's Hospital, Department of Ophthalmology, 30 Bond Street, Toronto, Ontario M5B 1W8, Canada
[e]McMaster University, Biomedical Engineering, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada

Abstract. Patches of atrophy of the retinal pigment epithelium (RPE) have not been described in rodent models of retinal degeneration, as they have the clinical setting using fundus autofluorescence. We hypothesize that prelabeling the RPE would increase contrast and allow for improved visualization of RPE loss *in vivo*. Here, we demonstrate a new technique termed "delayed near-infrared analysis (DNIRA)" that permits ready detection of rat RPE, using optical imaging in the near-infrared (IR) spectrum with aid of indocyanine green (ICG) dye. Using DNIRA, we demonstrate a fluorescent RPE signal that is detected using confocal scanning laser ophthalmoscopy up to 28 days following ICG injection. This signal is apparent only after ICG injection, is dose dependent, requires the presence of the ICG filters (795/810 nm excitation/emission), does not appear in the IR reflectance channel, and is eliminated in the presence of sodium iodate, a toxin that causes RPE loss. Rat RPE explants confirm internalization of ICG dye. Together with normal retinal electrophysiology, these findings demonstrate that DNIRA is a new and safe noninvasive optical imaging technique for *in vivo* visualization of the RPE in models of retinal disease. [DOI: 10.1117/1.JBO.19.7.076007]

Keywords: near-infrared; *in vivo* optical imaging; retinal pigment epithelium; indocyanine green.

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1 Introduction

Blinding eye disease, such as nonexudative (dry) age-related macular degeneration (AMD), and inherited retinal degenerations are characterized by patchy loss of the retinal pigment epithelium (RPE) and its overlying photoreceptors. Clinical evaluation of affected individuals identifies the presence of so-called "window defects" in the outer retina/RPE through which an observer gains unobstructed view to the underlying tissue.[1] In patients, these patches of RPE loss appear hypofluorescent when evaluated with fundus autofluorescence (FAF) imaging, a noninvasive technique that has transformed the ability to locate and quantify disease of the RPE.[2] FAF uses blue light to stimulate lipofuscin, a fluorescent byproduct of cellular activity and stress that accumulates in the RPE layer during aging and disease.[3,4] Despite its significant role in the clinic, FAF has been only recently applied to preclinical models of disease, but the observations of aged or mutant mice have identified punctate hyperfluorescent spots rather than regions of hypofluorescence.[5,6] We propose this may be due to insufficient optical contrast between normal and absent RPE in the rodent eye, resulting in no evident differences between areas of normal and absent cells. As such, we asked whether the RPE could be made more visible using a fluorescent dye that was compatible with current *in vivo* imaging systems such as confocal scanning laser ophthalmoscopy (cSLO), and whether such prelabeling would increase contrast and make areas of induced RPE loss evident.

Indocyanine green (ICG) is an FDA-approved dye used for ocular angiography due to its optical properties in the near-infrared (NIR) spectrum, resulting in the ability to view the deep choroidal blood vessels in addition to retinal vessels. ICG angiography is normally assessed during the transit phase or in the minutes thereafter to detect vascular abnormalities, such as leakage or anomalous anatomy.[7–9] However, in this study, we use ICG to observe the RPE layer *in vivo* in the days and weeks following systemic injection, and suggest that this technique is valuable in the preclinical modeling of disease and for the development of novel pharmaceutical, biological, or regenerative treatments.

2 Material and Methods

2.1 *Animal Studies*

Animals were handled in accordance with the Association for Research in Vision and Ophthalmology guidelines for the humane use of animals in ophthalmic research, and according to the Canadian Council on Animal Care guidelines. Animals were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg), and pupils were dilated with a single drop of 0.8% tropicamide in 5% phenylephrine hydrochloride solution (Diophenyl-T, Sandoz Canada Inc). GenTeal lubricating eye drops (Novartis, Canada) were repeatedly applied to the corneal surface during all procedures.

*Address all correspondence to: Shelley R. Boyd, E-mail: boyds@smh.ca

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2.2 *Confocal Scanning Laser Ophthalmoscopy*

In vivo images were acquired using a commercially available cSLO (Heidelberg Retinal Angiography, HRA-2, Heidelberg Engineering, Germany). Images were obtained in the red-free, FAF (488/500 nm excitation/emission), IR reflectance (830 nm), and ICG fluorescence (795/810 nm excitation/emission) channels.

2.3 *Indocyanine Green and Fluorescein Angiography*

ICG dye (Cardiogreen, Sigma) was freshly prepared prior to experimentation to a final stock concentration of 5.0 mg/ml in sterile water. A 24-gauge catheter was inserted into the tail vein, and ICG dye was infused at doses of 0.35 or 5.0 mg/kg. Images were taken prior to injection (baseline), during dye circulation and at various intervals thereafter out to 20 min. In a subset of animals, 200-kD fluorescein-dextran (Sigma) at 5.0 mg/ml was injected via tail vein catheter to yield a final dose of 5.0 mg/kg. ICG and fluorescein angiography were performed simultaneously in a subset of animals only; otherwise fluorescein was not injected. Angiographic images were obtained in the fluorescein and ICG channels with excitation and emission filters of 488/500 and 795/810 nm, respectively.

2.4 *Delayed Near-Infrared Analysis*

Delayed near-infrared analysis (DNIRA) images were obtained in the days and weeks after ICG injection using the ICG angiography settings, with excitation/emission filters in place, but without reinjecting the dye after day 0. Images were taken 2/3, 7/8, 21, and 28 days after angiography. Angiography was not performed again during the time course of the study.

2.5 *Sodium Iodate*

In a subset of animals ($n = 7$), the RPE toxin sodium iodate ($NaIO_3$, Sigma) was injected systemically at a dosage of 45 mg/kg body weight, via a 24-gauge catheter inserted into the tail vein. In these animals, ICG injection (0.35 mg/kg) and angiography were performed at day 0 immediately prior to $NaIO_3$ injection.

2.6 *Ex Vivo Retinal Pigment Epithelium Labeling*

Rat eyes were enucleated, and posterior eyecups devoid of overlying retina were isolated in RPMI-1640 cell culture media (Wisent Inc., St-Bruno, Canada). RPE monolayers were mechanically transferred from posterior eye cups onto polylysine-coated microscope slides by apposition for 1.5 h at room temperature. Regions of transferred RPE cells were outlined using a hydrophobic barrier pen, and their presence was confirmed by white-light microscopy using a Nikon Eclipse (Mississauga, Canada) TS100 microscope with DS-Fi1 digital sight capture imaging system. ICG at 0.25 mg/ml was added in cell culture media at 200 μl total volume per slide and incubated with RPE cells for 30 min at 37°C and 5% CO_2. Cells were washed with PBS and imaged a second time. RPE layers were, thereafter, permeabilized with 0.5% Triton X-100 for 15 min at room temperature, washed with PBS, and re-evaluated a final time by white-light microscopy. In a subset of animals, RPE monolayers were obtained 24 h following injection of systemic ICG or saline control, and the slides were observed using the cSLO system in the ICG and FAF channels to capture the associated RPE fluorescence.

2.7 *Immunofluorescence Microscopy*

Zona occludens protein 1 (ZO-1, 402200, Invitrogen) immunofluorescence was performed using a standard protocol. Briefly, cells were fixed with 4% PFA/PBS for 15 min and blocked with 1.25% BSA in TBS for 30 min at room temperature. Primary rabbit anti-ZO-1 was used at 2.5 μg/ml in 1.25% BSA/TBS and incubated for 1 h at room temperature. Cells were washed in TBS and incubated with goat anti-rabbit 488 (Invitrogen) at 5 μg/ml for 1 h at room temperature, washed, and counterstained with To-Pro-3 (Invitrogen). Some RPE monolayers were stained with 647 nm conjugated phalloidin (Invitrogen) for 20 min at room temperature and counterstained with Sytox green nuclear stain (Invitrogen). Slides were mounted with fluorescent mounting medium (Dako), and images were acquired using a Leica TCS SL confocal fluorescent microscope (Leica Microsystems, GmbH, Wetzlar, Germany), with Leica Confocal Software Version 2.61.

2.8 *Electroretinography*

The bright-flash ERG response was evaluated in animals using the Espion (Diagnosys LLC, USA) mini-Ganzfeld system following high (5.0 mg/kg) and low (0.35 mg/kg) dose administration of ICG, or saline control. Following anesthesia, animals were placed on an electrically silent heating pad and gold coil electrodes placed at the edge of the cornea after application of GenTeal lubricating drops. Following a short train of dim flashes (0.01 candelas/m^2, 1.0 Hz), the photopic b-wave response was evaluated using a single bright flash (3 candelas/m^2) that we previously determined consistently approximates the maximum b-wave amplitude.

2.9 *Statistical Analysis*

Three groups of ERG b-wave amplitudes were compared: control (no ICG, $n = 6$), low-dose ICG (0.35 mg/kg, $n = 8$), and high-dose ICG (5.0 mg/kg, $n = 10$). A two-tailed paired Student's t-test with a confidence interval (CI) of 95% was performed to compare the initial day 0 and final day 21 b-wave amplitudes for each group. For each eye, $t = 0$ values were subtracted from $t = 21$ values and the change in amplitude over time between groups was then compared. A small downward trend was noted in all groups including controls, which prompted a comparison of the slopes using analysis of covariance (ANCOVA). Normalized values were plotted against time, and a linear regression was determined for each group using GraphPad Prism 5.0 statistical software. "Compare slopes" function was used to perform ANCOVA using a CI of 95%.

3 Results and Discussion

Representative baseline (pre-ICG) cSLO findings in the SD rat eye are shown in Fig. 1(a), which, along with saline-treated animals in Fig. 2(a), serve as normal controls for all experimentations. Red-free (green dominant) imaging identifies the optic nerve head (ONH), radial blood vessels of the retina, and nerve fiber layer. Endogenous IR reflectance (830 nm) also identifies the vasculature with some imaging of the deeper choroidal vessels. Consistent with human studies, FAF of the rat eye

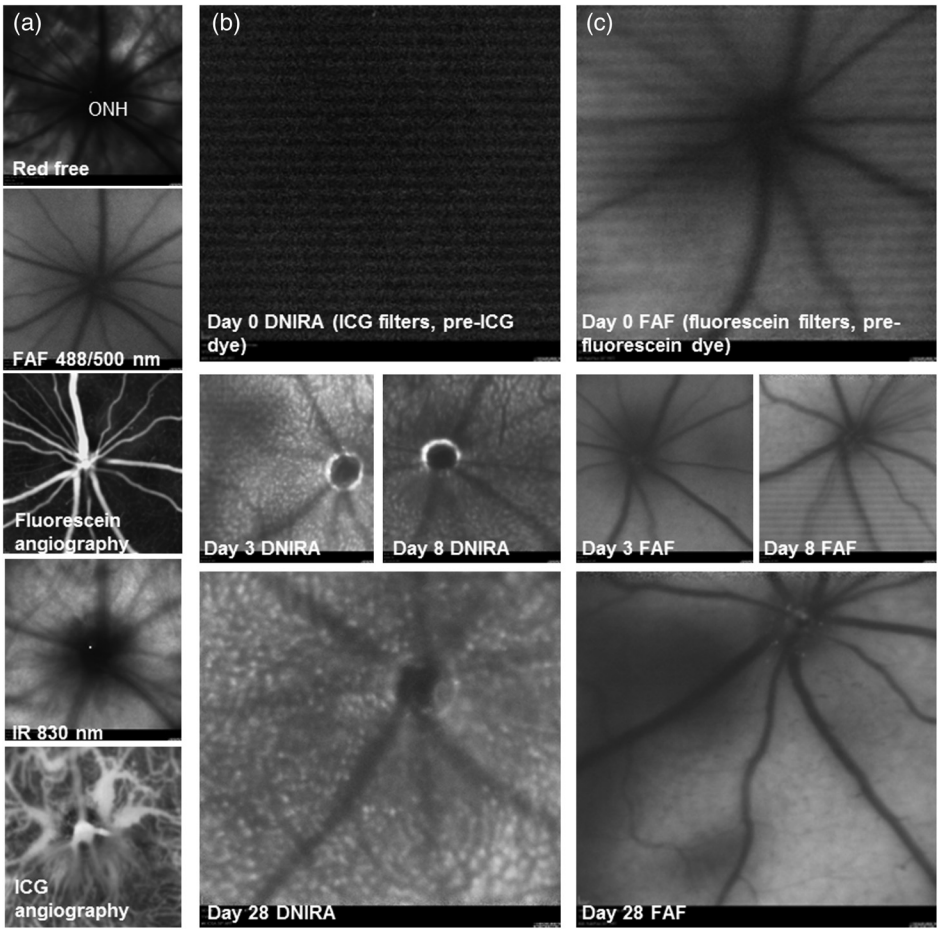


Fig. 1 Fluorescence detected by DNIRA in the 795/810 nm channel of the cSLO in the days following ICG dye administration. (a) Normal fundus images of wild-type SD rats obtained using red-free, FAF, and fluorescein angiography (488/500 nm), IR reflectance (830 nm), and ICG angiography (795/810 nm) wavelengths. (b) Time course of DNIRA at 795/810 nm with a single injection of 5 mg/kg ICG dye at day 0. No detectable signal is seen prior to dye injection. In the days after angiography (lower rows), there is an increased speckled or punctate background fluorescence compared to baseline. The signal remains strong and distributed throughout the fundus out to 28 days after injection. (c) FAF at 488/500 nm shows no change following fluorescein administration compared to prior any dye.

produces an image characterized by a faint, homogeneous glow that is lacking in detail, is obscured by the radial retinal blood vessels, and decreases nearing the ONH.[2,3] Fluorescein and ICG angiography show normal vasculature of the retina and choroid, respectively. Without ICG dye, but with the same NIR excitation and emission filters in place, no or negligible signal is observed in the eye [Fig. 1(b)]. Scan lines are evident, and the normally prominent retinal vasculature is barely or not detectable. Lack of NIR signal was observed in over 60 eyes. However, similarly obtained images acquired at days 3, 8, and 28 after a single injection of ICG dye demonstrate a delayed and persistent fluorescence in the NIR channel with the 795/810 nm excitation/emission filters. This persistent, delayed fluorescence is not observed in similar experiments performed following angiography with fluorescein-dextran dye analyzed with the fluorescein 488/500 nm excitation/emission filters in place, where the signal does not differ between baseline (preinjection) and any subsequent time point [Fig. 1(c)]. Qualitative analysis of the delayed NIR fluorescence after ICG generally shows a "mosaic" pattern of small speckles in the posterior pole that are external to the retinal vessels and obscure the view to the choroidal vasculature, suggesting its location between the retina and the choroid. Due to the high curvature of the rodent fundus compared with the human eye and the flat confocal plane of the cSLO, the view of the monolayer can vary with the plane of focus captured slightly above or below the RPE. This can result in blurring of the speckled appearance, and make the retinal or choroidal blood vessels more evident. The ONH and circumpapillary area do not fluoresce. These features are similar to fluorescence patterns obtained using FAF in patients.[3] We determined that the same plane of focus is optimal for ICG-dependent fluorescent imaging as that normally used for FAF, and termed our novel technique DNIRA.

To confirm our hypothesis that DNIRA was dependent on ICG labeling, we performed dose-response experiments. Figure 2 demonstrates that the higher the initial ICG dosage used for angiography, the brighter the DNIRA signal in the days thereafter. The fluorescence does not occur in the absence of previous ICG injection [Fig. 2(a)]. The high dose (5 mg/kg) required that the gain (sensitivity) of the cSLO be reduced to obtain suitable quality images, while the low dose (0.35 mg/kg) was less bright, and required that the gain to be set at higher levels. Comparative images taken with the gain set at the same level demonstrated a dose-dependent

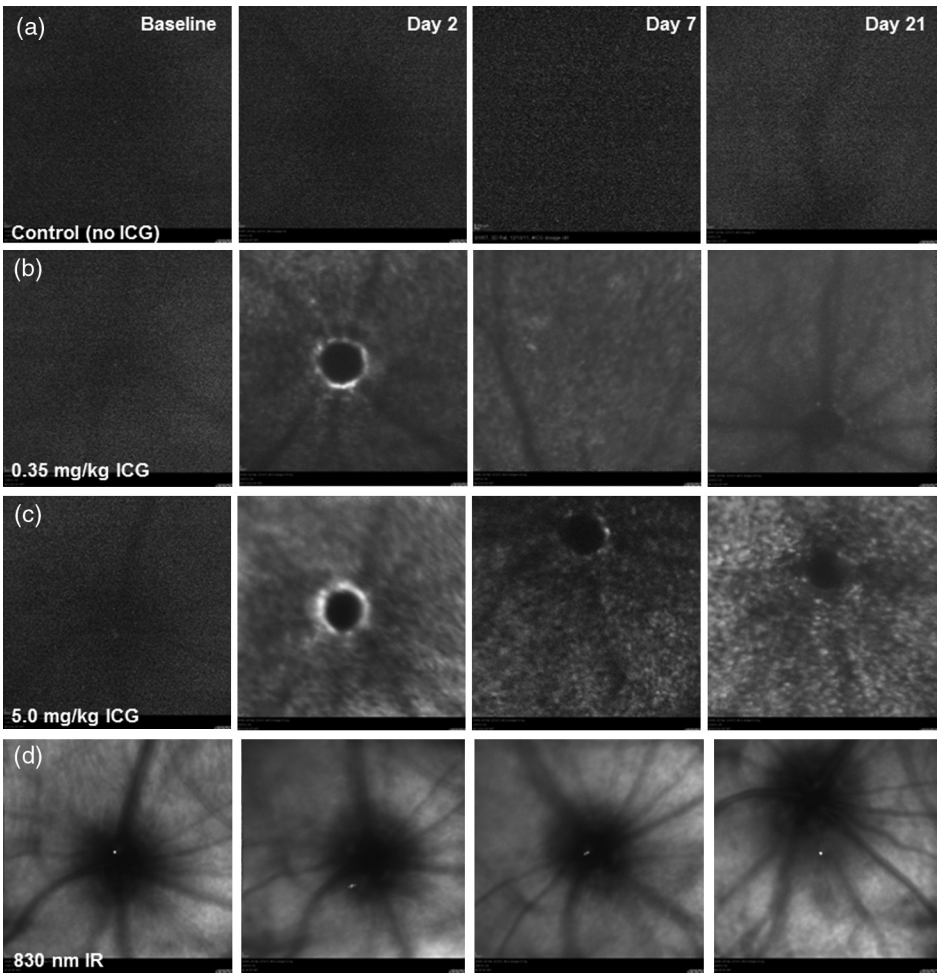


Fig. 2 DNIRA signal intensity is ICG dose and time dependent, and requires the 795/810 nm ICG excitation/emission filters. (a) Control cSLO images illustrate absence of 795/810 nm fluorescent signal without ICG dye injection. Control animals that do not receive ICG maintain the same level of background fluorescence with negligible signal over the 21-day period evaluated. (b) Using the same gain, DNIRA is readily detectable 2 days after ICG injection when given at the low dose of 0.35 mg/kg. With time there is a gradual fading from 2 to 21 days postinjection. (c) Using the same gain, 5 mg/kg ICG dose results in brighter (higher intensity) DNIRA signal at 2 days than the lower dose. This fluorescence persists largely unchanged out to 21 days. (d) The 830 nm IR reflectance channel, which does not utilize the ICG filters, does not show similar change despite administration of the dye.

increase in brightness [Figs. 2(a)–2(c)]. In the same experiment, we also show that by day 21 the speckled pattern faded in animals that received low-dose ICG [Fig. 2(b)]. By contrast, in animals that received high dose ICG, there was little appreciable difference between days 2 and 21 postinjection [Fig. 2(c)]. Further, we demonstrate that the IR reflectance imaging at 830 nm does not yield similar dose-dependent and time-dependent signals [Fig. 2(d)]. This indicates that the observed fluorescence is not intrinsic to the tissue in the NIR or IR wavelengths, but a property of the ICG dye, as it is dependent on the dye concentration and requires the correct excitation and emission settings be in place.

To further address our hypothesis that RPE cells are the source of *in vivo* fluorescence following systemic ICG injection, we incubated fresh RPE monolayers with ICG dye *ex vivo* and evaluated them by white-light imaging to detect their visible green color. Figure 3 outlines a schematic of the experiment, and confirms ICG internalization by explanted RPE monolayers. Transferred monolayers were evident using white-light microscopy and stained positive for actin-binding phalloidin and ZO-1 protein for tight junctions, confirming RPE integrity following transfer and prior to experimentation [Fig. 3(a)]. ICG was visualized as green using white-light microscopy only after dye incubation and not before [Fig. 3(b)]. The green color was no longer detectable in the RPE monolayers following permeabilization of their membranes, and reverted from green to colorless, suggesting that the internalized dye was released. As it was not possible to perform 795/810 nm microscopy on posterior eye cups due to the lack of instrumentation at this wavelength, we performed low-magnification imaging of RPE monolayers transferred to glass slides using the cSLO, after *in vivo* investigation [Fig. 4(a)]. Isolated RPE from ICG-injected rats sacrificed at least 24 h after dye injection shows a bright cellular fluorescence in the ICG channel of the cSLO. This fluorescence is absent in RPE from saline-treated rats, and similar to baseline *in vivo* imaging, the ICG channel shows no signal. The presence of RPE cells in the areas of ICG fluorescence is confirmed by concomitant faint fluorescence in the FAF channel, which is

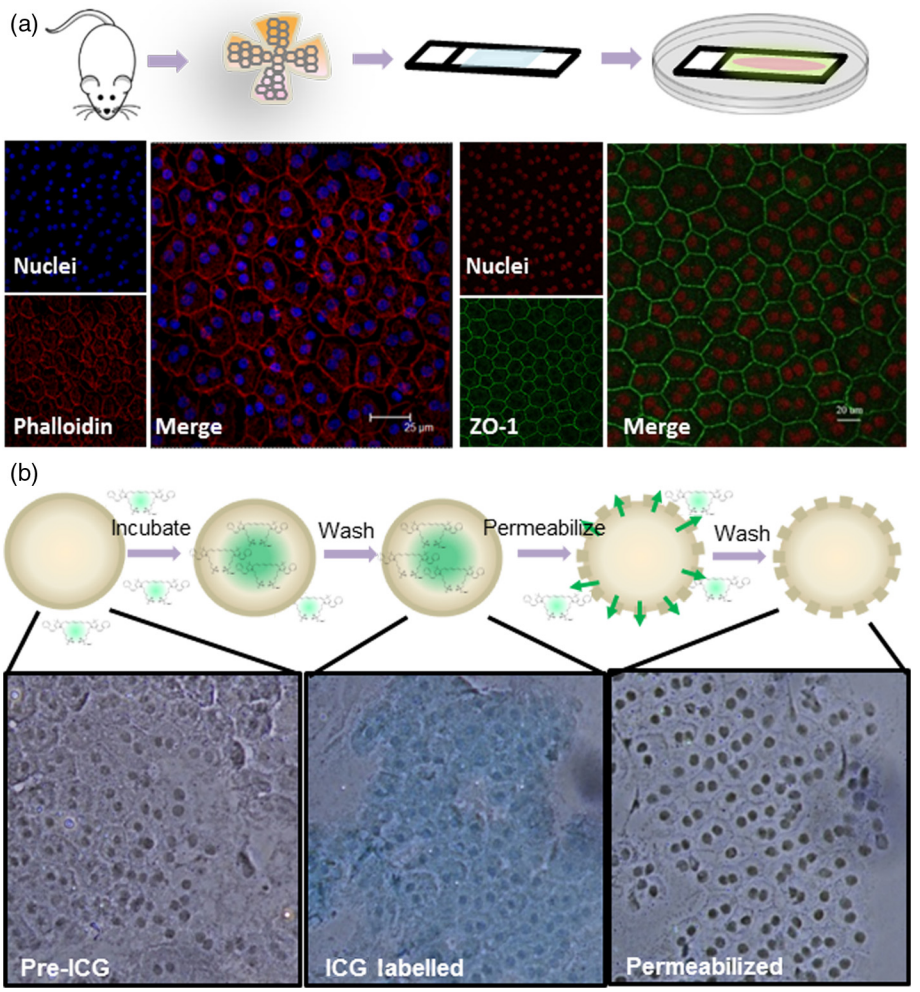


Fig. 3 RPE monolayers are capable of internalizing ICG dye *ex vivo*. (a) Diagram of RPE monolayer transfer shows that naïve rat posterior eyecups can be dissected in cell culture media, RPE transferred onto polylysine-coated slides, and incubated with ICG dye at physiological conditions. Morphology and integrity of transferred RPE are confirmed by 647 nm fluorescent-tagged phallodin (red, left) and ZO-1 protein (green, right). Nuclei are shown in blue (left) or red (right). (b) Diagram of RPE labeling and permeabilization shows cells as colorless when viewed with light microscopy prior to ICG incubation (left). RPE incubated with ICG dye internalize green color (center). Following permeabilization RPE release the ICG into solution and revert to colorless (right).

consistent with FAF emitted by RPE *in vivo*. This FAF signal is also evident in RPE from saline-treated animals and is not dependent on ICG dye. Accompanying white-light microscopy confirms the presence of the typical hexagonal pattern of binucleate RPE cells in the areas of fluorescence. These results are consistent with previously published studies reporting that cultured primary RPE cells or RPE cell lines take up ICG *in vitro*.[10,11] Furthermore, it has been shown that the injection of ICG for angiography can result in accumulation of dye at the RPE/Bruch's membrane complex in primates.[12] Together, these data confirm that RPE monolayers are capable of taking up ICG dye, strengthening our premise that the RPE is the source of the ICG fluorescence observed *in vivo* using DNIRA.

To further support this hypothesis, we proposed that DNIRA could be used to identify areas of RPE loss by coupling this technique with systemic injection of $NaIO_3$, which is a known RPE toxin.[13,14] Figure 4(b) confirms that DNIRA performed in the days after $NaIO_3$ injection identifies patches of profound hypofluorescence, evident as large dark areas within an otherwise continuous background of speckled fluorescence. Direct viewing through these patches permits clear visualization of the choroidal detail (enlarged box). Choroidal vessels are identified by their complexity, variable size, and nonradial pattern with respect to the ONH. By contrast, the speckled layer in non-$NaIO_3$-treated animals [Fig. 2] generally obscures the view of the choroidal vessels. These data are consistent with the notion that DNIRA detects ICG-labeled RPE, with the speckled fluorescent signal lost in areas of RPE loss.

Despite its known safety following systemic injection for clinical angiography in patients, previous studies have demonstrated that high-dose ICG dye can be toxic to RPE cells *in vitro*.[9,15,16] Similar concerns exist during ophthalmic surgery when ICG dye is injected intravitreally to help with visualization of diaphanous epiretinal membranes.[17,18] Given that we now suggest *in vivo* labeling of the RPE monolayer as a useful tool for investigating the rodent eye, we wished to determine

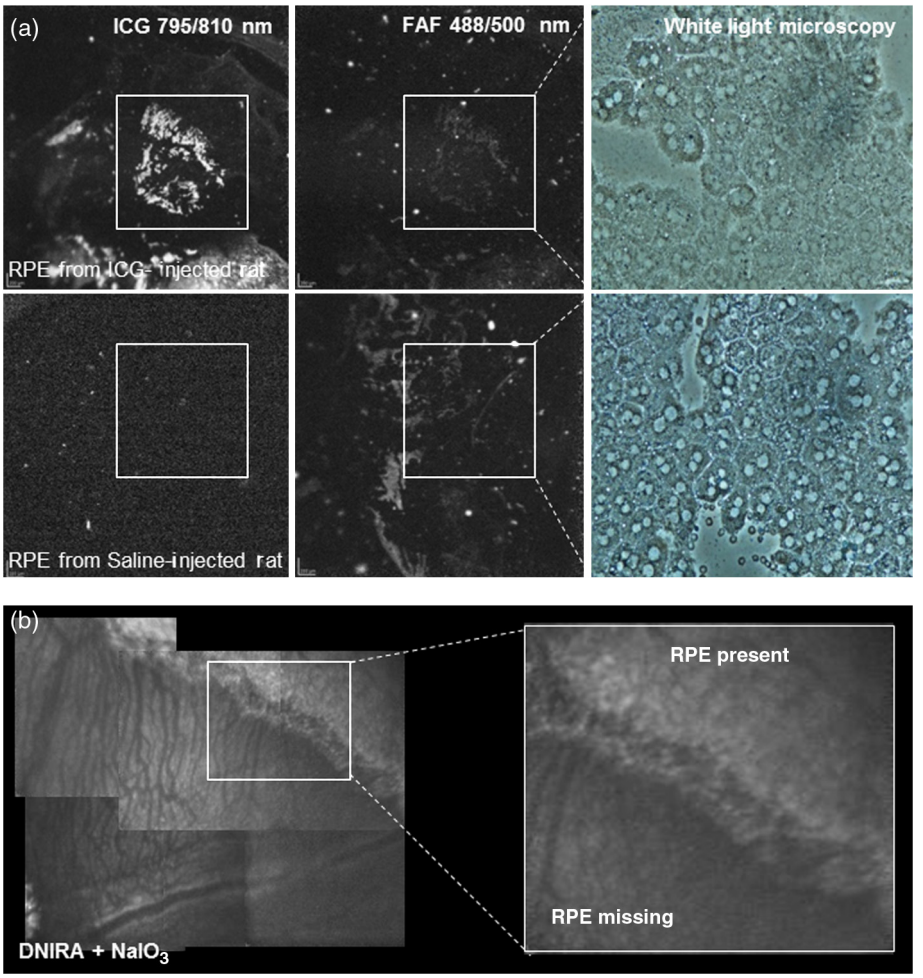
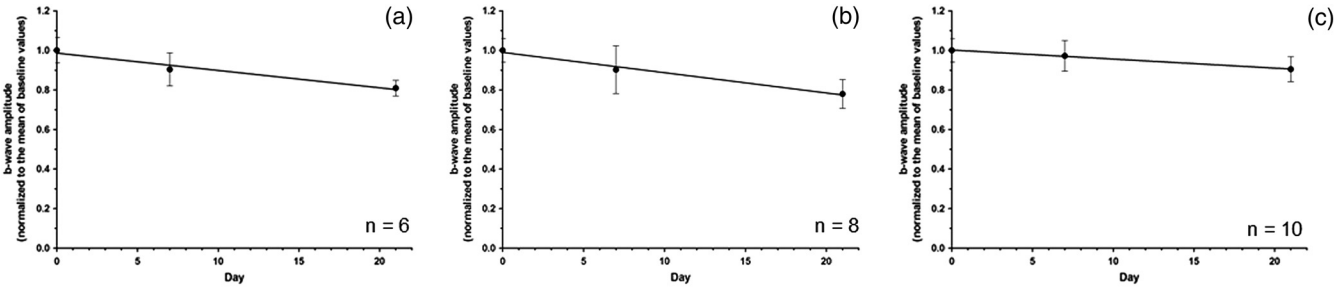


Fig. 4 The RPE monolayer is the source of *in vivo* ICG signal detected using DNIRA. (a) Monolayers isolated on polylysine slides from rats injected with ICG and viewed with the ICG channel of the cSLO display a bright cellular fluorescence that is absent in RPE layers isolated from control rats (white box). Similar analysis using the FAF channel of the cSLO displays faint fluorescence in both conditions confirming the presence of the cells on the slides dependent on their autofluorescent properties at 488 nm. White-light microscopy identifies the presence of hexagonal binucleate RPE cells in regions of fluorescence. (b) Following systemic injection of $NaIO_3$, the RPE is lost, and the speckled hyperfluorescent signal is also lost showing areas of hypofluorescence with choroidal vasculature readily evident in the ICG channel of the cSLO. Enlarged box (right) shows the junction of absent and present RPE using DNIRA.

whether it was toxic *in vivo* to the retina at the intravenous doses given. We therefore evaluated the bright-flash b-wave amplitude following high- and low-dose administration of ICG, and show that the ERG response does not differ between these two groups, nor between these two groups and saline-injected animals [Fig. 5]. A slight downward trend observed in all groups including controls prompted regression analysis using ANCOVA of normalized slopes. ANCOVA showed that there was no statistically significant difference among all three groups. The slight downward trend may be inherent to SD rats at this age and is not considered biologically significant.

Extravascular accumulation of ICG dye within choroidal stroma and the RPE/Bruch's membrane complex has been described using histological analysis of the nonhuman primate eye enucleated during the early, mid, and late phases of ICG angiography.[12] Such leakage is consistent with the highly fenestrated nature of the choroidal vessels.[19] In the present study, due to both the prolonged fluorescence of the RPE and the absence of ICG in the extravascular retina, we reason that RPE cells take up ICG through their basal surface, potentially through an ATP-dependent active transport process.[10,12] When injected into the systemic circulation, ICG binds to proteins such as albumin and lipoproteins such as LDL and HDL.[20] RPE cells are known to contain receptors for lipoproteins, and the uptake of cholesterol into the retina occurs mainly via the RPE.[21,22] It has also been shown that RPE cells can be effectively labeled for postenucleation study by injection of systemic rhodamine-conjugated LDL.[23] Presently, we show that the *in vivo* labeling of RPE can be accomplished by systemic injection of ICG that we propose may be due to the lipoprotein-binding characteristics of the dye and its uptake by basolateral RPE receptors.

In summary, we describe a novel technique, DNIRA, for identifying the RPE layer *in vivo* in the rodent eye. This technique capitalizes on a persistent extravascular NIR fluorescent signal that occurs after ICG injection, is dose dependent, persists for at



Group	Slope of Regression
Control (0 mg/kg ICG)	-0.004571 ± 0.004325
Low Dose (0.35 mg/kg ICG)	-0.01025 ± 0.005730
High Dose (5.0 mg/kg ICG)	-0.008768 ± 0.004245

Comparison of Slopes	P value	Significance
Control vs. Low	P = 0.4438	No
Control vs. High	P = 0.5045	No
Low vs. High	P = 0.8323	No
All 3 slopes	P = 0.7175	No

Fig. 5 ICG administration at low and high doses does not result in retinal toxicity *in vivo*. ERG response of the b-wave amplitude between t = day 0 and t = day 21 shows no significant decrease in the low (b, 0.35 mg/kg) or high (c, 5.0 mg/kg) ICG dose when compared to control (a). The amplitude of the dark-adapted bright flash response showed a slight decrease in all groups including control over the course of experimentation. Data were normalized to allow for slope comparison. ANCOVA showed no significance in the downward trend among all three groups.

least 4 weeks, and is eliminated in discrete patches when the RPE layer is lost. DNIRA is distinct from autofluorescence imaging in that it requires prior injection of a fluorescent dye so is not endogenous, and must be performed using the excitation/emission filters normally used for angiography. However, unlike angiography, photos are obtained in the days and weeks following dye injection, not during the transit stage or minutes thereafter. We suggest this to be a powerful new method for assessing preclinical models of disease, for identifying RPE toxicity during drug or biomaterial toxicity testing, and for evaluating therapeutic outcomes in pharmacological or regenerative studies.

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Natalie Pankova is a PhD candidate in the Department of Laboratory Medicine and Pathobiology at the University of Toronto, pursuing a direct entry PhD. She received her Honours BS degree from the University of Toronto in 2011, and spent a year working in the pharmaceutical and clinical industries before commencing a graduate degree specializing in retinal biology and immunology, with a focus on *in vivo* retinal imaging under the supervision of Shelley Boyd.

Shelley Boyd is a clinician-scientist in the Keenan Research Centre for Biomedical Science of St Michael's Hospital in Toronto. After graduate training in molecular neurobiology, she studied medicine, and she is a fellow of the Royal College of Surgeons of Canada, with specialization in ophthalmology. She is an assistant professor in the Departments of Ophthalmology and Vision Sciences, and Laboratory Medicine and Pathobiology at the University of Toronto, and has a cross-appointment to McMaster University's School of Biomedical Engineering.

Biographies of the other authors are not available.