Supplemental Document

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In situ autofluorescence lifetime assay of a photoreceptor stimulus response in mouse retina and human retinal organoids: supplement

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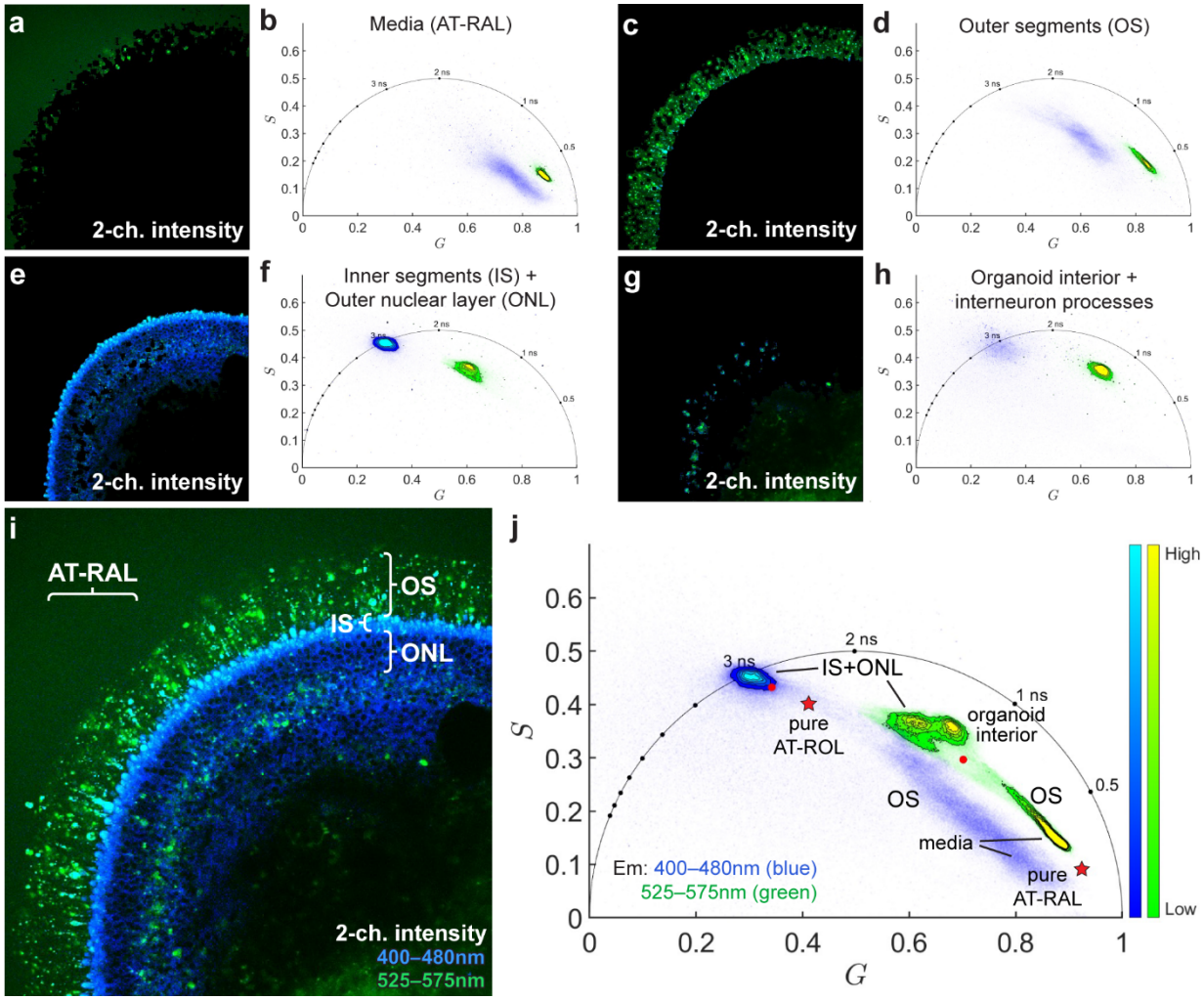

Fig. S1. Phasor-based segmentation of the intensity image for a retinal organoid treated with AT-RAL. Distinct groups of pixels on the phasor plot of the green channel were selected and separately mapped back to the intensity image to create masked 2-channel intensity sub-images. (a,b) Background (media) pixels and their corresponding phasor plot. (c,d) Outer segments (OS) and their corresponding phasor plot. (e,f) Inner segments (IS) and outer nuclear layer (ONL), and the disorganized deeper photoreceptors, and their corresponding phasor plot. (g,h) Organoid interior and interneuron processes, and their corresponding phasor plot. (i,j) Merged intensity image of all layers and its corresponding phasor plot. Red dots represent intensity-weighted phasor centroid of the organoid in either channel. Red pentagrams represent phasor locations of the pure species measured in solution.

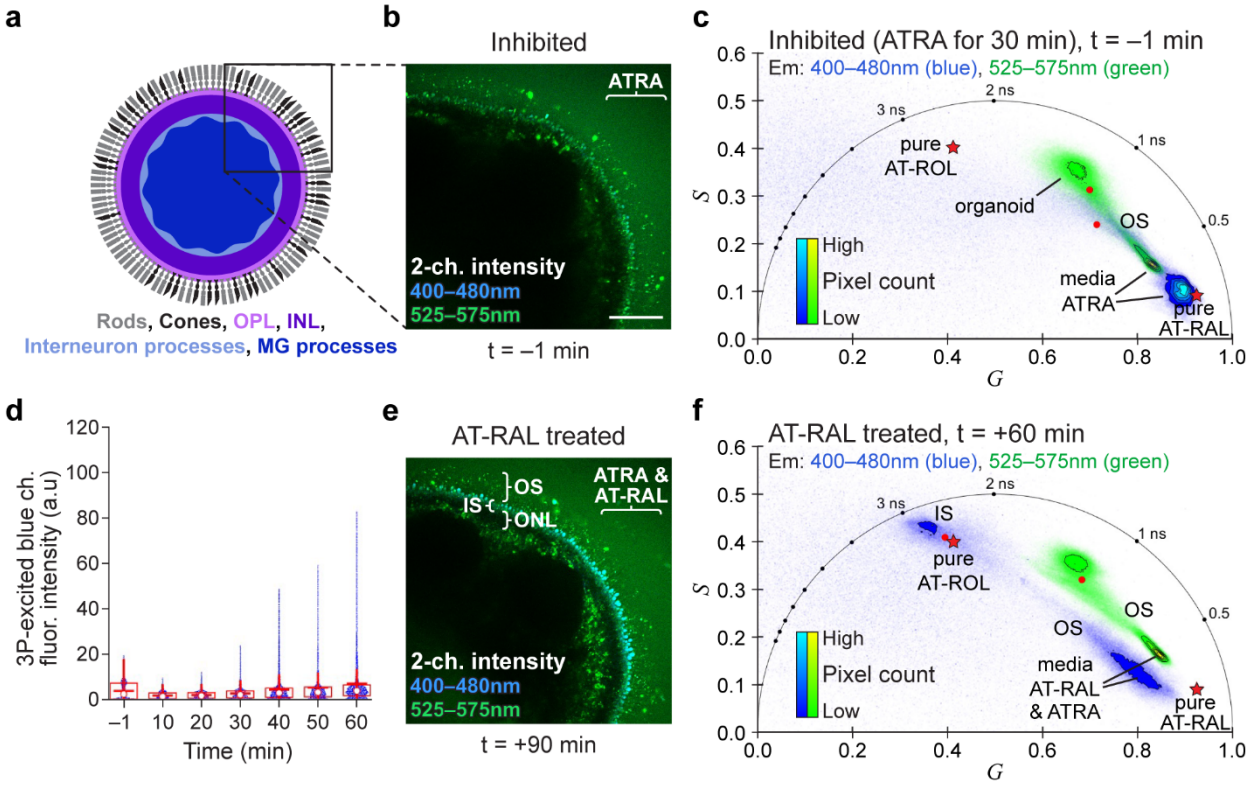

Fig. S2. Inhibition of the visual cycle using all-trans retinoic acid slows the conversion of AT-RAL to AT-ROL in retinal organoid. (a) Schematic of the 3D-cultured stem cell-derived retinal organoid. Box shows microscopy field of view. OPL: outer plexiform layer, INL: inner nuclear layer, MG: Müller Glia. (b) Two-channel fluorescence intensity image of dark-adapted and inhibited retinal organoid (day 245 in culture) after 30 mins of incubation in media with 100 µM ATRA (blue, 400-480nm; green, 525-575nm). 1040nm excitation with 15 mW average power. (c) Phasor representation of dark-adapted image shows abundant short lifetimes of ATRA in both emission channels and absence of long lifetime of AT-ROL in the blue channel. (d) Upon treatment of the organoid with exogenous 50 µM AT-RAL, which simulates strong photo-bleaching of visual pigments, the intensity of the 3P-excited fluorescence in the blue emission channel increases over the course of 1 hour, at a slower rate than the uninhibited organoid (in Fig. 5). White dot shows the median; red horizontal line shows the mean; box encompasses 25th to 75th percentile range; whiskers extend from the box to 1.5 times the interquartile range. Change in mean intensity with time is significant at the 5% significance level according to the linear trend test (t-statistic=2.6, p=0.045). (e) Two-channel fluorescence intensity image of the retinal organoid 60 min after treatment with AT-RAL shows a small increase in the blue channel signal, due to conversion of AT-RAL to AT-ROL, in the photoreceptor cells, particularly in the inner segments (IS). (f) Phasor representation of the treated organoid image reveals sparse long lifetime of AT-ROL in the blue channel, while the exogenous ATRA and AT-RAL in the media (i.e., background seen around the organoid and at the upper right corner of the intensity image) appear with their characteristic short lifetimes in both emission channels on the phasor plot. Red dots represent intensity-weighted phasor centroid of the organoid in either channel. Red pentagrams represent phasor locations of the pure species measured in solution. Scale bar: 100 µm.

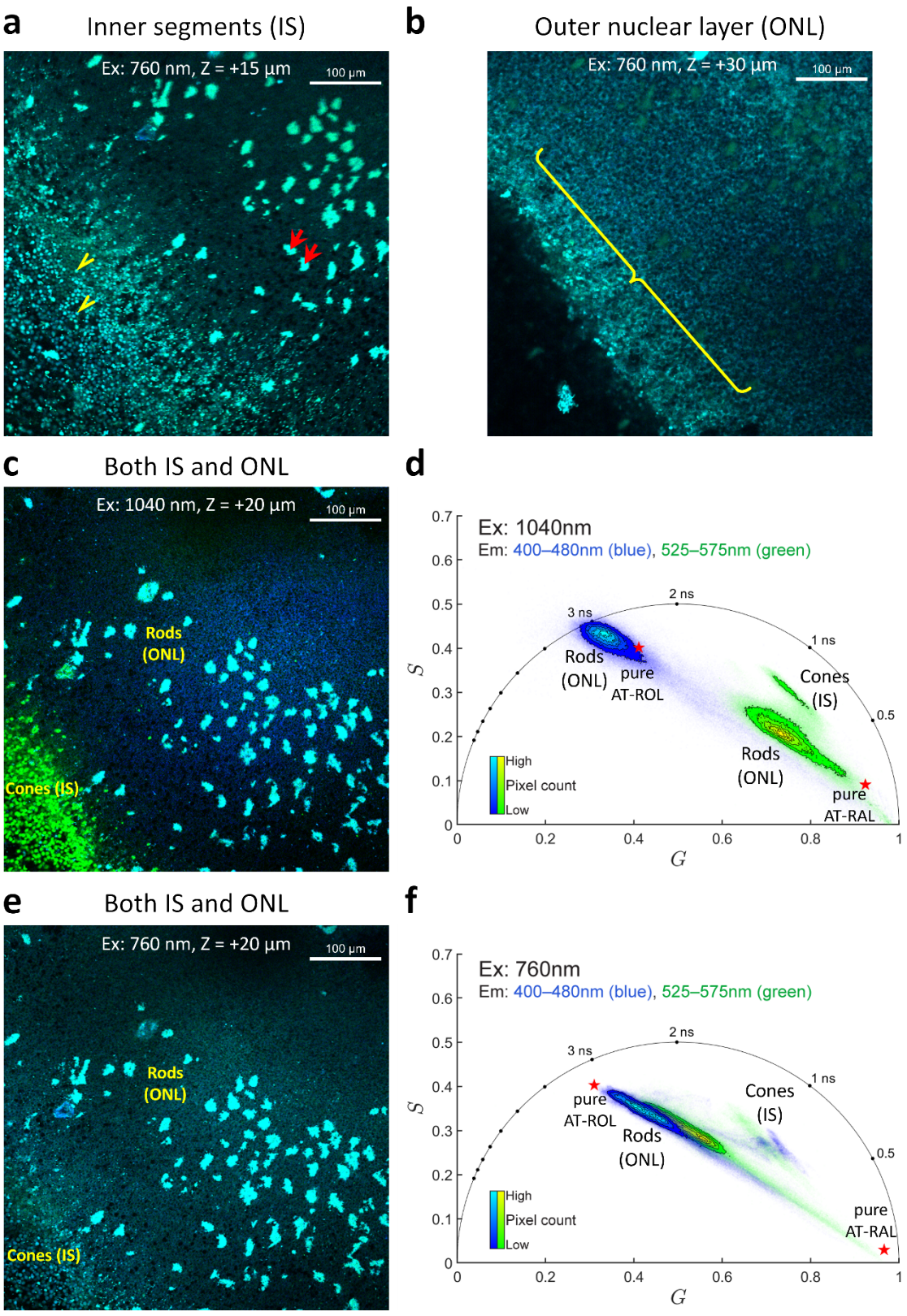

Fig. S3. Depth-resolved imaging shows FLIM contrast between cone and rod photoreceptors. (a) Two-channel autofluorescence (blue, 400-480nm; green, 525-575nm) intensity image of an RPE-dissociated mouse retina explant one hour after photobleaching with white light for one

minute, using 760 nm multiphoton excitation, shows the superficial layers (outer and inner segments in the lower left corner of the field of view) that have a high concentration of cone photoreceptors (yellow caret). Larger cyan blobs (red arrowheads) are pigmented debris left over from the RPE dissociation procedure. (b) The deeper section shows rod somas forming the outer nuclear layer in the middle and upper right side of the FOV (curly bracket). (c) Intensity image at an intermediate depth shows both IS and ONL under 1040 nm excitation. The brighter inner segments, presumed to be cones, are prominent in the green emission channel. Rod somas are prominent in the blue emission channel. (d) Phasor representation of the image in (c) shows rod somas expressing the long lifetime of AT-ROL the blue channel, and the short lifetime of AT-RAL in the green channel. The phasor representation of the cone inner segments separates from the rods and indicates presence of other fluorophores that are consistent with metabolic coenzymes (FAD and NAD(P)H) in the mitochondria. Cones do not express a detectable concentration of AT-ROL in the 3P-excited blue channel. (e) Two-channel autofluorescence intensity image of the same FOV under 760 nm two-photon excitation shows a lack of contrast between the two emission channels. (f) Phasor representation of the image in (e) shows a combination of AT-ROL and AT-RAL fluorescence from the rods in both emission channels, while the cone inner segments express a combination of AT-RAL and other metabolic fluorophores (FAD and NAD(P)H in the mitochondria) and separate from the phasor representation of the rods. Red pentagrams represent phasor locations of pure AT-RAL (lower right pentagram) and AT-ROL (upper right pentagram), as measured in solution.

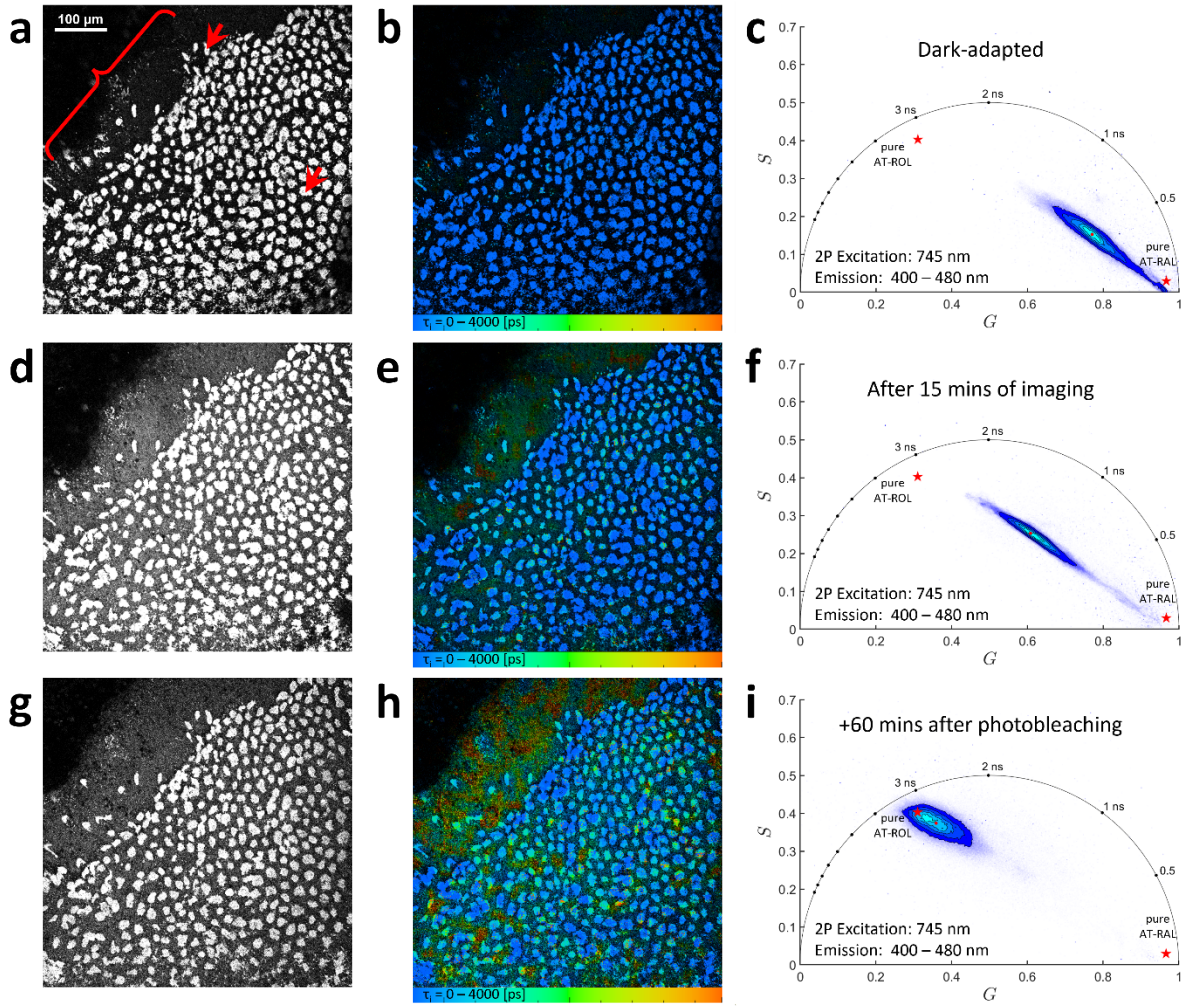

Fig. S4. AT-ROL is produced in dark-adapted mouse retina explant in response to multiphoton imaging and white light photobleaching of the sample. Two-photon FLIM was performed at 745 nm with 2 mW of power over a 500x500 µm field of view with a 20x/1.0NA WI objective lens, 512x512 pixels with a pixel dwell time of 4.8 µs. (a) Intensity image of the dark-adapted mouse retina explant in the 440/80 nm emission band. The large and bright cells (arrowheads) are left-over RPE cells from the dissociation procedure, and photoreceptors are seen in between the RPE cells and in a diagonal band in the upper left side of the field of view (curly bracket). (b) Corresponding fluorescence lifetime image of the dark-adapted sample shows short lifetimes in photoreceptors and RPE cells. (c) Corresponding phasor plot of the photoreceptor pixels in the dark-adapted sample (with the RPE cells masked out of the phasor analysis) shows short lifetimes consistent with AT-RAL. (d) Intensity image of the sample after 15 mins of 2P imaging without any other light exposure shows increase in fluorescence intensity in the photoreceptor pixels. (e) Lifetime image of the sample after 15 mins of 2P imaging shows increased lifetime of fluorescence in the photoreceptor cells while RPE cells maintain a short lifetime. (f) Phasor plot of the photoreceptor pixels shows increased lifetime along the axis connecting AT-RAL and AT-ROL loci, suggesting presence of a mixture of AT-ROL and AT-RAL in the photoreceptors. (g) Intensity image of the sample 60 mins after exposure to white light from the microscope condenser lamp for one minute to photobleach any remaining photopigments. (h) Lifetime image of the sample 60 mins after white light exposure shows longer fluorescence lifetime in the photoreceptors while RPE cells maintain shorter lifetimes. (i) Phasor plot of photoreceptor pixels shows the long lifetime of AT-ROL. Red pentagrams represent phasor locations of the pure AT-RAL (lower right pentagram) and AT-ROL (upper left pentagram), as measured in solution.