An unbiased method for evaluating changes in cell cycle progression and fate determination in the developing mouse retina

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Short Abstract: (50 words maximum)

Evaluation of cell cycle-related phenotypes following experimental manipulation requires both qualitative tissue imaging, coupled with high-throughput and unbiased quantification of DNA content. The method employs a correlative approach combining histology-based confocal microscopy with cell cycle analysis using flow cytometric evaluation of dissociated retinal explants.

Long Abstract: (150 words minimum, 400 words maximum)

Recent technical advances have allowed us to alter gene function in the postnatal mammalian retina. Of particular interest has been the use of plasmid-based vectors, which can be efficiently delivered to retinal progenitor cells via either *in vivo* or *in vitro* electroporation-based gene transfer (Matsuda and Cepko, 2004, 2007, 2008). This methodology provides a robust avenue for gene manipulation, including gain of function, expression of dominant negatives and gene silencing, as well as fate mapping with the use of fluorescent reporters. Conventional evaluation and quantification of transfected retinas involves a laborious method of confocal image acquisition, and follow-up "manual" quantification that is inherently susceptible to bias, low numbers of quantified cells, and loss of effect size (described in Pounds and Dyer, 2008). Here, we present a method of parallel explant transfection, preparation, and evaluation that is designed to allow researchers to compare high-quality histological data, with unbiased quantification of cell cycle progression and cell-type specification of transfected cells. The method is optimized for efficient and complete dissociation of retinal explants that can be used for flow cytometry and fluorescence-activated cell sorting (FACS). In addition, DNA staining of fixed cells is described, as well as procedures for cell cycle and DNA content analysis.

Procedure in brief: Retinas of postnatal mice are dissected, electroporated, and cultured as previously described (Donovan and Dyer, 2006; Matsuda and Cepko, 2004, 2007). Following treatment with one or more thymidine analogues, explants are either dissociated, or fixed and prepared for cryostat sectioning and confocal analysis. Nuclear content in fixed cells are evaluated using propidium iodide DNA staining, and flow cytometry. G0/G1, S, and G2 frequency distribution profiles, and ploidy analysis of GFP positive cells are performed using third-party algorithm software. Furthermore, high-throughput and unbiased assessment cell fates ratios are achieved through flow cytometry-based evaluation of explants co-electroporated with cell type-specific reporter constructs. This flow cytometry-based quantification method complements histological data, which itself, permits the profiling of cell distribution and morphological maturation of transfected progeny.

Protocol Text:

1.) Postnatal retinal explant dissection and in vitro electroporation

Preparation

1.1.1) *Dissection and electroporation*: ***Note: All retinal dissection and electroporation steps should take place inside a sterile laminar-flow or microzone cabinet***. Prepare counter space for decapitation and enucleation in close proximity to a sterile cabinet – include scissors, no. 5 forceps and 70% ETOH. The cabinet should contain a dissecting microscope and light source, two pairs of no.55 forceps, 1 pair of no.5 forceps, 1000 ul pipette with wide bore tips, 200 ul pipette with tips, 20 ul tips, prepared DNA solutions (see below), 100 ul electroporation chamber attached to electroporator, explant dissection dish containing pre-warmed HBSS without calcium or magnesium (-/-), media dish containing pre-warmed DMEM, explant wash dish containing pre-warmed HBSS(-/-).

1.1.2) *Explant culture dish preparation (modified from Matsuda and Cepko, 2008)*: Prepare 6-well culture dishes for explants culturing: 2.5 ml per well of explant media (Neurobasal media containing 1X Glutamax and 1X B27 supplement). Place a single polycarbonate membrane in each well with the shiny surface facing up. Place in incubator.

1.1.3) *DNA preparation*: calculate the volume of DNA required to attain a **final** concentration of 1-1.5 ug/ul. For electroporation of 2-4 explants, prepare a total volume of 150 ul DNA in HBSS (+/+). For 5-8 explants, prepare 200 ul of DNA solution.

Procedure

1.2.1) Following decapitation of P1 mice outside of the biosafety cabinet, eyes should be quickly enucleated, briefly dipped in 70% ethanol, and placed into sterile HBSS(-/-). Retinas are dissected using two, sterile no. 55 forceps. First, puncture the corneal-scleral interface with forceps, and progressively tease away the cornea. The retina can be removed from the eye cup, and the lens and ciliary epithelium/vitreous removed from the retina. Immediately transfer the retina to pre-warmed DMEM-high glucose using a wide bore pipette.

1.2.2) Rinse retina in pre-warmed HBSS (-/-) and transfer to a 100 ul electroporation chamber. Remove HBSS and replace it with 100 ul of plasmid solution (1 ug/ul plasmid in HBSS(+/+). Using the no. 5 forceps inserted into a 20 ul pipette tip, orient the retina such that the outer (scleral) surface faces the anode (or negative pole). Electroporate using the following parameters: Mode = Low-Voltage (LV), Set voltage to 30 Volts, Pulse length = 50 ms, Number of pulses = 5, Pulse interval = 950 ms. Optional: Rotate retina such that the ventral surface faces the anode – re-electroporate, thus transfecting the peripheral retina that typically curls over during dissection and handling. Remove DNA solution and return it to the original tube containing unused DNA (DNA ratio will be partially replenished using this method). Fill electroporation chamber with 100 ul HBSS(-/-). Transfer retina back to DMEM high-glucose. When all retinas (2-4 per plasmid) are complete, transfer explants onto polycarbonate membranes and cover with approximately 15 ul of explant media. Perform 50% media changes and re-apply 15 ul of explant media daily.

2.) Preparation of retinal explants for histology

Preparation

2.1.1) *Fixation, cryoprotection, embedding and cryostat sectioning*: Prepare 4% PFA and 0.1M PBS and place on ice. Fill three, 25 mm culture dishes with approximately 5 mm of OCT compound, and setup cryo- molds containing OCT compound under a dissecting microscope.

Procedure

2.2.1) *Fixation and cryoprotection*: Transfer cultured explants into microtubes containing chilled 0.1M PBS. Remove PBS and replace it with 1.5 ml chilled 4% PFA. Incubate at room temperature for 25 minutes. Remove PFA and wash explants with chilled PBS, then replace PBS with PBS containing 30% sucrose. Place explants in 4 degrees C until explants sink to the bottom of the tube (12-20 hours).

2.2.2) *Embedding and cryosectioning*: Transfer cryoprotected explants into the 1st of 3 dishes containing OCT. Mix explants thoroughly and progressively transfer to the 2nd and 3rd dishes. Incubate explants in final OCT dish at room temperature for 1 hour. Following incubation, transfer explants into cryo-molds containing OCT compound, and arrange explants such that they are situated in appropriate positions for subsequent identification. Place cryo-mold on dry ice until completely frozen, and place in cryostat at -17 degrees C. Mount and cut at 14 um thickness, and melt sections onto adhesive-coated slides. Dry slides overnight at room temperature, and store at -20 degrees C until used.

3.) Preparation of retinal explants for flow cytometry

Preparation

3.1.1) *Dissociation and fixation*: This procedure is optimized for explant dissociation using the MACS dissociation kit. Prepare 1 ml of Solution 1 for every reaction, which should contain 2-4 explants to be dissociated together and analyzed as a pooled sample. Prepare solutions for MACS dissociation as follows: **Enzyme Mix 1** (per 1 ml) - Add 1.3 ul of 50 mM β-mercaptoethanol to 950 ul Solution 2, and then add 5 ul Solution 1; **Enzyme Mix 2** (per reaction) – 15 ul Solution 3 + 7.5 ul Solution 4. Prepare 100 ml of 0.1M PBS; 100 ml of PBS-BSA (0.5g/100 ml); 1 ml of 1% PFA per reaction; and 1 ml of 70% ETOH per reaction. Place all solutions on ice.

3.1.2) *Propidium iodide staining:* Prepare 1 ml of PBS containing 0.1% Triton X-100 for every sample to be stained.

3.1.3) ***Optional:*** *Thymidine-analogue labeling:* The use of one or more thymidine-analogues can provide detailed data on cell birth. *In vitro* working concentrations for three analogues are as follows: BrdU = 5 ug/ml; IdU = 2.5 ug/ml; CldU = 1 ug/ml. To prepare (CldU for example): weigh 0.25 g 2`-Chlorodeoxyuridine (CldU) and suspend it in 1 ml HBSS(-/-). Heat to 50 degrees C, and vortex at regular intervals until completely dissolved. Pulse cultures containing 2.5 ml media with 1 ul CldU for 30 minutes prior to dissociation. Mix thoroughly and apply 15 ul media to explants.

Procedure

3.2.1) *Explant dissociation*: Suspend explants in 3 ml PBS. Add 1ml Enzyme Mix 1 and place in 37 degree C water bath for 15 minutes, agitating tubes every 5 minutes. Add 15 ul Enzyme Mix 2, and dissociate retinas into coarse pieces using a 5 ml pipette that has been pre-wetted with PBS-BSA (pipette up and down approximately 10 times). Incubate for 10 minutes in water bath with agitation. Add 7.5 ul Enzyme Mix 2, and dissociate coarse pieces using a 1000 ul pipette tip until all coarse pieces disappear (approximately 25 times). Using a fire-polished Pasteur pipette, further triturate approximately 25 times until the solution appears completely clear. Centrifuge tubes at 3500 g for 10 minutes. Gently pour off supernatant. A small volume (approximately 50 ul) of Enzyme Mix will remain over the pellet.

Optional: preparation for microscopic analysis of dissociated cells: Apply 50 ul of dissociated cells per well in a 16 well, Permanox chamber slide containing 100 ul explant media. Incubate for 2 hours in a CO_2 incubator. Remove media, briefly wash with ice cold HBSS and fix cells for 15 minutes with -20 degree C methanol. Note: Place slides at 4 degrees C during fixation. Remove methanol and replace with ice cold 4% PFA, and fix for a further 15 minutes. Replace PFA with PBS and store at 4 degrees C until staining.

3.2.2) *Cell fixation*: With 1 ml of 1% PFA in a pipette, begin vortexing pelleted cells, and introduce PFA dropwise until the entire 1 ml is delivered. Immediately triturate cells until solution appears clear (approximately 25 times). Place sample on ice for 15 minutes. Following PFA fixation, centrifuge cells (3500g for 10 min), pour off supernatant, and repeat vortex/dropwise/trituration using 70% ETOH, and incubate on ice for 30 min. After fixation, centrifuge cells at 2500 g for 12 minutes, and pour off supernatant.

3.2.3) *Propidium iodide staining*: Resuspend cells in 1 ml PBS containing 0.1% Triton X-100 and 10 ul RNAseA (stock RNAse = 10 mg/ml) and triturate until solution is clear (approximately 25 times). Incubate for 5 minutes at room temperature, add 5 ul propidium iodide (stock solution = 50 ug/ml), and incubate further for 30 minutes at room temperature. Following incubation, centrifuge cells (2500 g for 10 min), resuspend cells in 2 ml PBS-BSA making sure to triturate until fully dissociated (approximately 25 times) and transfer into 5 ml Falcon tubes for flow cytometry.

4.) Cell cycle analysis using flow cytometry

Preparation

4.1.1) *Appropriate controls:* Cell cycle evaluation of transfected cells requires the ability to discriminate between non-transfected (GFP-negative) and transfected (GFP-positive) cells, as well as cells the representative fluorescence for propidium iodide. It is crucial, therefore, to include the following control samples: 1) unstained, untransfected control; 2) GFP transfected positive control that is unstained for propidium iodide; 3) propidium iodide stained cells without GFP transfection; and 4) your GFP containing experimental samples that are stained with propidium iodide (**summarized in Figure 2**).

4.1.2) *Create following plots and histograms for data acquisition:*

1. Plot: FORWARD vs. SIDE scatter

2. Plot: FL1-H vs. FL2-H

3. Plot: FL2-A vs. FL2-W

4. Plot: FL1-H vs. FL2-A

5. Frequency distribution histogram: FL2-A counts

6. Frequency distribution histogram: GFP counts

7. Frequency distribution histogram: Propidium iodide

Setup

4.2.1) *Accurate cell detection and setup*

Run the untransfected, unstained control cells on "low" flow rate, and set the forward and side scatter voltages such that the majority of cells reside in the middle of the FSC vs. SSC plot. ***Note: using the low flow setting minimizes noise, and is required for clean cell cycle data***. Using the FL1-H vs. FL2-H plot, adjust the FL1-H voltage such that unstained cells reside between 10^0 and 10^1 (Figure 3B). Remove unstained cells and run the GFP positive-only sample. In this view, you may need to adjust the compensation of FL1-FL2 % to ensure there are no false positive PI fluorescent events.

4.2.2) *Gating for singlet cells*

Run the PI positive/GFP negative sample and adjust the following parameters using the FL2-A vs. FL2-W density plot: Adjust the voltage of FL2-H, as well as the Amp Gain of both the FL2-A and FL2-W channels such that the G1 population resides at approximately 200 on each axis (Figure 3C). This will give you a linear relationship between FL2-A and FL2-W and should be used to gate for singlet cells and exclude doublets or aggregates from your analysis. In the FL2-A vs. FL2-H plot, identify the highly saturated G1 population (arrowhead in Figure 3C), and draw a singlet gate as shown (Figure 3C). To generate a DNA content profile in your FL2-A frequency distribution histogram, apply your singlet gate to the FL2-A histogram using your navigator window. A cell cycle profile demonstrating the G0/G1; S; G2/M peaks should now be apparent (modeled example in Figure 3D). Minor changes to the singlet gate may be required in order to optimize your cell cycle profile.

4.2.3) *Gating for GFP cells*

To perform a cell-autonomous analysis, you will need to gate for GFP positive cells using your FL1-H vs. FL2-A plot. Draw a gate on the FL1-H axis above 10^1 to exclude unstained cells (Figure 3 E), and apply this gate to the FL2-A histogram. The resulting DNA profile will reveal your GFP positive cell population. ***Note: valuable housekeeping data, such as transfection efficiencies can be acquired here.***

Analysis

4.3.4) *Cell cycle analysis using FlowJo software:*

4.3.5) *Analyzing G1/S/G2 populations using FlowJo:* The proportions of retinal cells contributing to each phase of the cell cycle can be determined using 3rd party software programs; this protocol describes cell cycle analysis using FlowJo. The program will attempt to overlay a line of best fit on top of the cell cycle profile produced from your FCS analysis files. We find that the Watson method is most effective for modeling retinal explant data. The line of best fit can be optimized to fit your distribution by manually adjusting the alignment, while using the root mean squared (RMS) value as an estimate of fit (Figure 4A-B). To perform this, grossly align the line of best fit to the left edge and peak of the G1 population. It may also be necessary to set constraints on the location of the G2 peak if it is not easily identifiable (Figure 4C). For example, although the G2 peak should be located approximately 2.0 times the value of the G1 peak, some minor adjustments (+/- 0.2) can be employed in order to properly model your data.

5.) Cell fate analysis using flow cytometry

Detection of cell type specification and differentiation can be achieved via the use of reporter constructs as previously described (Matsuda and Cepko, 2008). Briefly, we use a panel of dsRED expression vectors in which dsRED is under the control of cell-type specific reporter sequences. These sequences are derived from genes that are specific to particular cell types, and are therefore only expressed in these cells. Examples of retinal cell fate reporters are as follows: CRALBP = Müller glia; CaBp5 = bipolar cells; NRL = photoreceptors. These constructs can be co-electroporated with transfection control plasmids (GFP or GFP-expressing knockdown vectors, for example) to determine the proportion of cell types produced as a function of your experimental manipulation.

Preparation

5.1.1) *Appropriate controls:* Experimental setup for cell fate determination requires the ability to discriminate between non-transfected cells, and GFP, dsRED, and GFP/dsRED expressing cells. Include the following control and experimental samples: 1) non-transfected (GFP and dsRED-negative); 2) GFP transfected positive control; 3) dsRED-transfected positive control; 4) your GFP/dsRED co-electroporated experimental samples.

5.1.2) *Create following plots and histograms for data acquisition:*

1. Plot: FORWARD vs. SIDE scatter

2. Plot: FL1-H vs. FL2-H

3. Frequency distribution histogram: dsRED-positive cell counts

4. Frequency distribution histogram: GFP-positive cell counts

Setup

5.2.1) *Accurate cell detection and setup*

Run the untransfected, unstained control cells and adjust forward and side scatter as described in 4.2.1. ***Note: with careful attention, GFP/dsRED analysis can be performed using low, medium or high flow settings.*** To establish your negative populations for both the GFP and dsRED channels, you will need to adjust the FL1-H and FL2-H voltages using the FL1-H vs. FL2-H acquisition plot. Make adjustments such that your negatively stained cell population resides between 10^0 and 10^1 on both axes (Figure 5A).

5.2.2) *Gating for GFP and dsRED cells*

Run the GFP positive/dsRED negative sample in the FL1-H vs. FL2-H acquisition plot (Figure 5B), and adjust the compensation (FL2-FL1%) to place all events below 10^1 on the FL2-H axis. Repeat the process using the GFP negative/dsRED positive sample (Figure 5C), and adjust the compensation (FL1-FL2%) to place all events below 10^1 on the FL1-H axis.

Analysis

5.3.1) *Analyzing GFP and dsRED populations*

Statistics for GFP/dsRED data can be generated using the CellQuest software. Divide the FL1-H vs. FL2-H plot into quadrants based on a negative population at $< 10^1$ on each axis (Figure 5D). Quadrants represent data as follows: bottom left - negative for fluorescence; bottom right -GFP positive/dsRED negative; top right – GFP/dsRED double positive; top left – GFP negative/dsRED positive. Percentages are reported in each quadrant.

6.) Representative Results: An example of retinal explant histology electroporated at P1, and harvested 24h later (Figure 6A). Transfected cells expressing GFP are evident throughout the neuroblastic layer (NBL) at 24 hours following *in vitro* electroporation and culture. Immunocytochemical counter-staining for Ki-67 (pan-competent retinal progenitors – RED) and $p27^{kip1}$ (postmitotic cells – BLUE) illustrate the proliferative NBL and differentiating cell layers, respectively. Complementary, flow cytometric data reveal changes in retinal cell cycle profiling at 24 hours following transfection with either scrambled control or shRNA knockdown vectors (Figure 6B). Differences in cell-autonomous vs. non-cell-autonomous effects can be isolated when comparing ungated cells, to those gated for GFP (Figure 6C). Cell fate specification and differentiation analyses using flow cytometric (Figure 6D) and histological (Figure 6E) data provide a robust assessment of cell production and maturation.

7.) Discussion: The method presented here provides a highly powerful and reliable quantitation of phenotypes related to cell cycle dynamics, and fluorescent reporters in transfected postnatal retinal cells. This high-throughput screen can be extended to assay the retina by running entire

samples through flow cytometric analysis. One additional advantage to this method is the ability to gate and isolate transfected cells through the use of fluorescent reporter vectors. For example, many of our manipulations involve the use of shRNA knockdown vectors containing a reporter sequence for GFP. By gating for GFP, we can attempt to resolve cell-autonomous and non-cell-autonomous effects, as well as evaluate important housekeeping parameters such as transfection efficiency.

One key to the success of this method, however, is preservation of fluorescence intensity through the explant processing procedure. Consider that our typical transfection efficiencies at P0 are 5-6% of the total retinal cell population. Given that the vast majority of transfectable cells at any given time are restricted to progenitors engaged in the cell cycle (Matsuda and Cepko, 2008), 5-6% of the total retinal population represents a significant number of data points. Losses in fluorescence signal intensity can, however, result in significant loss of data, as marginally fluorescent cells fall below threshold of inclusion. To combat this, preliminary evaluation of vector efficiency and fluorescence signal in test explants should be performed using histological preparations. In addition, experimental explants can be grossly evaluated before dissociation by quickly viewing under a fluorescent microscope. These procedures will help to optimize aspects such as plasmid titers, which can contribute to overall fluorescence intensity.

The extent to which the experimenter can dissociate explants while maintaining high cell numbers greatly affects the efficiency of subsequent flow analysis. We find that maintaining a modest level of aggression when triturating cells, while increasing the number of times one triturates helps to maintain cell yields, while generating a sufficient population of singlet cells. Falling short of fully dissociating cells will rob your ability to produce adequate cell cycle profiles, as gating and removal of cell doublets and aggregates is essential in performing this analysis.

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Disclosures: None

Table of reagents and equipment:

Name of the reagent	Company	Catalogue number	Comments (optional)
Dissection and *in vitro* electroporation			
Hank's Balanced Salt Solution (-) Calcium Chloride, (-) Magnesium Chloride	GIBCO	14175	Denoted HBSS (-/-) in text
Hank's Balanced Salt Solution (+) Calcium Chloride, (+) Magnesium Chloride	GIBCO	14025	Denoted HBSS (+/+) in text
Dulbecco's Modified Eagle Medium (DMEM) high-glucose	GIBCO	11960	
Neurobasal Medium (-) glutamine	GIBCO	21103	
Platinum Block Petridish Electrode	Protech International	CUY520-P5	
ECM-830 square wave electroporator	BTX		
no. 55 Dumostar forceps	Fine Science Tools	11295-51	
no. 5 Dumont forceps	Fine Science Tools	11251-30	
Dissecting microscope	Nikon	SMZ-800	
Light source	Fiber-Lite	M1-150	
100x20 mm culture dish	Sarstedt	83.1802	
6-well culture dish	Starstedt	83.1839	
Polycarbonate Track-Etch Membranes	Whatman	110606	25mm, 0.2 um
B27 supplement	GIBCO	17504044	
Glutamax	GIBCO	35050-061	
1000 ul wide-bore pipette tips	Axygen	T-1005-WB-C-R	
200 ul pipette tips			Generic brand
20 up pipette tips			Generic brand
Explant Dissociation and staining			
1.5 ml microtubes	Axygen	MCT-150-C	
15 ml Falcon tubes	BD Biosciences	352097	
5m and 10 ml Falcon	BD Biosciences	35-7551	

serological pipettes			
1000 ul pipettes			
Fire-polished Pasteur pipette			Approx. diameter 0.6 mm
MACS cell dissociation kit – Postnatal Neurons	Miltenyi Biotec	130-094-802	
50mM β-mercaptoethanol			
Albumin (from bovine serum)	Sigma-Aldrich	A7906	
0.1M phosphate-buffered saline (PBS)			
1% paraformaldehyde in 0.1M PBS	Electron Microscopy Sciences	157-8	8% stock PFA
70% ETOH			
0.1M PBS + 0.1% Triton X-100			
RNAse A			
Propidium Iodide	BD Pharmingen	51-66211E	
DRAQ-5	Biostatus	DR5 – 5 mM	
5 ml Polystyrene Round-Bottom Tubes for flow-cytometry	BD Biosciences	35-2058	
Plating of Dissociated cells for microscopic analysis			
16-well Permanox chamber slides	Nunc	177437	
Methanol			Cool in -20 degrees C
4% paraformaldehyde in 0.1M PBS	Electron Microscopy Sciences	157-8	8% stock PFA
Explant preparation for histology			
HBSS (-/-)	GIBCO	14175	
4% paraformaldehyde in 0.1M PBS	Electron Microscopy Sciences	157-8	8% stock PFA
0.1M PBS			
0.1M PBS with 30% sucrose			
O.C.T. Compound	Tissue-Tek	4583	
Adhesive coated slides	Newcomer Supply	5070	75x25x1.0 mm
Flow cytometry and FACS			

Flow cytometer	BD		BD Facscalibur

Figures

Figure 1 – Flow chart outlining the possible experimental approaches used via this protocol

A correlative approach that combines histological and flow cytometric analyses provides a powerful means with which we can evaluate changes in cell cycle and cell fate. DNA content analysis and thymidine analogue pulse/chase experiments can be used to explore cell cycle deficits with a high degree of temporal precision. Cell type-specific fluorescent reporter plasmids provide a sensitive output for early specification and maturation of newly generated progeny.

Figure 2 – Experimental design checklist for retinal flow cytometry

Figure 3 – Flow cytometric setup and procedures

Using unstained cells, forward and side scatter (A) and negative control gating for GFP and propidium iodide (B) are established. Singlet discrimination is performed (C) by isolating singlet cells in G0/G1 (arrowhead), S, and G2/M phase. A cell cycle profile is generated and analyzed via a frequency distribution histogram of the FL2-A channel (D). The G0/G1 peak (green), s-phase plateau (yellow) and G2/M peak (blue) collectively make up the cell cycle profile. GFP gating (red gate in E) and GFP profiling (inset in E) enable the isolation and analysis of transfected cells for cell-autonomous effects.

Figure 4 – FlowJo setup for cell cycle analysis of retinal explants

(A) Default Watson alignment will generate a preliminary model that requires manual alignment and changes to constraints (shown in C). The relative mean squared (RMS – red highlight) can be used as a measure of fit. (B) An example of closely modeled retinal explant data using the G1 peak as a reference.

Figure 5 – Retinal cell fate analysis using flow cytometry

(A) Unstained cells. (B) GFP-positive/dsRED negative cells with positive events above 10^1 on the FL1-H axis. (C) dsRED-positive/GFP-negative cells with positive events above 10^1 on the FL2-H axis. (D) Subpopulations of GFP-positive and GFP/dsRED-positive cells are resolved in lower right and upper right quadrants, respectively.

Figure 6 – Representative results for cell cycle analysis of retinal explants

An example of histological data (A) in which immunolabeling provides a means to evaluate the position and morphology of proliferating (Ki-67 – RED) and postmitotic ($p27^{kip1}$ - BLUE) retinal cells expressing GFP (NBL – neuroblastic layer; iNBL – inner neuroblastic layer; GCL – ganglion cell layer). (B) Cell cycle data showing changes in retinal proliferation following shRNA-mediated gene knockdown. Arrowhead denotes major S-phase shoulder. (C) An example of cell-autonomous effects revealed by GFP gating of transfected retinal cells. Flow cytometric data from gene knockdown retinas were acquired, and represent the percentage cells present in either G0/G1, S, or G2/M. Percent change data were calculated by normalizing to control vector counterparts. A significant cell-autonomous effect can be seen in GFP-gated, G2/M phase cells (bracket). (D) Robust flow cytometric data showing proportions of specific retinal cell types being produced (yellow quadrant) relative to all transfected cells (yellow + green compartments). (E-F) Cell distribution and morphological data can be acquired from explant sections containing shRNA-GFP-expressing cells. These data can be elaborated to include co-transfection with cell type specific reporter plasmids for retinal cells. Examples: NRL-dsRED photoreceptors in (E); CRALBP-dsRED Müller glia in (F). Topro-3 nuclear counter stain (BLUE).

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Figure 1

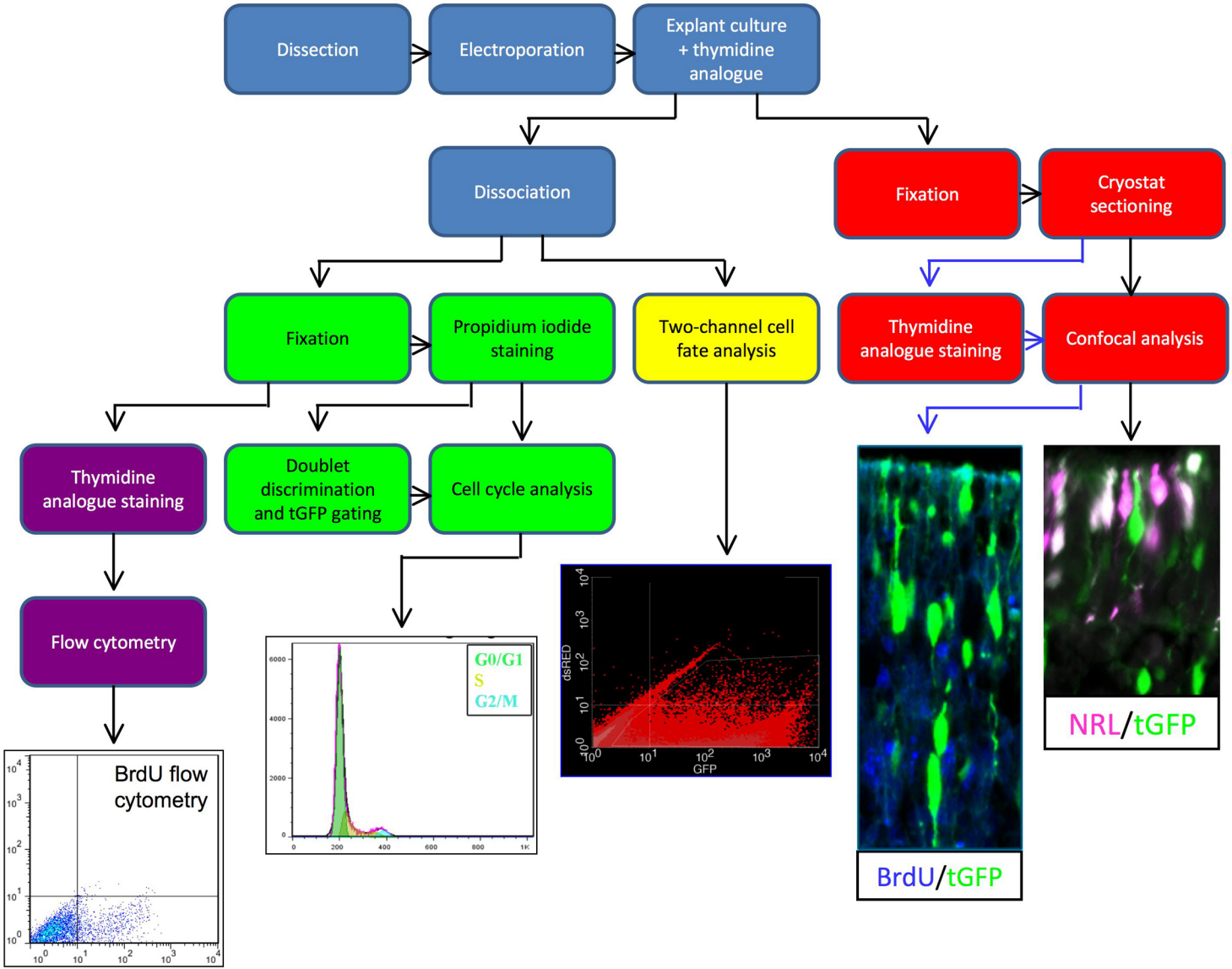

Dissection
Electroporation
Explant culture + thymidine analogue
Dissociation
Fixation
Cryostat sectioning
Fixation
Propidium iodide staining
Two-channel cell fate analysis
Thymidine analogue staining
Confocal analysis
Thymidine analogue staining
Doublet discrimination and tGFP gating
Cell cycle analysis
Flow cytometry
BrdU flow cytometry
G0/G1
S
G2/M
dsRED
GFP
BrdU/tGFP
NRL/tGFP

Figure 2

Experimental Design Checklist for Flow Cytometry:

Cell cycle analysis (GFP reporter)

Un-transfected	Un-transfected PI stained	GFP-only	GFP + PI (experimental tissue)

BrdU flow cytometry

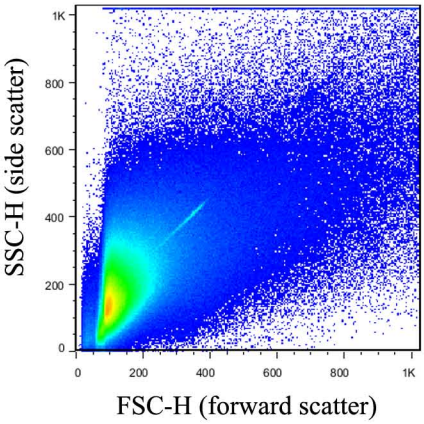
Un-transfected	Un-transfected BrdU stained		GFP-only		GFP + PI (experimental tissue)
	No BrdU in culture	(+) BrdU in culture	No BrdU in culture	(+) BrdU in culture	

Two-channel cell fate analysis (GFP and dsRED reporters)

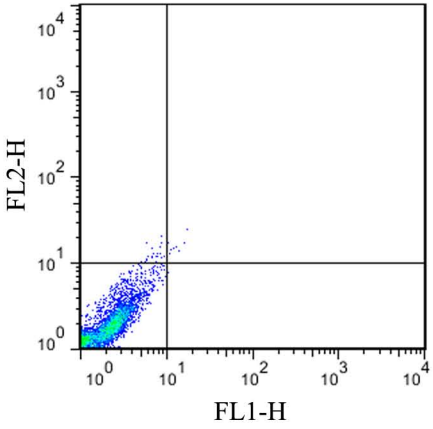
Un-transfected	GFP-only	dsRED-only	GFP + dsRED (experimental tissue)

Figure 3

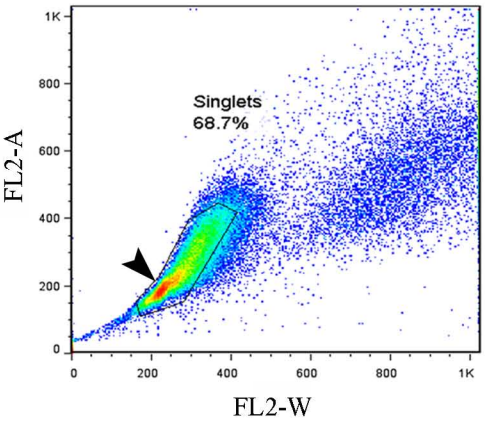
A. Unstained cells: forward and side scatter gating



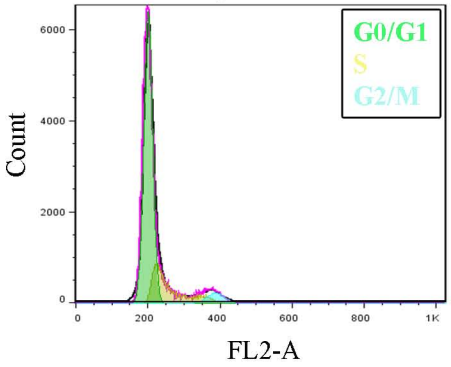
B. Unstained cells: negative control gating



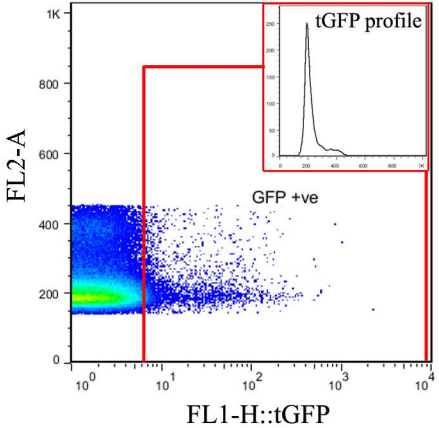
C. Propidium iodide stained: singlet discrimination



D. Retinal explant cell cycle profile



E. tGFP gating

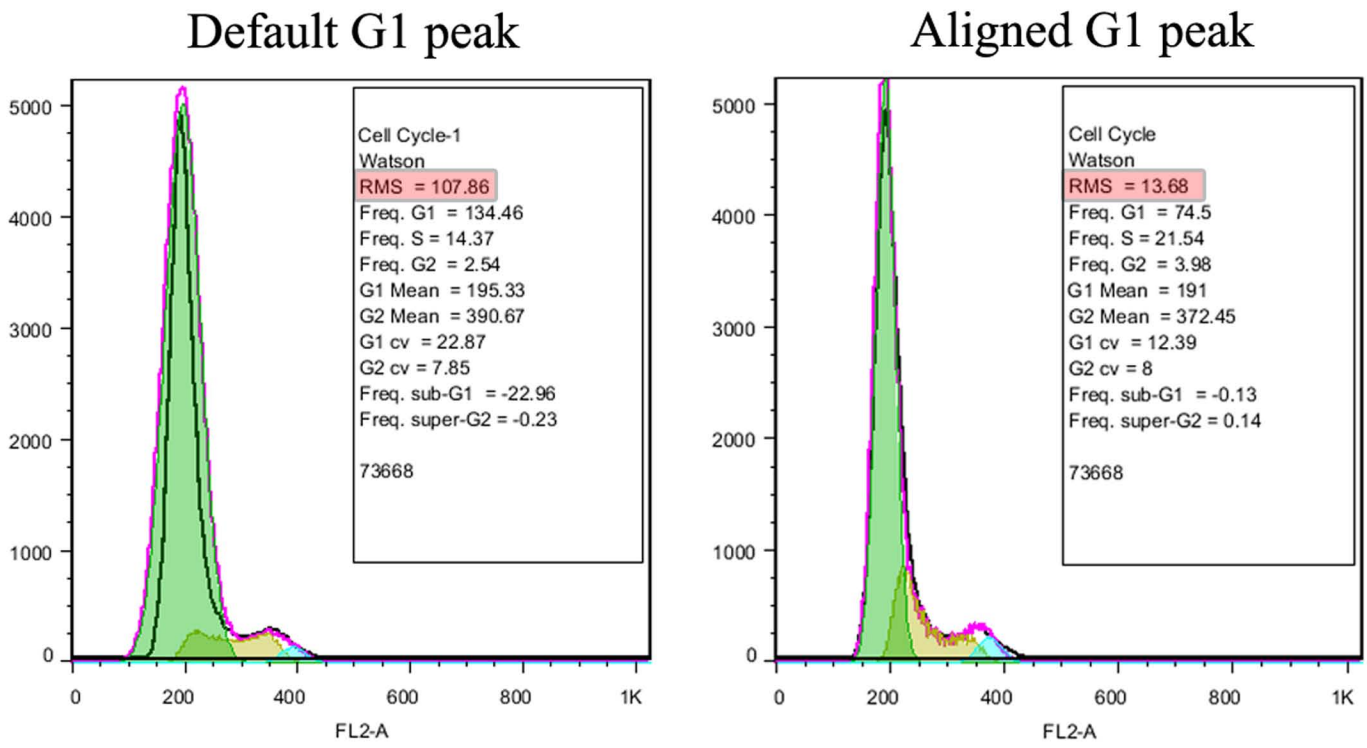


F.

	Ungated	tGFP gated
Watson		
RMS	35.05	1.91
Freq. G1	51.73	48.02
Freq. S1	23.71	14.47
Freq. S2	10.88	19.58
Freq. S3	1.27	4.28
Freq. S4	0	0.08
Freq. G2	10.55	3.94
G1 Mean	189	191
G2 Mean	378	382
G1 cv	8.93	8.9
G2 cv	15.3	8.9
Freq. sub-G1	2.64	6.04
Freq. super-G2	-0.23	1.89

Figure 4

A.


Default G1 peak
Aligned G1 peak
Cell Cycle-1
Watson
RMS = 107.86
Freq. G1 = 134.46
Freq. S = 14.37
Freq. G2 = 2.54
G1 Mean = 195.33
G2 Mean = 390.67
G1 cv = 22.87
G2 cv = 7.85
Freq. sub-G1 = -22.96
Freq. super-G2 = -0.23
73668
Cell Cycle
Watson
RMS = 13.68
Freq. G1 = 74.5
Freq. S = 21.54
Freq. G2 = 3.98
G1 Mean = 191
G2 Mean = 372.45
G1 cv = 12.39
G2 cv = 8
Freq. sub-G1 = -0.13
Freq. super-G2 = 0.14
73668
5000
4000
3000
2000
1000
0
0
200
400
600
800
1K
FL2-A

B.

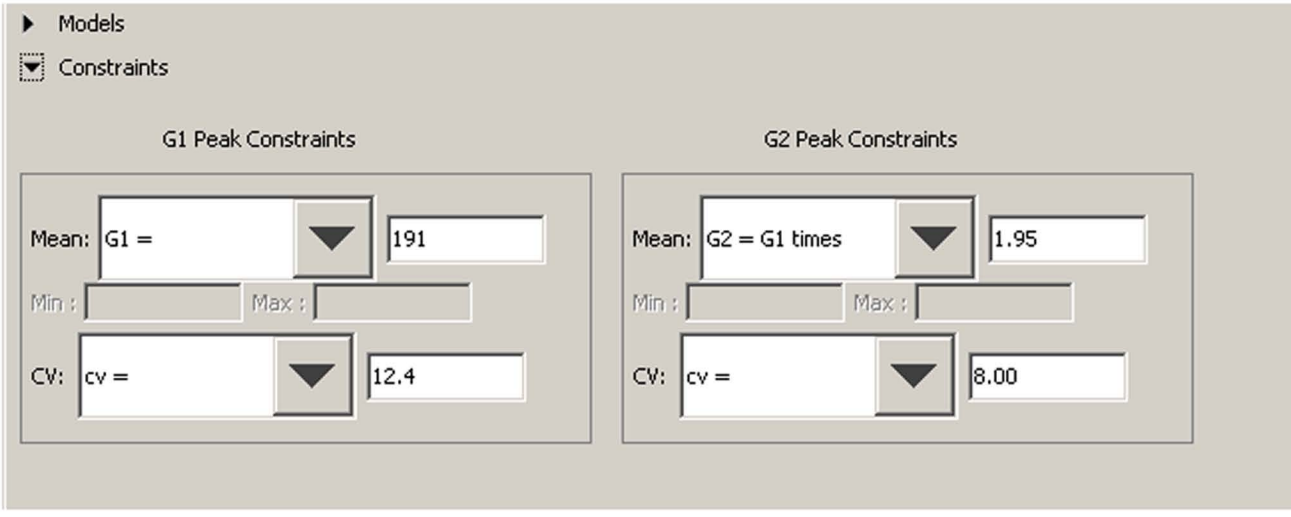

Models
Constraints
G1 Peak Constraints
G2 Peak Constraints
Mean: G1 =
191
Min :
Max :
CV: cv =
12.4
Mean: G2 = G1 times
1.95
Min :
Max :
CV: cv =
8.00

Figure 5

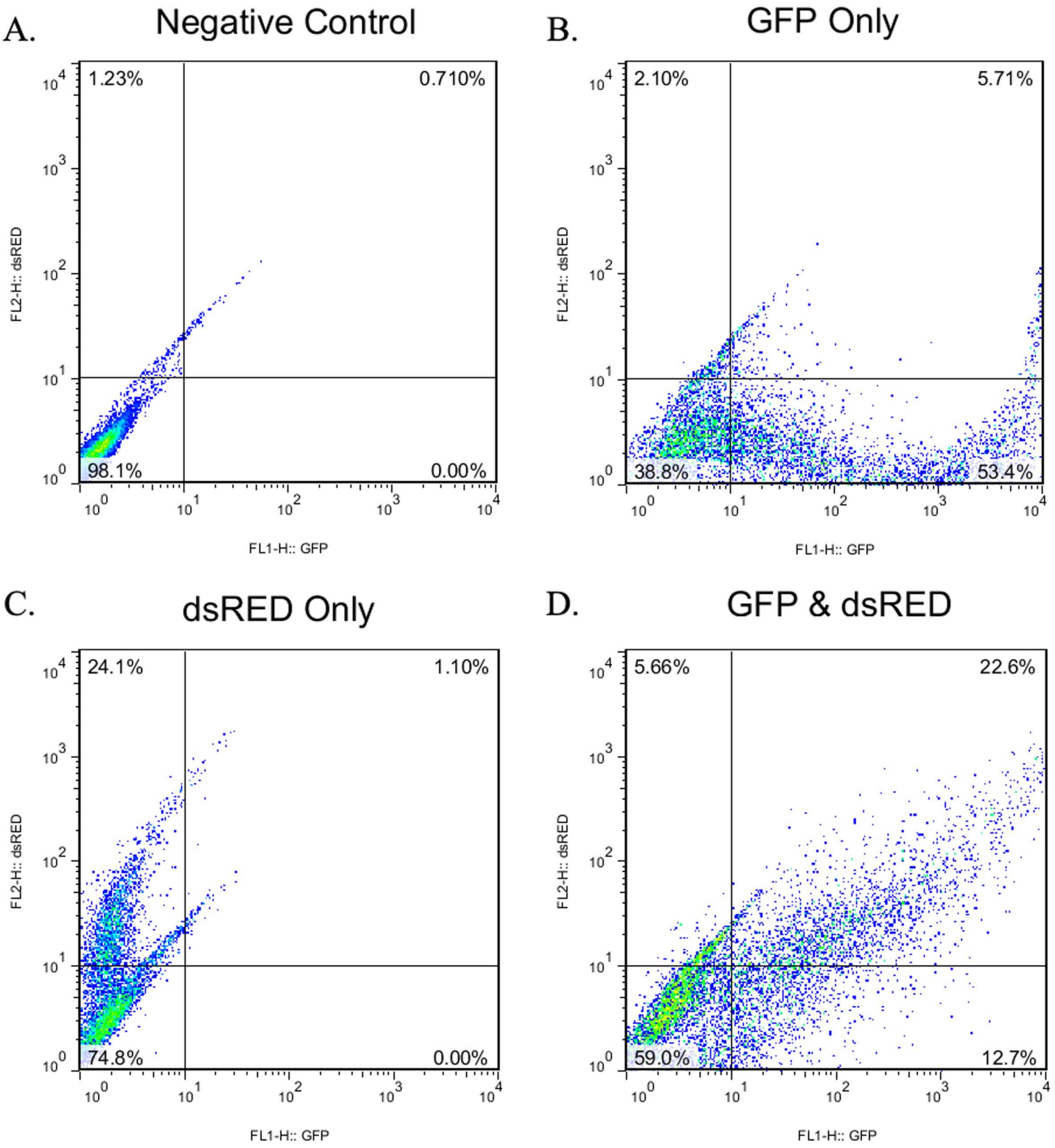
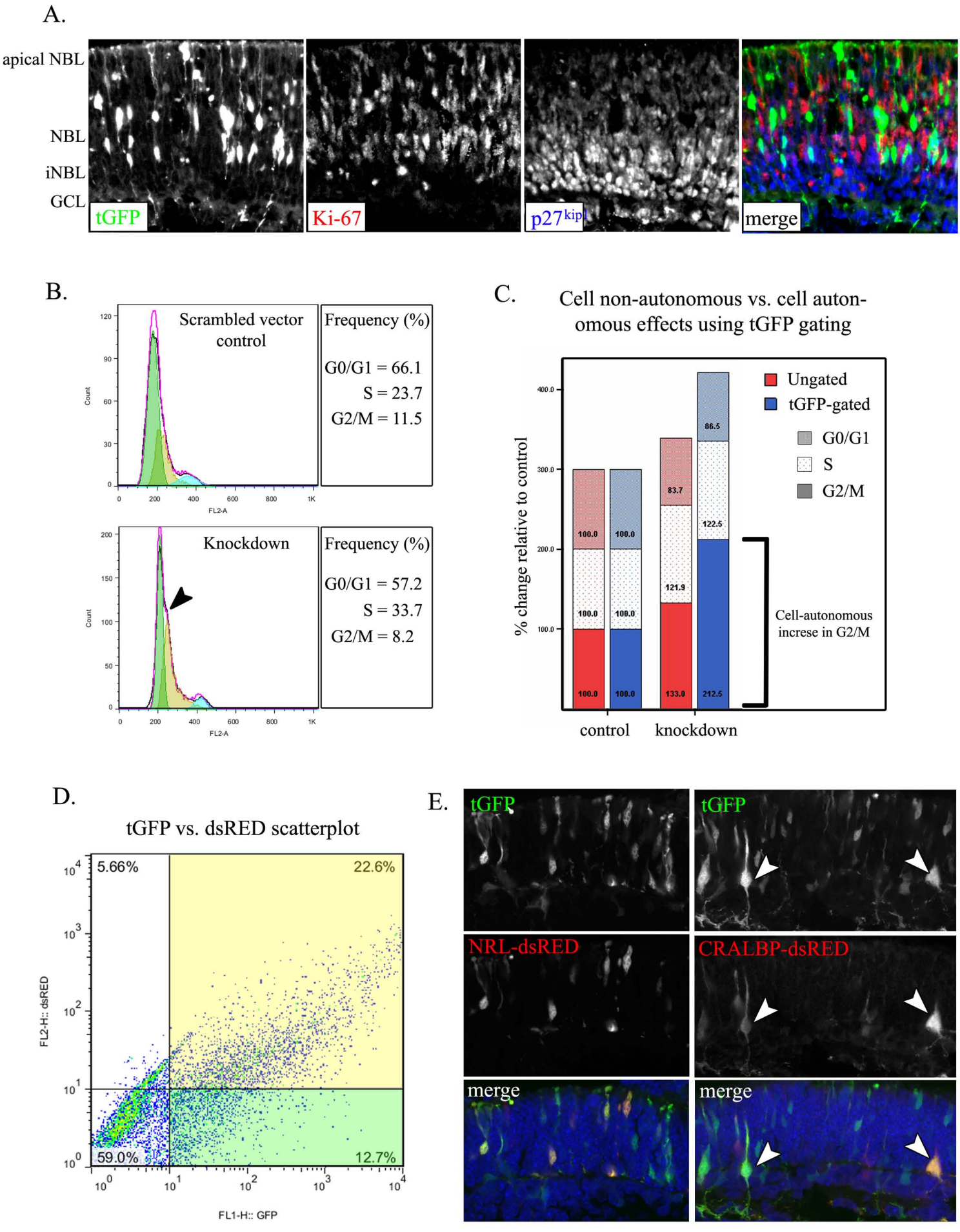

A.
Negative Control
1.23%
0.710%
98.1%
0.00%
B.
GFP Only
2.10%
5.71%
38.8%
53.4%
C.
dsRED Only
24.1%
1.10%
74.8%
0.00%
D.
GFP & dsRED
5.66%
22.6%
59.0%
12.7%
FL2-H:: dsRED
FL1-H:: GFP

Figure 6


A.
apical NBL
NBL
iNBL
GCL
tGFP
Ki-67
p27kip
merge
B.
Scrambled vector control
Frequency (%)
G0/G1 = 66.1
S = 23.7
G2/M = 11.5
Knockdown
Frequency (%)
G0/G1 = 57.2
S = 33.7
G2/M = 8.2
Count
FL2-A
C.
Cell non-autonomous vs. cell autonomous effects using tGFP gating
% change relative to control
Ungated
tGFP-gated
G0/G1
S
G2/M
100.0
100.0
100.0
100.0
100.0
100.0
83.7
121.9
133.0
86.5
122.5
212.5
Cell-autonomous increse in G2/M
control
knockdown
D.
tGFP vs. dsRED scatterplot
5.66%
22.6%
59.0%
12.7%
FL2-H:: dsRED
FL1-H:: GFP
E.
tGFP
tGFP
NRL-dsRED
CRALBP-dsRED
merge
merge