Additional File 1

Retinoic acid delays initial photoreceptor differentiation and results in a highly structured mature retinal organoid

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Table S1: Primers used for qPCR analysis of gene expression

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon	Efficiency
ARR3	CCCAGAGCTTTGCAGTAACC	CACAGGACACCATCAGGTTG	189 bp	1.79
BRN3A	AGAAGCAGAAGCGGATGAAA	CAGAGAATGGGTGGAGGAAA	194 bp	1.90
CRX	TGATGGTGGGATTGGAAAAT	GCCTTGTGAACTCCACCAAT	248 bp	2.04
GAD2	TCGTCAGATTCCAAGTGCTG	TAGGGCATTTCTACCCGTTG	169 bp	1.81
GLAST1	AACCCATCGACAGTGAAACC	TCCCTTGTGTTTTGGAGGAC	184 bp	1.93
LIM1	GCCAAAGAGAACAGCCTTCACTC	GGTCGTCATTCTCGTTGCTACC	148 bp	1.94
NRL	GGCTCCACACCTTACAGCTC	ATGGCCTCTTCAGGACTCAG	170 bp	1.96
NR2E3	CACTTCATGGCCAGCCTTAT	CTGGAGAACACAGGCAGGTT	215 bp	1.80
OPN1MW	GAACCAGGTCTATGGCTACTTCG	TCTCACATTGCCAAAGGGCTT	154 bp	2.04
OTX2	GCAGAGGTCCTATCCCATGA	CTGGGTGGAAAGAGAAGCTG	211 bp	1.95
PKCα	GTGGCAAAGGAGCAGAGAAC	TGTAAGATGGGGTGCACAAA	151 bp	1.92
RAX	GTCCCTAAGCGTGCTTTCAG	CATGCCAGGGTCTTGGTACT	200 bp	1.99
RCVRN	AGCTCCTTCCAGACGATGAA	CAAACTGGATCAGTCGCAGA	150 bp	2.02
RHO	ACAGGATGCAATTTGGAGGGC	GCTCATGGGCTTACACACCA	111 bp	2.06
GRK1	GCCCGTGAAGTACCCTGATA	TTGGAGTCTGGGATGAAAGG	198 bp	2.02
SIX3	CAGAAGACGCATTGCTTCAA	GCCGGTTCTTAAACCAGTTG	151 bp	2.00
VSX2	CTGACTCTGGACCATGCTGA	GAGCTGGGAAGGAGGACTCT	189 bp	1.87
GAPDH	AACCATGAGAAGTATGACAAC	CTTCCACGATACCAAAGTT	112 bp	2.02

Table S2: Primary and secondary antibodies used for immunofluorescence studies

Antibody	**Dilution**	**Company Cat #; RRID**
Goat polyclonal anti-Arrestin 3	1/200	Novus Biologicals Cat# NBP1-37003; AB_2060085
Mouse monoclonal anti-CRX, clone 4G11	1/2000	Abnova Cat# H00001406-M02;AB_606098
Mouse monoclonal anti-NR2E3/PNR, clone H7223	1/150	R&D Systems Cat# PP-H7223-00; AB_2155481
Rabbit polyclonal anti-Opsin, Red/Green	1/500	Millipore Cat# AB5405; AB_177456
Rabbit polyclonal anti-Recoverin	1/2000	Millipore Cat# AB5585; AB_2253622
Mouse monoclonal anti-Rhodopsin, clone 4D2	1/250	Millipore Cat# MABN15; AB_10807045
Donkey anti-Mouse IgG (H+L), Alexa Fluor 488	1/500	ThermoFisher Scientific Cat# A-21202; AB_141607
Donkey anti-Rabbit IgG (H+L), Alexa Fluor 594	1/500	ThermoFisher Scientific Cat# A-21207; AB_141637
Donkey anti-Goat IgG (H+L), Alexa Fluor 594	1/500	ThermoFisher Scientific Cat# A-11058; AB_2534105

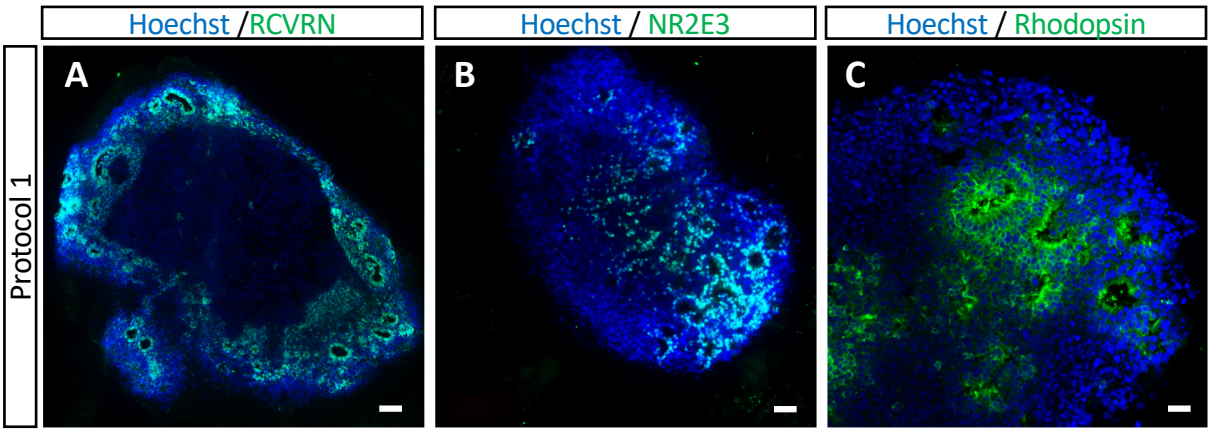

Figure S1: Expression of photoreceptor markers in non-laminated Protocol 1 organoids. A) Representative image of RCVRN expression in green, which is localized to rosette-like structures of the non-laminated retinal organoids at day (D) 105 of differentiation. Scale bar = 100 µm. **B-C:** Representative images of NR2E3 (**B**) or rhodopsin (**C**) expression in green in rosette-like structures of late-stage retinal organoids at D300 of differentiation. Nuclei are stained with Hoechst in blue. Scale bars= 50 µm.

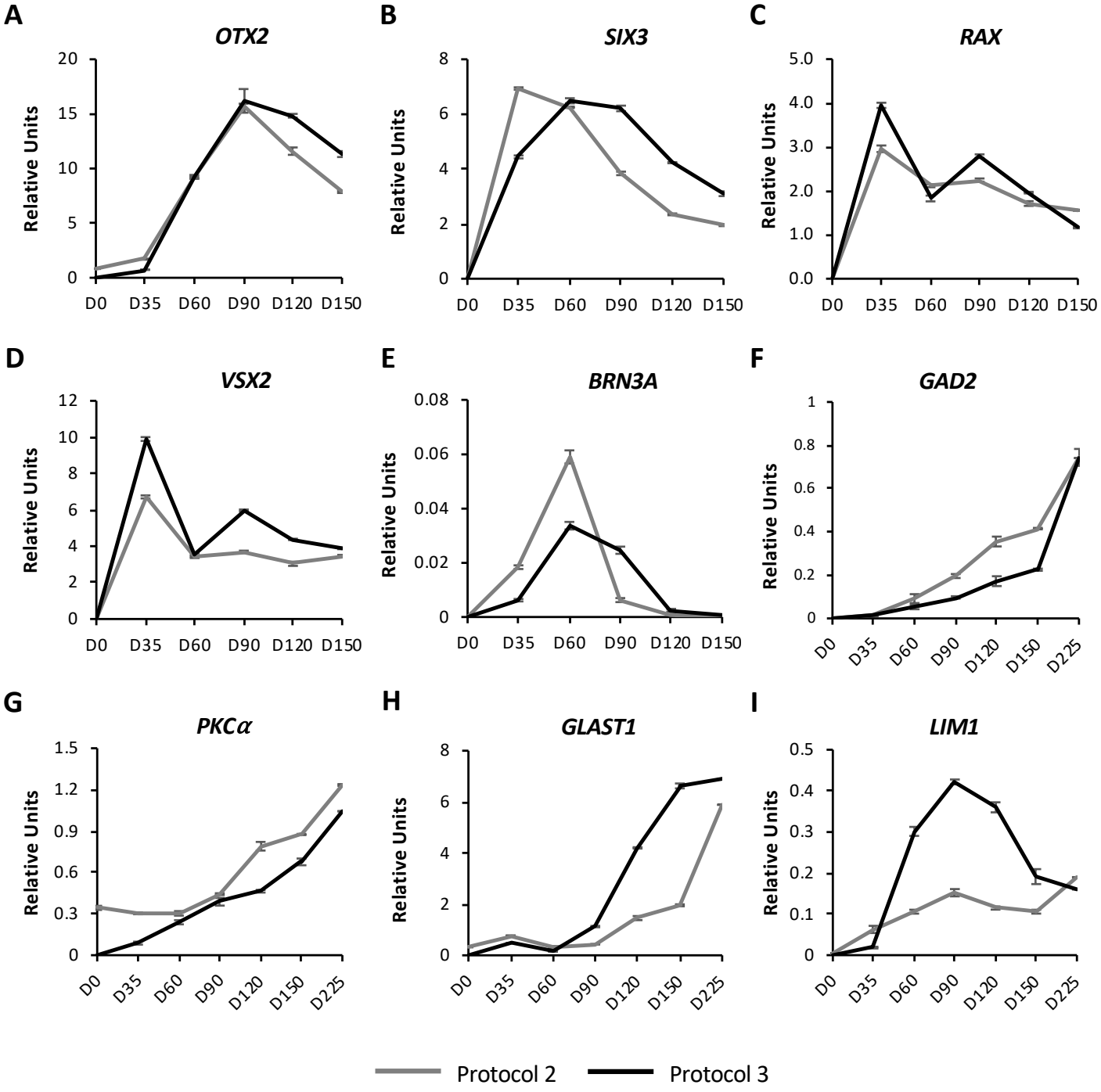

Figure S2: Temporal gene expression of eye-field specification and non-photoreceptor markers in Protocol 2 and Protocol 3 organoids. Pools of 25-30 retinal organoids differentiated with Protocol 2 or Protocol 3 were collected at different time points (D35, D60, D90, D120, D150 and/or D225) and relative gene expression was measured by qPCR for the eye-field specification markers: *OTX2* (**A**), *SIX3* (**B**), *RAX* (**C**) and *VSX2* (**D**). Relative gene expression was also measured for the retinal ganglion cell marker *BRN3A* (**E**), the amacrine cell marker *GAD2* (**F**), the bipolar cell marker *PKCα* (**G**), the Müller cell marker *GLAST1* (**H**) and the horizontal cell marker *LIM1* (**I**). Grey lines represent Protocol 2 and black lines Protocol 3. Data is normalised to the housekeeping gene *GAPDH* and shown as mean ± SEM, n=3.

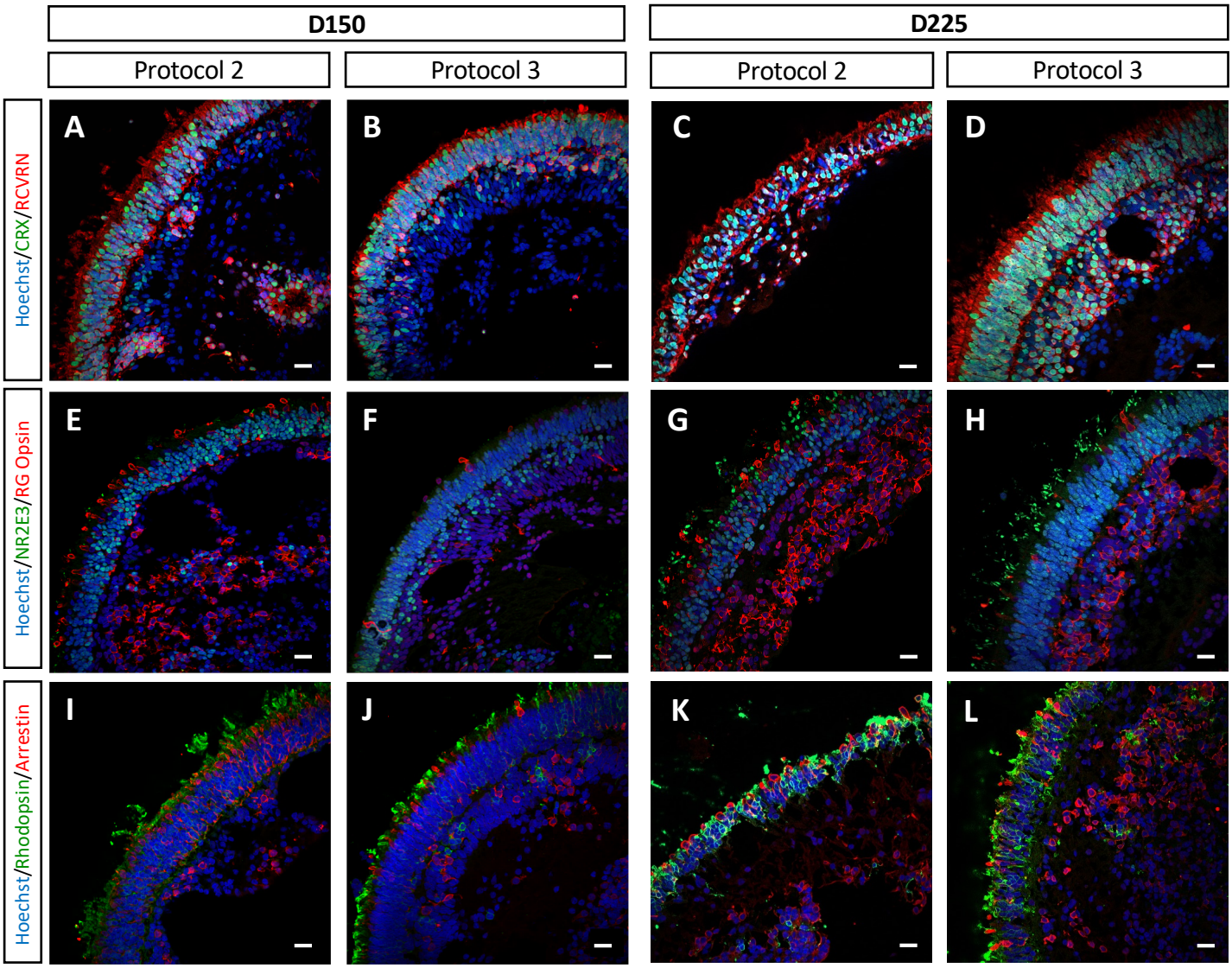

Figure S3: Immunofluorescence studies of Protocol 2 and Protocol 3 organoids. The same images shown in Figure 3 with the additional channel showing the Hoechst-stained ONL (in blue). Retinal organoids were analysed at D150 (**A**, **B**, **E**, **F**, **I**, **J**) and D225 (**C**, **D**, **G**, **H**, **K**, **L**) of differentiation. CRX in green and RCVRN in red in organoids generated with Protocol 2 (**A**, **C**) and Protocol 3 (**B**, **D**). NR2E3 in green and RG opsin in red in organoids generated with Protocol 2 (**E**, **G**) and Protocol 3 (**F**, **H**). Rhodopsin in green and arrestin in red in organoids generated with Protocol 2 (**I**, **K**) and Protocol 3 (**J**, **L**). Scale bars = 20 µm.

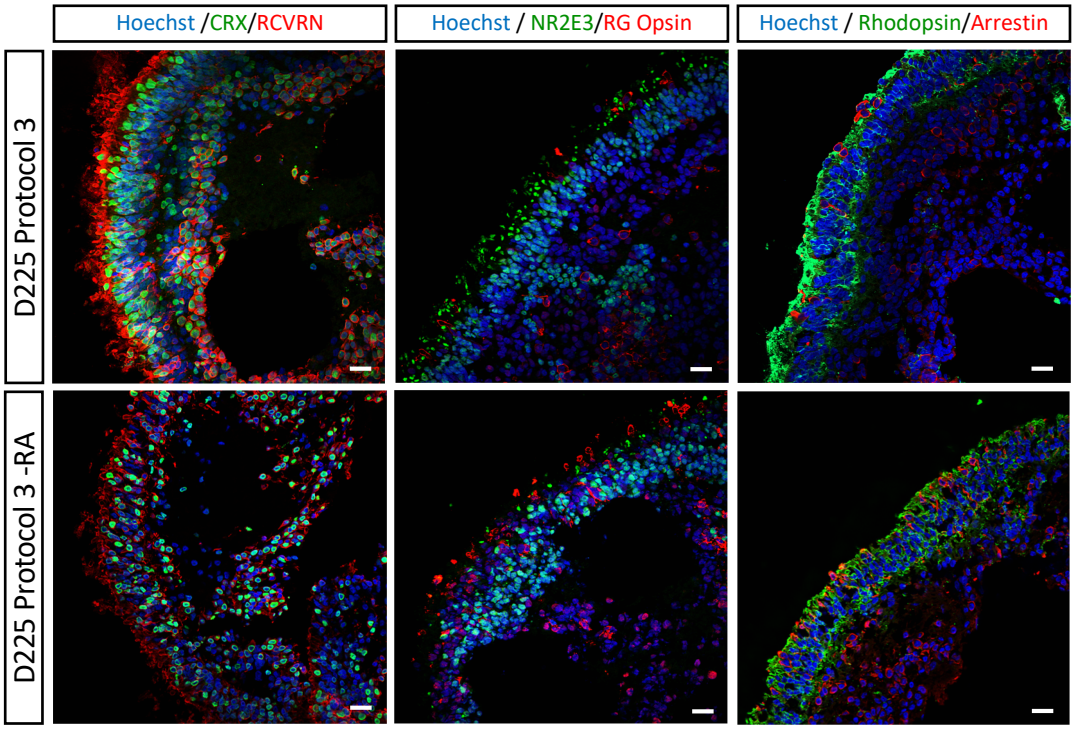

Figure S4: Immunofluorescence studies of Protocol 3 organoids with or without RA. The same images shown in Figure 4 with the additional channel showing the Hoechst-stained ONL (in blue). Left-hand panels, CRX in green and RCVRN in red; middle panels, NR2E3 in green and RG opsin in red; right-hand panels, rhodopsin in green and arrestin in red. Scale bars = 20 µm.

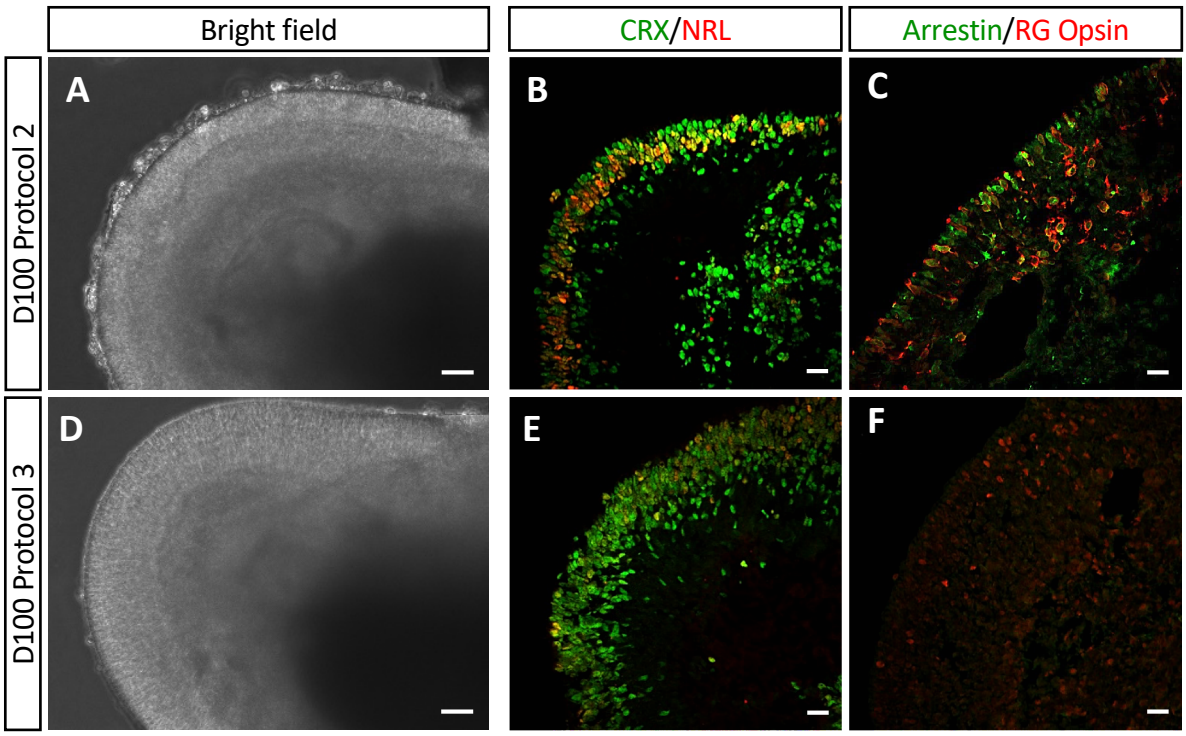

Figure S5: Cones in early-stage Protocol 2 and Protocol 3 organoids. Representative bright-field images of retinal organoids at D100 differentiated with Protocol 2 (**A**) or Protocol 3 (**D**). Scale bars = 50 µm. Representative IF images of CRX in green and NRL in red in Protocol 2 (**B**) and Protocol 3 (**E**) organoids. Representative IF images of arrestin in green and RG opsin in red in Protocol 2 (**C**) and Protocol 3 (**F**) organoids. Scale bars = 20 µm.