Intravitreal fluorogold tracing as a method to label retinal neurons and the retinal pigment epithelium

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Techniques to label the neuroretina and the retinal pigment epithelium (RPE) have been the topic of many studies for years. The reliability and reproducibility of these techniques are essential to investigate retinal alterations of ocular pathologies and possible treatments. In particular, the study of the integrity of the RPE is of great importance in pathologies such as retinitis pigmentosa or age-related macular degeneration (Gu et al., 2012; Fisher and Ferrington, 2018; Zhang et al., 2019). The most common approaches use antibodies to identify different cell populations. Recently, we have described a novel technique to study the integrity of the neuroretina and the RPE, as well as the functionality of the RPE in rats (Valiente-Soriano et al., 2020).

This technique is based in the intravitreal administration of fluorogold (FG). FG is a fluorescent retrograde axonal tracer that is taken up by the axonal terminals (Schmued and Fallon, 1986) and that is widely used to trace retinal ganglion cells (RGCs) from their retinorecipient areas or the optic nerve (Salinas-Navarro et al., 2010; Nadal-Nicolas et al., 2015). Once FG is administered in the vitreous, it is transported trans-neuronally from the inner to the outer retina until it reaches the RPE. Importantly, only neurons and the RPE but not the retinal glia, are labeled. As the tracer passes through the retinal neurons, it accumulates in their somas labeling them and their processes in a time-specific manner. Thus, the retina must be analyzed at specific time-points after the administration of FG depending on the population of interest.

In this perspective, we revise the course of FG tracing in intact retinas (**Figure 1**).

Intravitreal tracing with FG is a fairly quick process and 5 minutes after its administration, the somas of neurons in the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) are already traced (**Figure 1A–A''**). In the plexiform layers, neuronal processes and synaptic terminals are also delineated (**Figure 1A**, yellow arrows). In the GCL, some traced-somas are RGCs since they co-localize with the RNA-binding protein with multiple splicing protein (RBPMS) (Rodriguez et al., 2014), while the rest must be displaced amacrine cells which, together with RGCs are the neurons located in the GCL. This is the best time point to visualize RGC axons. Indeed, RGC axons are now beautifully traced (**Figure 1A–A''**) but lose the tracing rather quickly. In fact, RGC axons have lost most of the tracing 15 minutes after FG administration (**Figure 1B–B''**) and are no longer traced after 6 hours (**Figure 1** in Valiente-Soriano et al., 2020). The same course is observed for the processes in the plexiform layers.

Fifteen minutes of tracing labels more somas in the ONL where photoreceptors (rods and cones) lay, reaching their outer segments and finally, the RPE (**Figure 1B–B''**). The tracing in the ONL and the outer segments is also transient, diminishing gradually between 1 and 24 hours (**Figure 1** in Valiente-Soriano et al., 2020) when no outer segments and only few and faintly traced photoreceptor somas are observed (**Figure 1C–C''**).

In sections analyzed 24 hours after the tracing, the RPE is clearly labeled but, as said above, the outer retina is mainly devoid of FG. Interestingly, many somas in the inner retina remain labeled (see RGCs in the GCL and rod-bipolar cells in the INL immunodetected in **Figure 1C**).

The disappearance of the tracer from the photoreceptors is due to one of the most important functions of the RPE (Sparrow et al., 2010), the phagocytosis of shed photoreceptors' outer segment membrane. This phagocytosis explains why the tracer disappears from the outer retina but remains in the inner regions. Because the tracing of RPE occurs when the RPE cells phagocytose the outer segments of photoreceptors, this approach is very useful to study the functionality of the RPE in models of retinal degeneration (Valiente-Soriano et al., 2020).

The accumulation of FG in the RPE cells is better observed in flat mounts than in sagittal sections (compare panels C and D in **Figure 1**). Immunodetection of zonula occludens, a marker of tight junction protein 1 that delimits the morphology of the RPE cells (Zech et al., 1998; Georgiadis et al., 2010), shows that FG accumulates inside the cytoplasm avoiding the nuclei and extracellular spaces (**Figure 1D–D''**). Thus FG tracing reveals the characteristic honey-comb morphology of the RPE cells and their one or two nuclei per cell (Bonilha, 2008; Bhatia et al., 2016).

As shown above, this tracing technique depends on the time of administration because it is a sequential process that goes from the inner to the outer retina. Therefore, if the neuronal structures of the inner retina are the focus, 5 minutes are sufficient for labeling. For the study of the photoreceptors, somas and internal and external segments included, the appropriate time for the administration of the tracer is 15 minutes and, for the optimum labeling of the RPE 24 hours suffices. Although longer times can be used and, in fact, the tracer remains in the RPE and inner retina up to 30 days, shorter times (5 minutes to 24 hours) are advisable, since 30 days of tracing causes oxidative stress in the inner retina (Valiente-Soriano et al., 2020).

The advantages of tracing intravitreally are that for the first time we can investigate *in vivo*, rather quickly and fairly easily, functional transport between retinal neurons, the phagocytic status of the RPE, and the anatomical remodeling that occurs in retinal dystrophies. In addition, this tracing can be combined *ex vivo* with immunodetection, thus maximizing the number of analyses that can be carried out per retina.

In conclusion, intravitreal administration of FG traces retinal neurons and the RPE in a sequential and time dependent manner. Using this approach, the morphology and integrity of the retina and RPE, as well as the functionality of the RPE, can be safely and quickly assessed in rat models of retinal pathologies.

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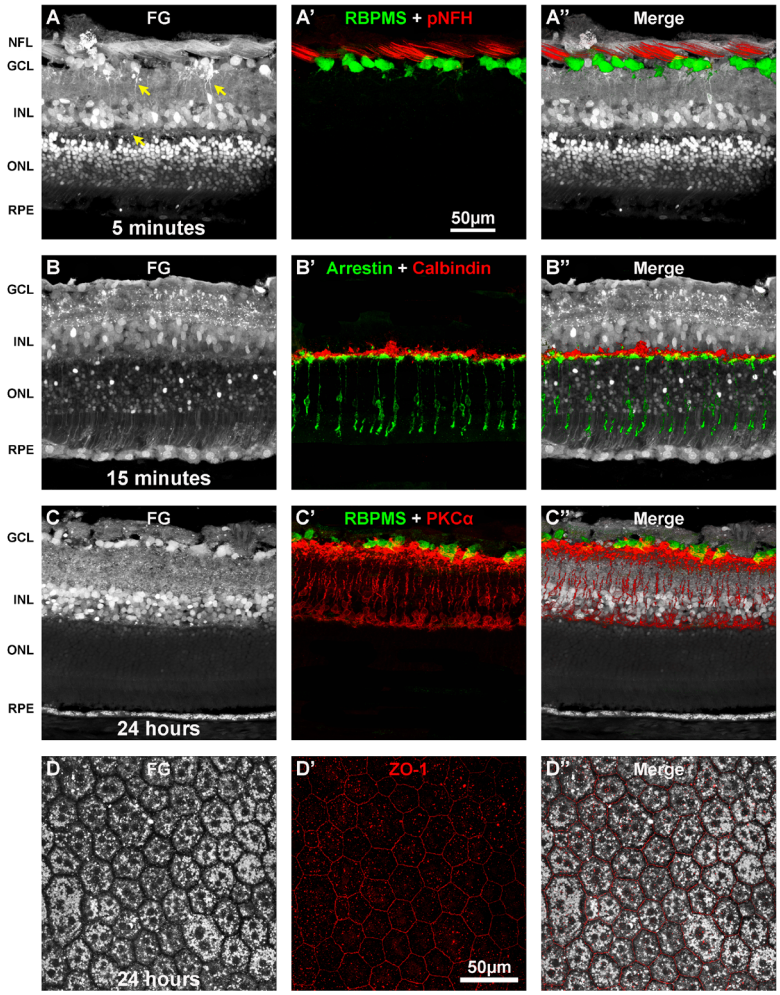

Figure 1 | **Time course of retinal labeling by intravitreal injection of fluorogold (FG).**
FG tracing in representative rat retinal sections analyzed at 5 (A–A'') or 15 (B–B'') minutes or 24 hours (C–C'') after intravitreal injection of FG. Yellow arrows in A point to neuronal processes and synaptic terminals in the plexiform layers. In these sections different populations have been immunoidentified: RGC somas (A', C', RBPMS), RGC axons (A', pNFH), horizontal cells (B', calbindin), cone photoreceptors (B', arrestin), rod bipolar cells (C', PKCα), and RPE cells (D', ZO-1). GCL: Ganglion cell layer; INL: inner nuclear layer; NFL: nerve fiber layer; ONL: outer nuclear layer; RBPMS: RNA-binding protein with multiple splicing protein; RGC: retinal ganglion cells; RPE: retinal pigment epithelium. Reprinted from Valiente-Soriano et al. (2020). The scale shown in panel A' refers to retinal sections from A to C'' and the scale shown in D' refers to flat retinal magnifications from D to D''.

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References

Bhatia SK, Rashid A, Chrenek MA, Zhang Q, Bruce BB, Klein M, Boatright JH, Jiang Y, Grossniklaus HE, Nickerson JM (2016) Analysis of RPE morphometry in human eyes. Mol Vis 22:898-916.

Bonilha VL (2008) Age and disease-related structural changes in the retinal pigment epithelium. Clin Ophthalmol 2:413-424.

Fisher CR, Ferrington DA (2018) Perspective on AMD pathobiology: A bioenergetic crisis in the RPE. Invest Ophthalmol Vis Sci 59:AMD41-AMD47.

Georgiadis A, Tschernutter M, Bainbridge JW, Balaggan KS, Mowat F, West EL, Munro PM, Thrasher AJ, Matter K, Balda MS, Ali RR (2010) The tight junction associated signalling proteins ZO-1 and ZONAB regulate retinal pigment epithelium homeostasis in mice. PLoS One 5:e15730.

Gu X, Neric NJ, Crabb JS, Crabb JW, Bhattacharya SK, Rayborn ME, Hollyfield JG, Bonilha VL (2012) Age-related changes in the retinal pigment epithelium (RPE). PloS One 7:e38673.

Nadal-Nicolás FM, Salinas-Navarro M, Vidal-Sanz M, Agudo-Barriuso M (2015) Two methods to trace retinal ganglion cells with fluorogold: from the intact optic nerve or by stereotactic injection into the optic tract. Exp Eye Res 131:12-19.

Rodriguez AR, de Sevilla Müller LP, Brecha NC (2014) The RNA binding protein RBPMS is a selective marker of ganglion cells in the mammalian retina. J Comp Neurol 522:1411-1443.

Salinas-Navarro M, Alarcón-Martínez L, Valiente-Soriano FJ, Jiménez-López M, Mayor-Torroglosa S, Avilés-Trigueros M, Villegas-Pérez MP, Vidal-Sanz M (2010) Ocular hypertension impairs optic nerve axonal transport leading to progressive retinal ganglion cell degeneration. Exp Eye Res 90:168-183.

Schmued LC, Fallon JH (1986) Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. Brain Res 377:147-154.

Sparrow JR, Hicks D, Hamel CP (2010) The retinal pigment epithelium in health and disease. Curr Mol Med 10:802-823.

Valiente-Soriano FJ, Salinas-Navarro M, Di Pierdomenico J, García-Ayuso D, Lucas-Ruiz F, Pinilla I, Cuenca N, Vidal-Sanz M, Villegas-Pérez MP, Agudo-Barriuso M (2020) Tracing the retina to analyze the integrity and phagocytic capacity of the retinal pigment epithelium. Sci Rep 10:7273.

Zhang Q, Chrenek MA, Bhatia S, Rashid A, Ferdous S, Donaldson KJ, Skelton H, Wu W, See TRO, Jiang Y, Dalal N, Nickerson JM, Grossniklaus HE (2019) Comparison of histologic findings in age-related macular degeneration with RPE flatmount images. Mol Vis 25:70-78.

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