

# An Immunohistochemical Analysis of Hypoxia in Multi-layer Avascular Retina and Vascular Pecten Oculi of the Developing Chicken

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**Abstract**

The retina lines the internal surface of the back of the eye. In order to function, the retina requires a supply of oxygen. Retinal hypoxia often precedes proliferative diabetic retinopathy in humans. In vertebrate eyes, there is a blood supply between retina and choroid, which supports the outer retinal layers. In humans, additional blood vessels enter the retina from the vitreal side and ramify in the plexiform layers. However, in chicks, such plexiform blood vessels are absent. The spatial oxidative state distribution in multi-layer avascular chick retina was studied using the oxygen-sensing reagent EF5. As development of the chick retina proceeds, different oxidation states in different layers were found.

The hypoxic staining was almost evenly distributed throughout the tissues for Embryonic day 5 chick retina. For E7 chick retina, the hypoxic stain was concentrated in a subset of ganglion cells. The inner portion of the inner nuclear layer and ganglion cell layer were hypoxic for E11 chick retina. For E14 chick retina, hypoxia was observed at the ganglion cell layer, but additional reaction was seen in the inner portion of the inner nuclear layer and the outer plexiform layer. There was also some reaction in the region of budding photoreceptors. The Ganglion cell layer, and the Inner Nuclear Layer were more hypoxic for E19 chick retina. In most cases, the regions of hypoxia did not colocalize with the positions that have high mitochondrial density.

Pecten oculi is a corrugated flap of tissue that extends along the back of the eye and is embedded in the vitreous body of the avian eye. It also contains a highly efficient glucose transporter. Hypoxia upregulates glucose transport activity. We found that pecten oculi is much less hypoxic than retina tissue nearby. There are more mitochondrial densities in pecten oculi than in retina.

**Key words:** immunohistochemistry, hypoxia, retinal development, pecten oculi

## Introduction

The retina is a multi-layered tissue which lines the internal surface of the back of the eye.

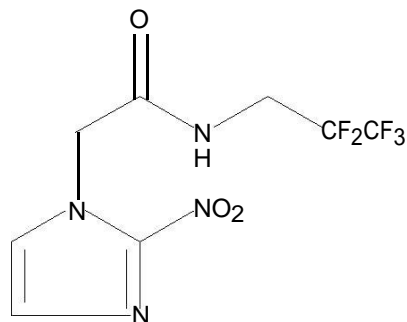
It requires a supply of oxygen and nutrients in order to function. In vertebrate eyes, there is a blood supply between retina and choroids to support the outer retinal layers. Retinal hypoxia often happens before proliferative diabetic retinopathy in humans.<sup>1, 2</sup> In mammals including humans, additional blood vessels enter the retina from the optic nerve and ramify in the plexiform layers. However, in lower vertebrates including chicks, such blood vessels are absent.

Buono and Sheffield<sup>3, 4</sup> showed that as the retina develops, the inner layers become adapted to anaerobic metabolism, both by the redistribution of mitochondria, and by the use of the anaerobic form of lactic dehydrogenase (LDH).

In view of the lack of a blood supply, and these metabolic studies, we have initiated a study of the spatial oxidative state distribution in multi-layer avascular chick retina.

In order to test the oxidative state of different layers in avascular retina, EF5/Cy3 immunohistochemical staining was used.

EF5, which is a pentafluorinated derivative of etanidazole (**Figure 1**), can react with cellular macromolecules to form adducts (EF5 reduction-protein conjugates). These adducts are formed at a much greater rate in hypoxic than aerobic cells (Lord E.M. et al., 1993). Their binding to hypoxic cells has been shown to be a method for determining the relative amount of intracellular oxygen concentration.



**Figure 1:** The chemical structure of EF5 [2-(2-nitro-1-H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide].

Exposure of tissues to EF5, followed by immunolocalization with highly specific antibodies (ELK3-51), can indicate levels of hypoxia within the tissues.<sup>5-12</sup>

In our studies, regions of high EF5 metabolism in chick retina of different developmental ages were visualized immunochemically using fluorochrome Cy3 (N,N'-(dipropyl)-tetramethyl-indocarbocyanine) conjugated to the ELK3-51 antibody.

Mitochondrial carboxylases use covalently bound biotin as a co-enzyme. Because biotin is bound extraordinarily tightly by avidin, labeled avidin can be used as a mitochondrial tracer.<sup>13</sup> Avidin conjugated to Alexa 488, which can specifically bind to biotin containing carboxylases in the mitochondria, was used to localize these structures.

7-AAD (7-aminoactinomycin D), which can specifically bind to DNA, was used as a nuclear marker.

While all vertebrate eyes do share a similar basic organization, avian eyes contain a unique structure, the pecten oculi. It is a corrugated flap of pigmented tissue that extends along the back of the eye and is embedded in the vitreous body of the avian eye. The pecten oculi contains connective tissue, pigment and blood vessels. In addition, there is a population of glial cells,

called peripapillary glial cells, which line the base of the pecten oculi. The blood vessels are continuous with those of the choroid layer of the avian eye. The pigmented cells are derived from the pigment epithelium of the retina.<sup>14</sup> This structure has been known for over a century, but its functions are still not clearly known.<sup>15-17</sup> The pecten with the unique morphological characteristics has been advantageous as an *in vivo* model for the blood-brain barrier investigation.<sup>15, 18, 19</sup>

Since the unique and somewhat major difference between the eyes of mammals and birds is that the complex and intricate system of retinal blood vessels is absent in the chicken, we hypothesize that the pecten oculi may be a structure providing oxygen supply which can compensate somewhat for the relatively low oxygen level in the inner chicken retina.

The oxidative state of the pecten oculi was examined with EF5/Cy3. We found that pecten oculi has a much higher oxygen level than the retina tissue nearby. The position, where the pecten oculi enters the retina and where the choroid layer has a blood vessel going through, has a much higher oxygen level than the retina tissue nearby, which does not contain any blood vessels.

In addition, Avidin-Alexa 488, was also used to examine the pecten oculi. The result shows that there are more mitochondria in the pecten oculi than in the retina tissue nearby. In most cases, the regions of high oxygen level colocalized with the positions that have high mitochondrial density.

## Materials and Methods

### *Animals:*

White leghorn chicken embryos (CBT Farms, Chestertown, MD) were raised in a forced draft air incubator at 37 °C in 80% humidity to the desired age. Embryonic

age was determined by counting the number of days after the fertilized eggs were placed in the incubator.

### *Experimental Protocol:*

Embryonic chickens of five days, seven days, eleven days, fourteen days, and nineteen days were selected. EF5 (10 mM) in 0.01M phosphate buffered saline (PBS) was injected into vitreous humor of the left eye (0.01 mL for E5, 0.02 mL for E7, 0.03 mL for E11, 0.04 mL for E14, 0.05 mL for E19) and additional volume to a total of 0.2 mL was injected into the tissues near the eyeball of the chicken embryo. The EF5 injected embryonic chickens were incubated for half an hour in the incubator at 37 °C.

Embryonic chickens were sacrificed by decapitation and their eyeballs were detached. The lenses and vitreous humor were removed. The left eyeballs were fixed in 2% paraformaldehyde in PBS at 4 °C overnight. Then the eyeballs were soaked in 30% sucrose as a cryoprotectant overnight.

OCT (an abbreviation for “optimal cutting temperature” solution) was placed in the vitreal cavity, and eyeballs were put one by one on the stages of the cryostat at -20°C. Additional OCT was added to the eyeballs until all of the tissue was covered. Then the OCT covered eyeballs were left in the microtome for one hour. Once the tissue had sufficiently frozen after one hour, the eyeballs were sectioned at -20 °C for 20µm cross-sections and the sections were transferred to gelatin-coated slides for later staining and imaging.

### *Immunohistochemical Analysis:*

The prepared sections of eye were rinsed in PBS for ten minutes to remove the OCT. The sections were then incubated in blocking solution Nonfat milkpowder 1 g, Albumin 1.5 g, NP-40 0.3 mL in 100 mL PBS for one hour to block nonspecific

endogenous antigens. Then the tissue sections were rinsed in PBS for ten minutes.

The slides were then put into a Petri dish and incubated/labeled with Cy3-conjugated ELK3-51 antibody (37.5 µg/mL). The Petri dish was covered and put into refrigerator at 4°C for 24 hours. After staining, the tissues were rinsed in PBS twice (each for 10 mins.).

Sections from eyeballs of five days, eleven days, fourteen days and nineteen days were incubated with Avidin-Alexa 488 (20 µM) for ten minutes. The sections of seven days, fourteen days and nineteen day eyes were incubated with 7-AAD (32 µM) - for one hour. After staining, the sections were

rinsed in PBS twice (each for 10 mins.). The cover slips were mounted on the slides and the edges were sealed with nail polish to prevent drying. The prepared slides with eyeball were then ready for imaging.

*Imaging with confocal microscope:*

According to the excitation and emission spectrum for different stains. (Table 1) - The slides with stained retinas were then scanned in Leica SP1 Confocal Microscope. The images were processed with Image J, a public domain image analysis program (-<http://rsb.info.nih.gov/ij/>) to determine the relative density of EF5 staining of different stage chicken retina.

Reagent	Excitation Peak	Emission Peak
Alexa 488 Avidin	495 nm	519 nm
ELK3-Cy3	552 nm	570 nm
7-AAD	552 nm	655 nm

**Table 1: The excitation and emission wavelengths of different stains.**

*Image processing:*

In order to determine the relative density of EF5 staining of different stage chicken retina a straight line was drawn across the images stained with EF5/Cy3/ELK3-51. The line width was then changed to 150 pixels to form a rectangle. The rectangle was used to select the region for analysis of EF5/Cy3 intensity. The “plot profile” option was used to determine the values. The values were then transferred to Excel for further analysis and graphing. Two distinct regions across the retina were analyzed separately, and the results combined.

**Results**

As development of the chick retina proceeds, progressive changes in oxidation

state in different layers were found.

*The chicken retina of five days embryogenesis.*

For embryonic five days (E5) chick retina, the tissues are thin and have not been differentiated into different layers. The oxygen levels were examined with EF5/Cy3 staining.

The gain was increased in order to observe the details of the tissue. The staining was almost evenly distributed throughout the tissues.

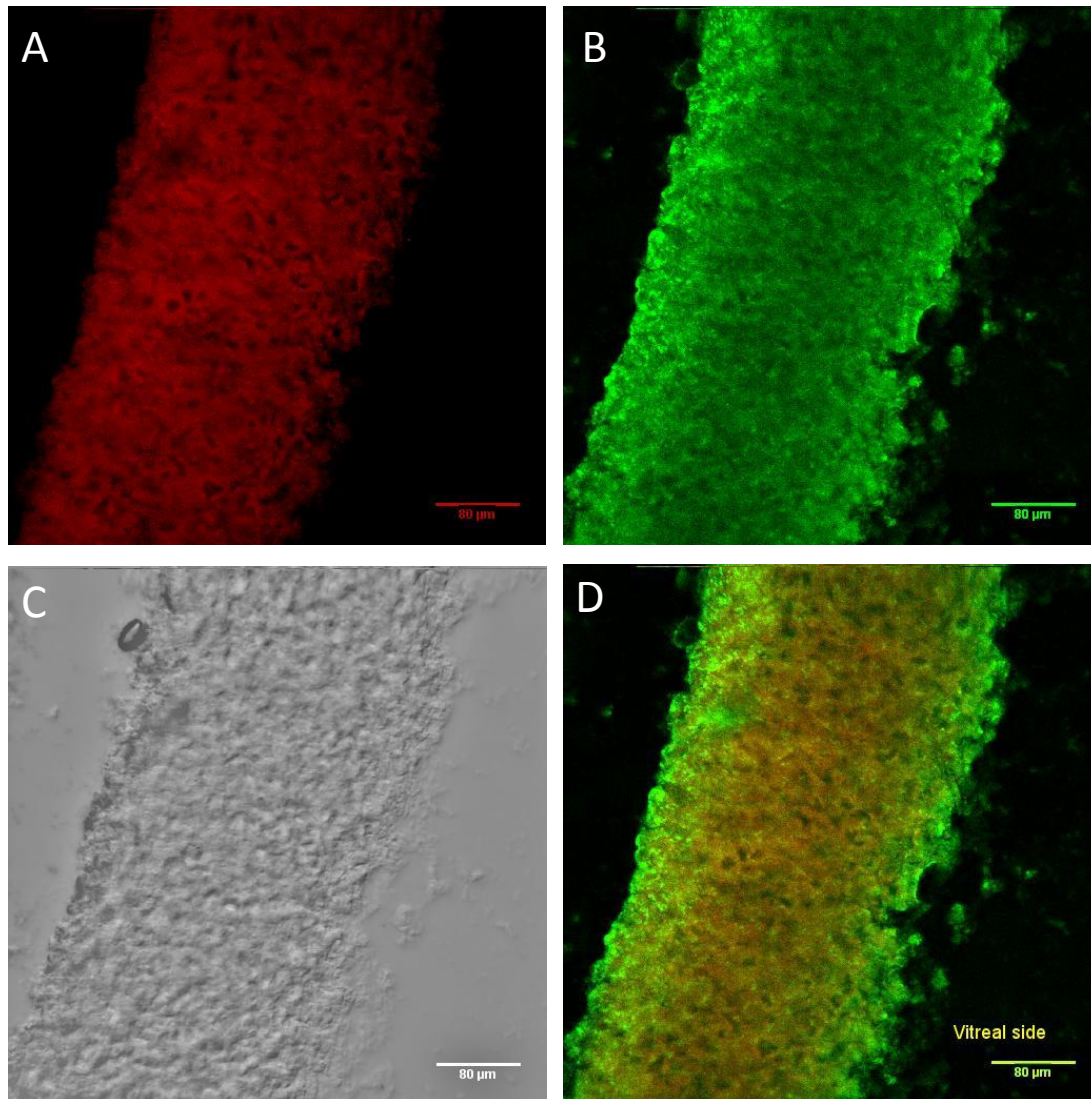
Avidin-Alexa 488, which can specifically bind to biotin containing carboxylases in the mitochondria, was used to localize these structures. The staining is distributed evenly in most of the tissues and there are more densities at the vitreal and PE

edges.

The EF5/Cy3 staining image, Avidin-Alexa 488 staining image, transmission merge image and the merged

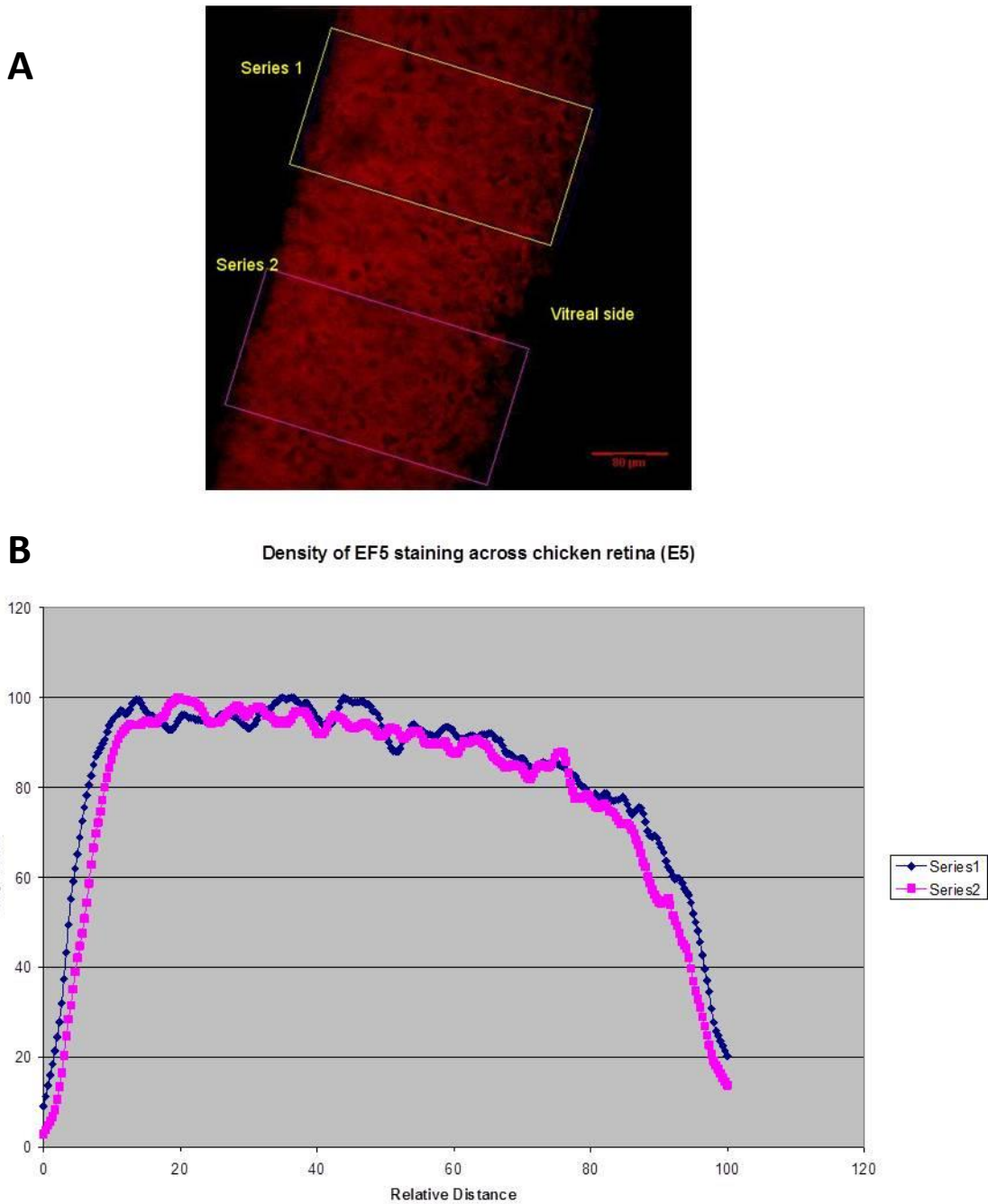
image are shown in **Figure 2**.

The density analysis of EF5 staining across the retina is shown in **Figure 3**.



**Figure 2: Retinal images of E5 retina.**

(A) EF5/Cy3 indicate levels of hypoxia (B) Avidin-Alexa 488, localize mitochondria (C) Transmission image. (D) The merge image of EF5/Cy3 and Avidin-Alexa 488 staining.



**Figure 3: Density analysis of EF5 staining (Embryonic five days).**

(A) The image of EF5 staining. The squares (series 1 and 2) on the image show the regions which are analyzed. (B) The density analysis of EF5 staining (Embryonic five days) from outer layer (Photoreceptor cell layer) to inner layer (Ganglion cell layer).

*The chicken retina of embryonic seven days.*

For embryonic seven days (E7) chick retina, the tissues had just started to differentiate. EF5/Cy3 staining was

concentrated in a subset of ganglion cells indicating increased hypoxia.

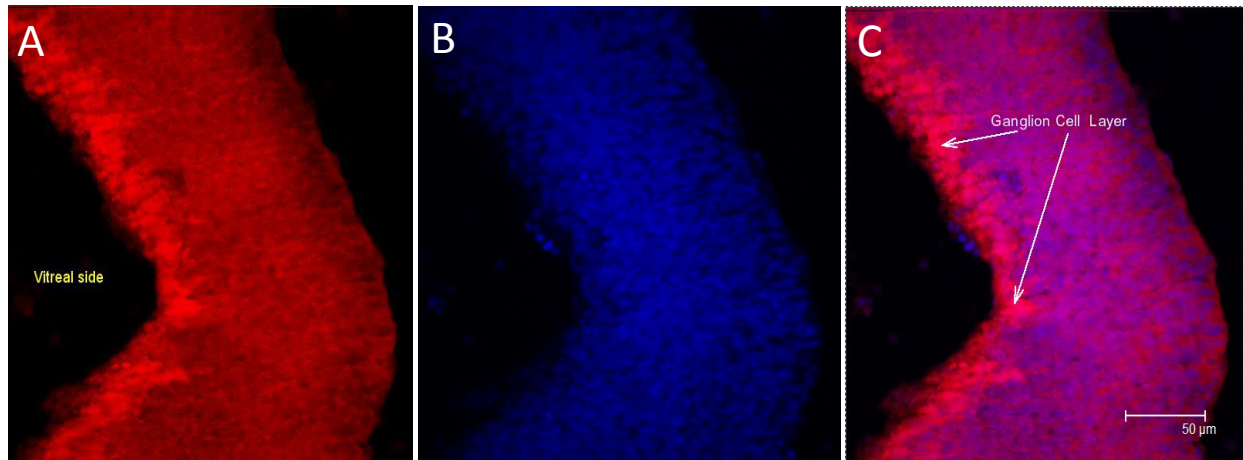
7-AAD, which can specifically bind to DNA, was used to localize nuclei. The staining is almost evenly distributed across

the retina, which shows the tissues have not been differentiated into plexiform different layers.

The images of EF5/Cy3 staining, 7-

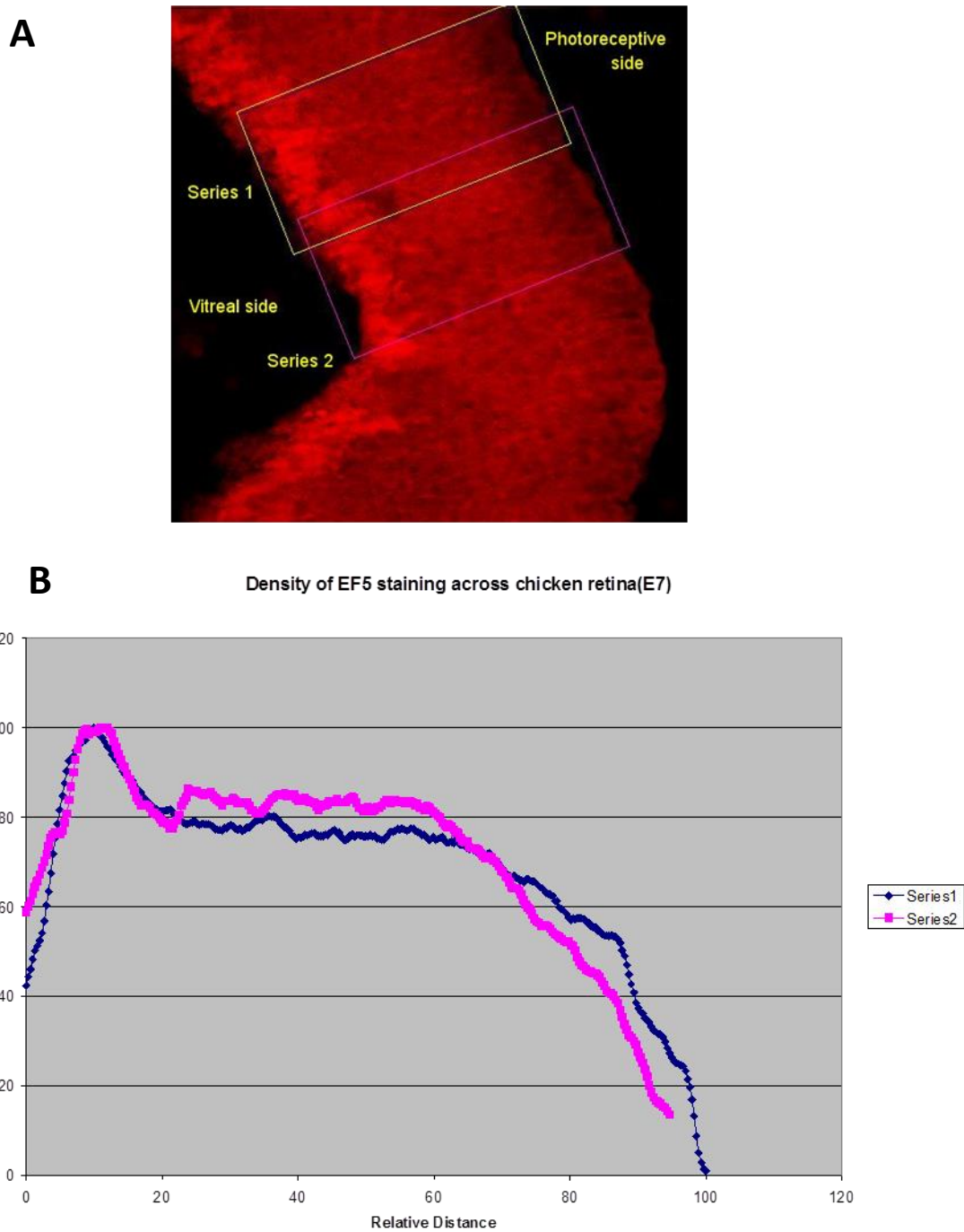
AAD staining and merge images are shown in **Figure 4**.

The density analysis of EF5 staining is shown in **Figure 5**.



**Figure 4: Retinal images of embryonic seven days.**

(A) EF5/Cy3 staining. (B) 7-AAD, which can specifically bind to DNA, was used to localize nuclei. (C) The merge image of EF5/Cy3 and 7-AAD staining.



**Figure 5: Density analysis of EF5 staining (Embryonic seven days).**

(A) The image of EF5 staining. The squares (series 1 and 2) on the image show the regions which are analyzed. (B) The density analysis of EF5 staining (Embryonic seven days) from inner layer (Ganglion cell layer) to outer (Photoreceptor cell layer) layer.

*The chicken retina of embryonic eleven days.*

For embryonic eleven days (E11) chick retina, the tissues are partially

differentiated. The oxidative states were examined with EF5/Cy3 staining. The hypoxia (more staining) is observed in single cells in the inner portion of the inner



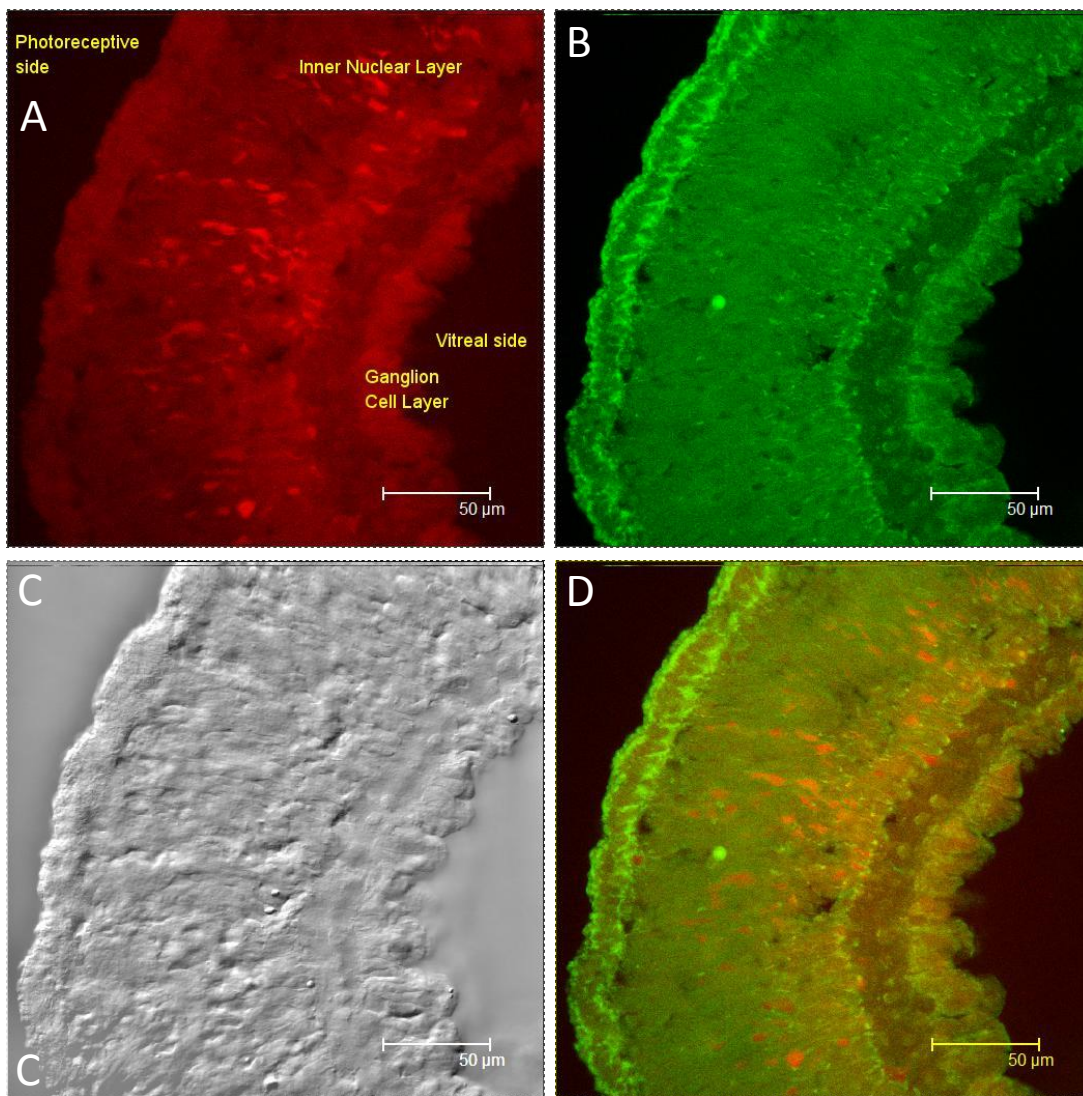
nuclear layer and ganglion cell layer.

Avidin-Alexa 488 was used to localize mitochondria. There are different layers of stain. From outer to inner retina, the stain is seen in the photoreceptor cell layer, at the border of the outer plexiform layer, at the border of inner nuclear layer and in the ganglion cell layer. There is conspicuous absence of stain in the center of both the outer nuclear layer and inner nuclear layer.

In most cases, the regions of hypoxia did not colocalize with the positions that have high mitochondrial density.

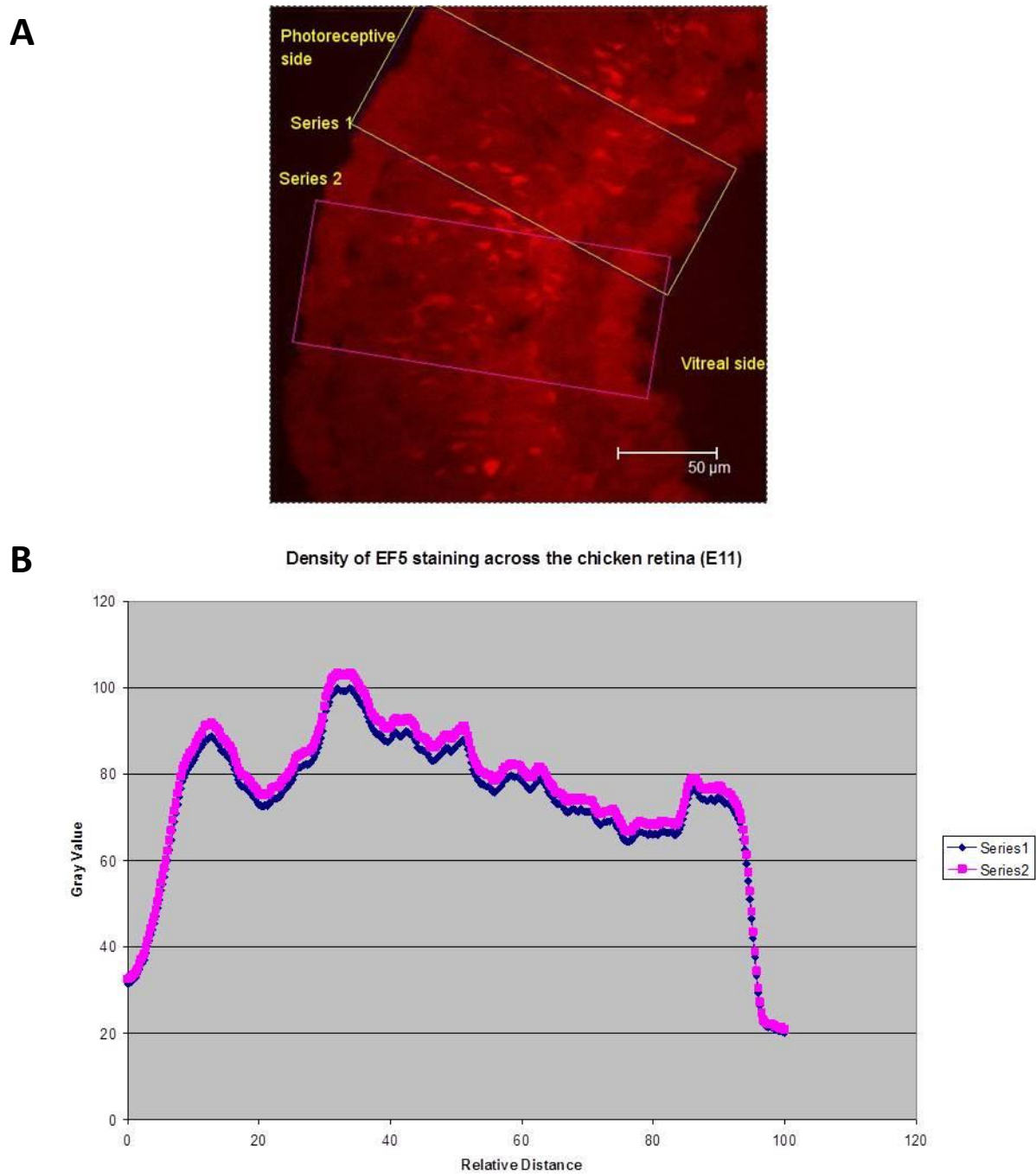
The EF5/Cy3 staining image, Avidin-Alexa 488 staining image, transmission image and the merge image are shown in **Figure 6**.

The density analysis of EF5 staining is shown in **Figure 7**. The general trend, as indicated by the scan, does not clearly show the single cell's stain.



**Figure 6: Retinal images of embryonic eleven days.**

(A) EF5/Cy3 staining, which can indicate levels of hypoxia within the tissue. (B) Avidin-Alexa 488, which can specifically bind to biotin containing carboxylases in the mitochondria, was used to localize these structures. (C) Transmission image. (D) The merge image of EF5/Cy3 and Avidin-Alexa 488 staining.



**Figure 7: Density analysis of EF5 staining (Embryonic eleven days).**

(A) The image of EF5 staining. The squares (series 1 and 2) on the image show the regions which are analyzed. (B) The density analysis of EF5 staining (Embryonic seven days) from outer (Photoreceptor cell layer) to inner layer (Ganglion cell layer).

*The chicken retina of embryonic fourteen days.*

At 14 days, the embryonic chick retina is still differentiating. Hypoxia is

observed at the ganglion cell layer, but additional reaction was seen in the inner portion of the inner nuclear layer and the outer plexiform layer. There was also some

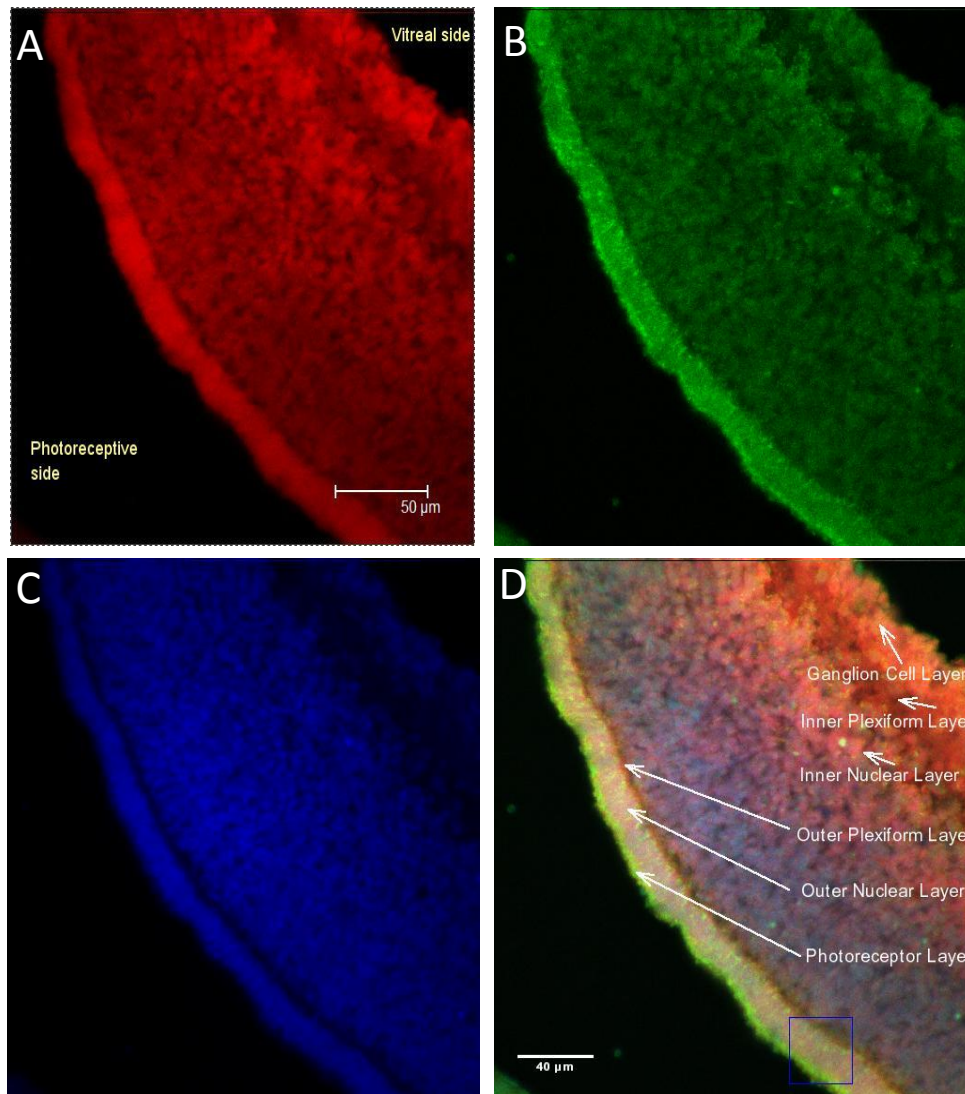
reaction in the region of budding photoreceptors. In most cases, the regions of hypoxia do not colocalize with the positions that have high mitochondrial density.

The images of EF5/Cy3 staining, Avidin-Alexa 488 staining, 7-AAD and merge image are shown in **Figure 8**.

In the enlarged images, (enlarged from the square showing in the merge image in **Figure 8**) it can be seen more clearly that the regions of hypoxia do not colocalize with the positions that have high mitochondrial density. As shown in the merged enlarged image, the outer plexiform

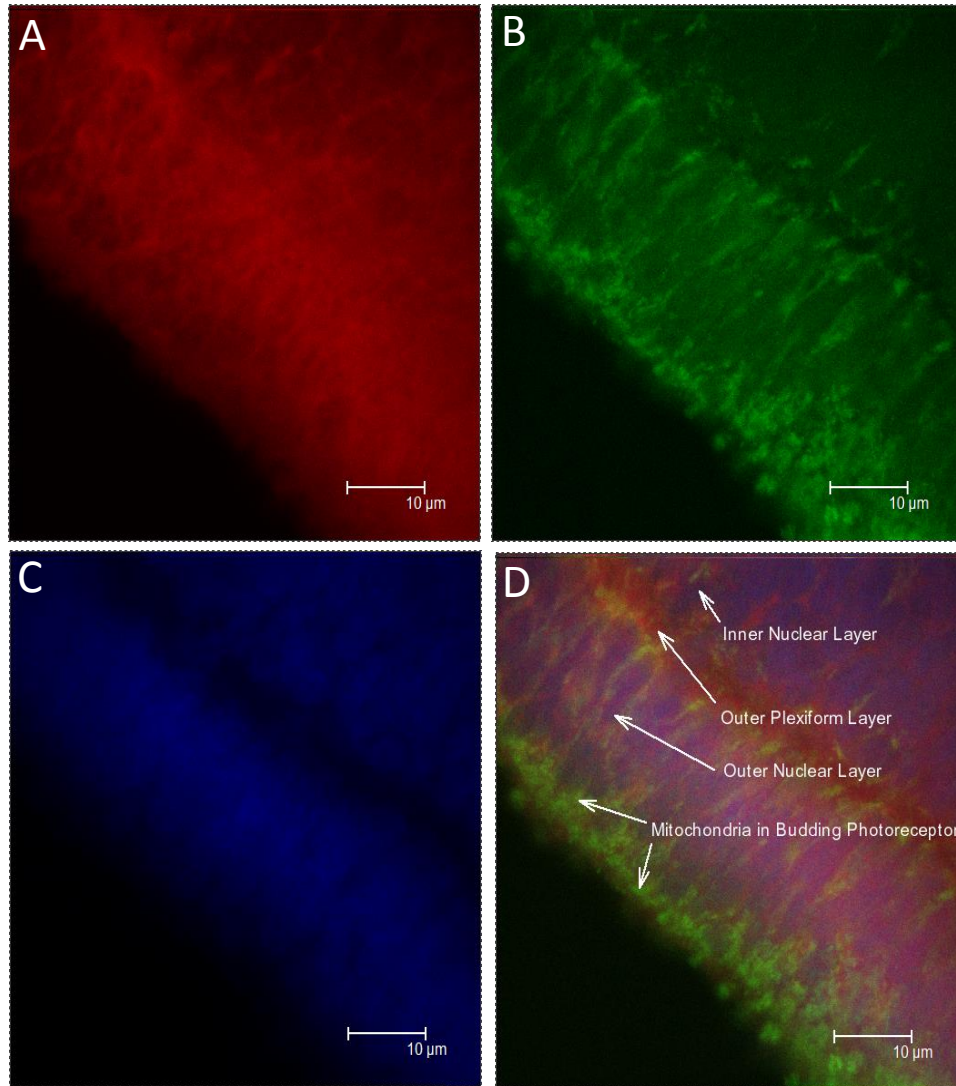
layer is more hypoxic and most mitochondria are in the budding photoreceptor. There are some mitochondria at the border of the outer nuclear layer. The relatively hypoxic outer plexiform layer is at the right side of the outer nuclear layer and photoreceptor cell layer which contain the mitochondria. The regions of hypoxia do not colocalize with the positions that have high mitochondrial density. The enlarged images are shown in **Figure 9**.

The density analysis of EF5 staining is shown in **Figure 10**.



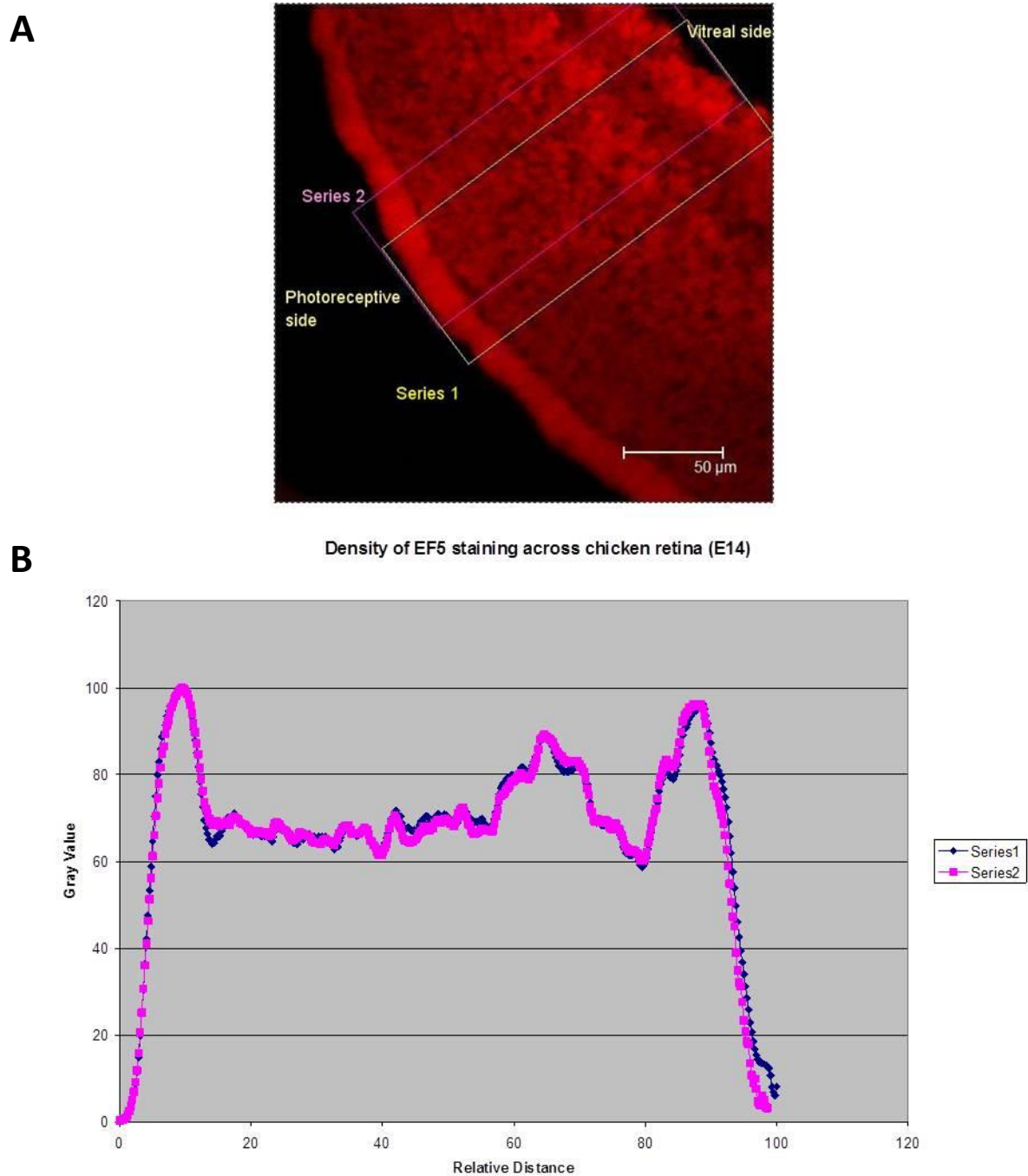
**Figure 8: Retinal images of embryonic fourteen days.**

(A) EF5/Cy3 staining, which can indicate levels of hypoxia within the tissue. (B) Avidin-Alexa 488, which can specifically bind to biotin containing carboxylases in the mitochondria, was used to localize these structures. (C) 7-AAD, which can specifically bind to DNA, was used to localize nucleus. (D) The merge image of EF5/Cy3, Avidin-Alexa 488 staining and 7-AAD staining.



**Figure 9: Retinal images of embryonic fourteen days. (Enlarged)**

Enlarged images which were stained with different reagents. **(A)** EF5/Cy3 staining, which can indicate levels of hypoxia within the tissue. **(B)** Avidin-Alexa 488, which can specifically bind to biotin containing carboxylases in the mitochondria, was used to localize these structures. **(C)** 7-AAD, which can specifically bind to DNA, was used to localize nucleus. **(D)** The merge image of EF5/Cy3, Avidin-Alexa 488 staining and 7-AAD staining.



**Figure 10: Density analysis of EF5 staining (Embryonic 14 days).**

(A) The image of EF5 staining. The squares (series 1 and 2) on the image show the regions which are analyzed. (B) The density analysis of EF5 staining (Embryonic 14 days) from outer (Photoreceptor cell layer) to inner layer (Ganglion cell layer).

*The chicken retina of embryonic nineteen days.*

For embryonic nineteen days (E19) chick retina, there are different layers. The

oxygen levels were examined with EF5/Cy3 staining. The ganglion cell layer and the outer portion of the inner nuclear layer were more hypoxic (more staining) than other layers.

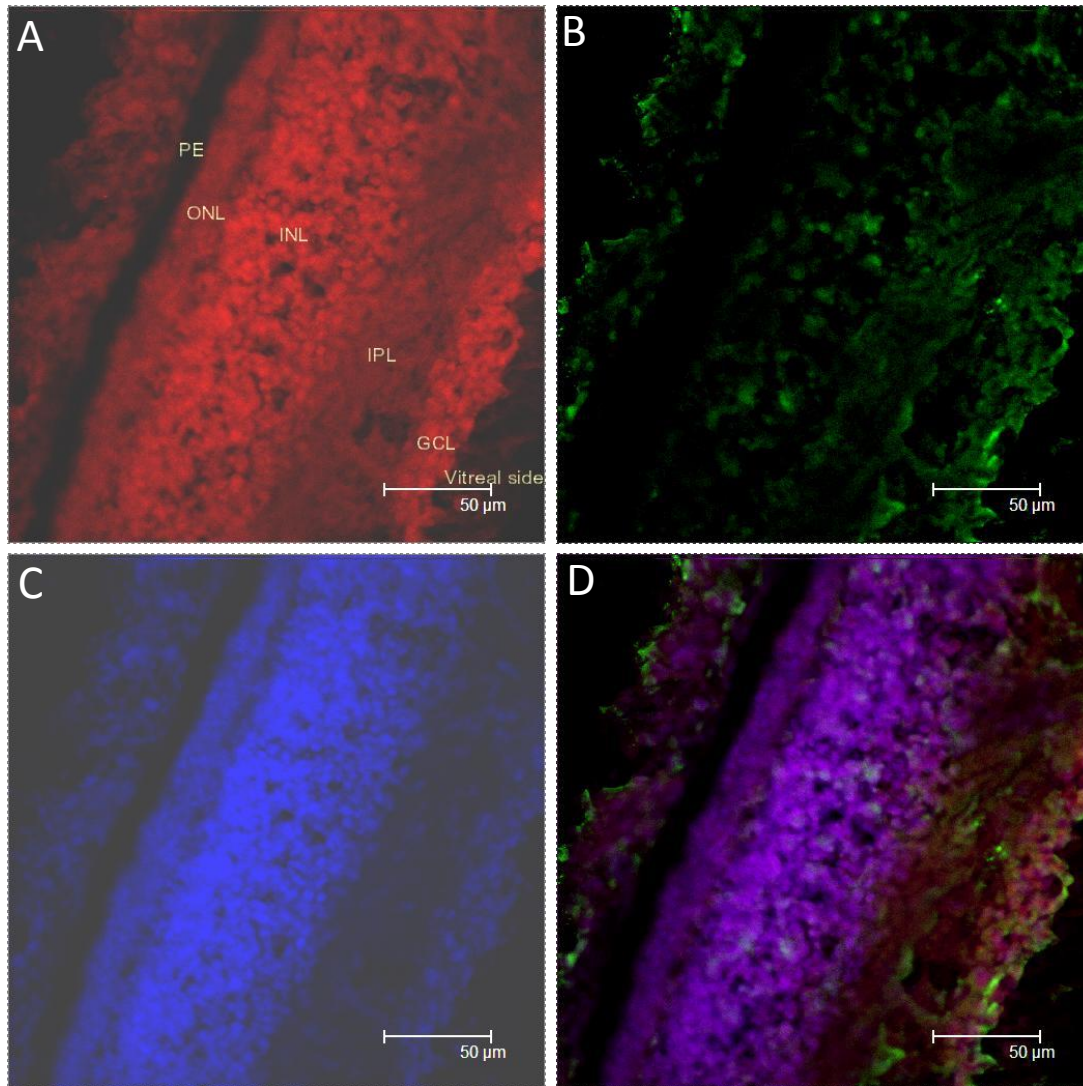
Avidin-Alexa 488 was used to localize mitochondria. There is very little binding of the avidin throughout the retina. The expected high density of mitochondria in the photoreceptor inner segments is masked by the pigment epithelium. In most cases, the regions of hypoxia did not

colocalize with the positions that have high mitochondrial density.

7-AAD was used to localize nuclei. The staining was distributed in different nuclear layers, which shows the tissues were differentiating into different layers.

The images of EF5/Cy3 staining, Avidin-Alexa 488 staining, 7-AAD staining and merge image are shown in **Figure 11**.

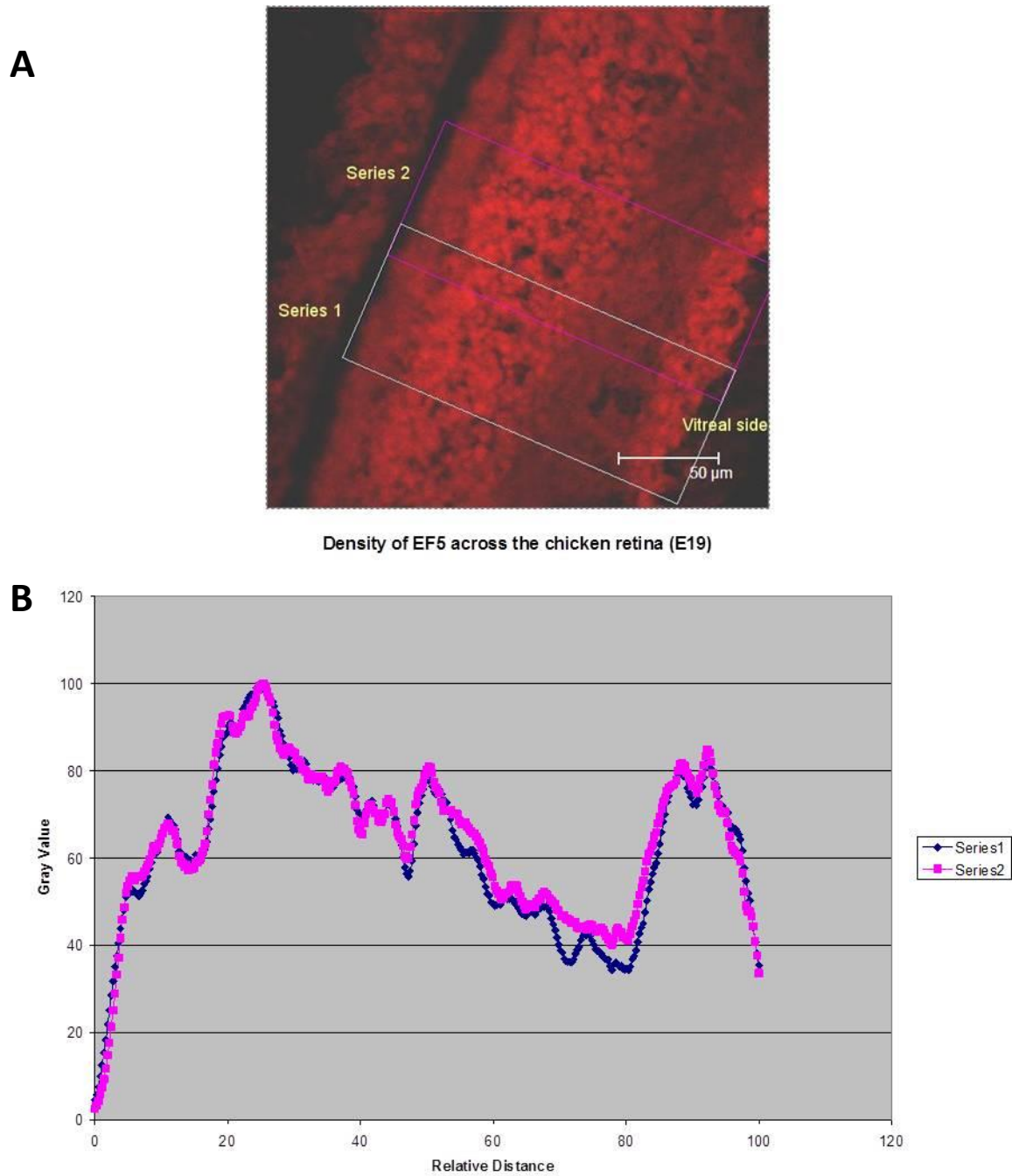
The density analysis of EF5 staining is shown in **Figure 12**.



**Figure 11: Retinal images of embryonic nineteen days.**

Figure shows the retinal images of embryonic nineteen days which were stained with different stains. (A) EF5/Cy3 staining, which can indicate levels of hypoxia within the tissue. (B) Avidin-Alexa 488, which can specifically bind to biotin containing carboxylases in the mitochondria, was used to localize these structures. (C) 7-AAD, which can specifically bind to DNA, was used to localize nucleus. (D) The merge image of EF5/Cy3, Avidin-Alexa 488 staining and 7-AAD staining.





**Figure 12: Density analysis of EF5 staining (Embryonic 19 days).**

(A) The image of EF5 staining. The squares (series 1 and 2) on the image show the regions which are analyzed. (B) The density analysis of EF5 staining (Embryonic 19 day) from outer layer (Photoreceptor cell layer) to inner layer (Ganglion cell layer).

*Pecten oculi of embryonic nineteen days.*

Pecten oculi is a corrugated flap of pigmented tissue that extends along the back

of the eye and is embedded in the vitreous body of the avian eye. The pecten oculi contains connective tissue, pigment, blood vessels and peripapillary glial cells. The

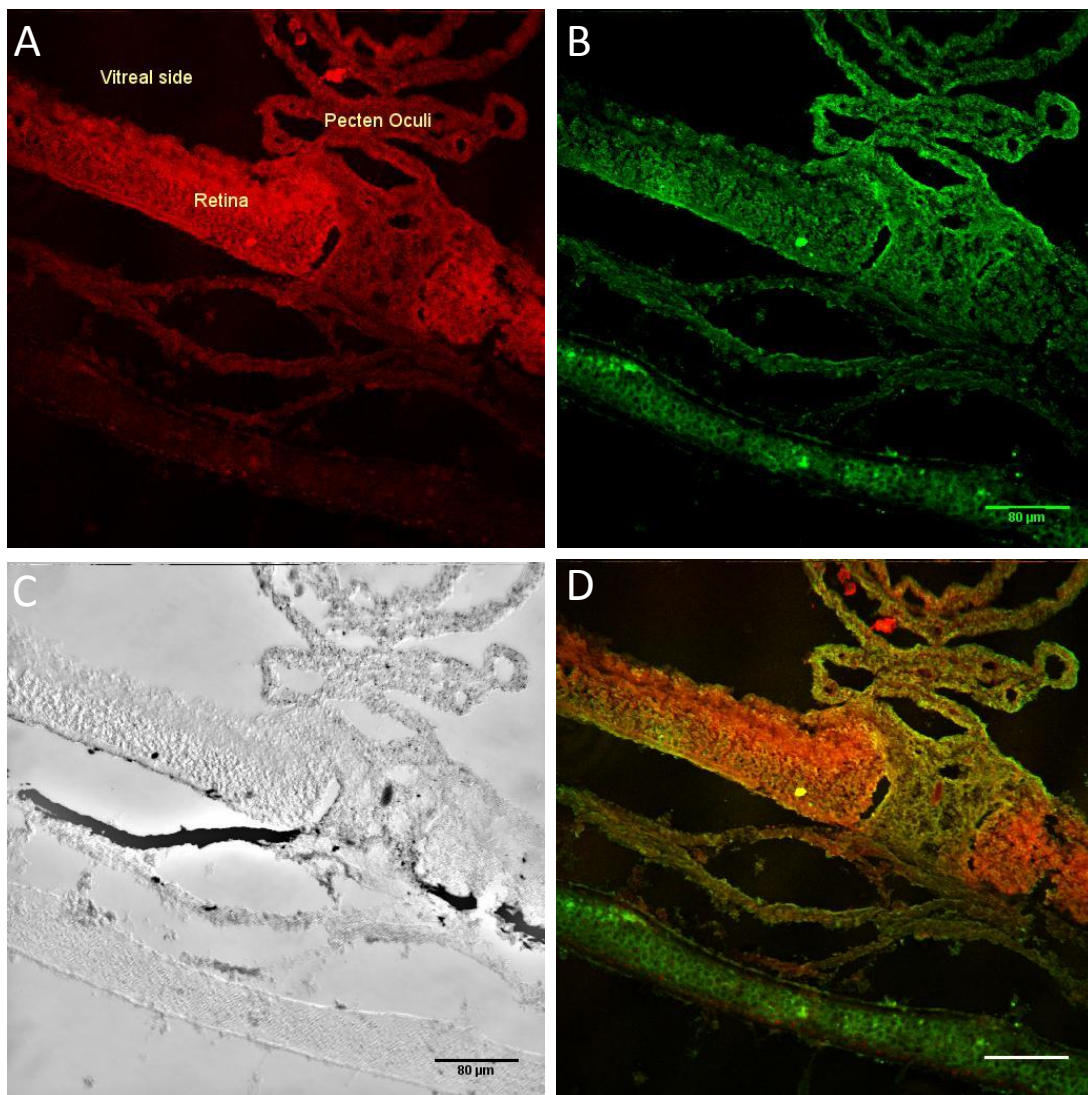
connective tissue and blood vessels are continuous with those of the choroid layer of the avian eye. The pigment cells in the pecten oculi are derived from the pigment epithelium of the retina.<sup>14-17</sup>

The oxidative state of the pecten oculi was examined with EF5/Cy3. We found that pecten oculi is much less hypoxic than retina tissue nearby. Avidin-Alexa 488 was used to localize mitochondria. There are

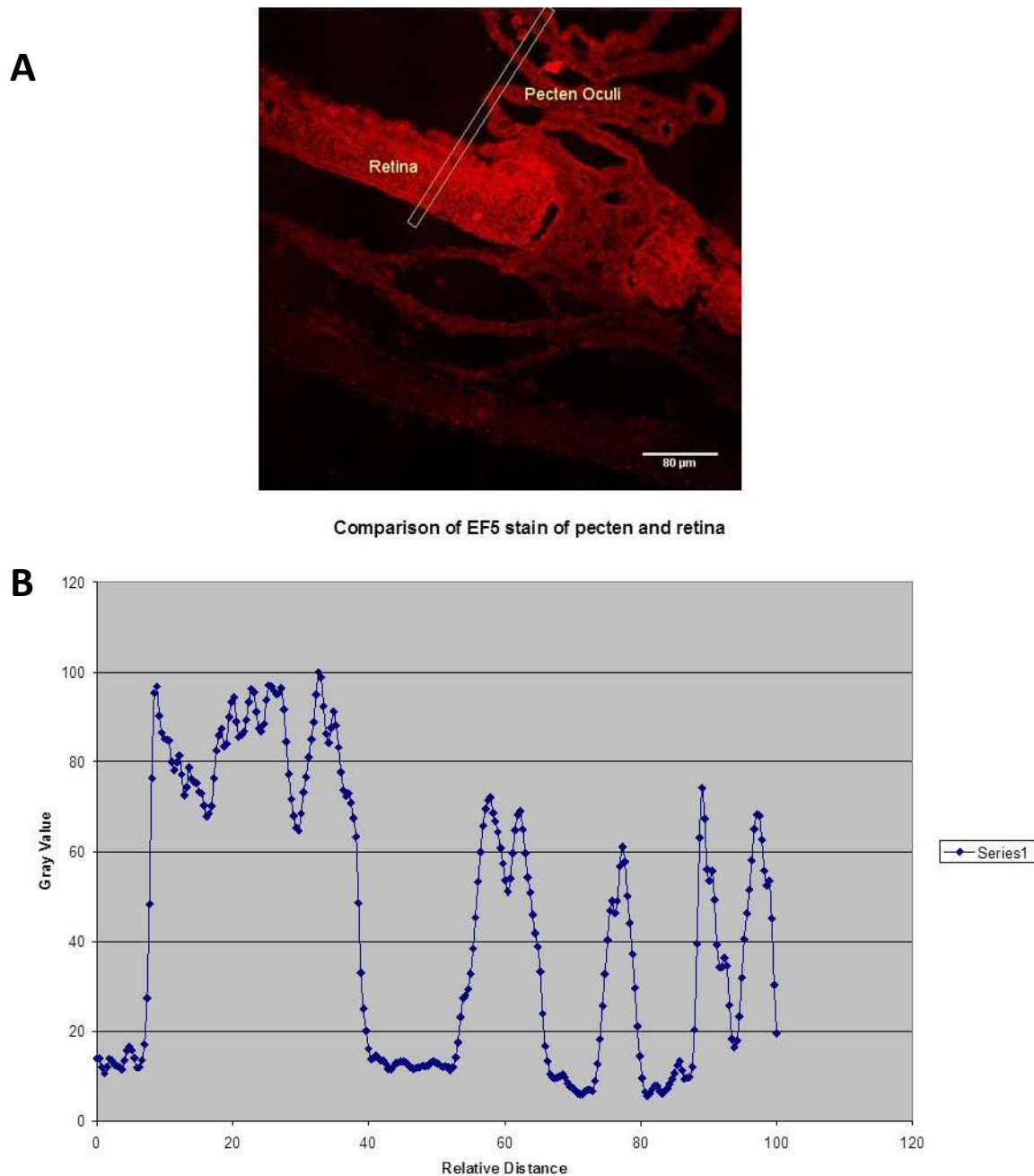
intense mitochondrial densities on the periphery of the pecten oculi, which are absent in retina. In most cases, the regions of hypoxia did not colocalize with the positions that have high mitochondrial density.

The images of EF5/Cy3 staining, Avidin-Alexa 488 staining, and merge image are shown in **Figure 13**.

The density analysis of EF5 staining is shown in **Figure 14**.



**Figure 13: The pecten oculi and retinal images of embryonic nineteen days.** (A) EF5/Cy3 staining, which can indicate levels of hypoxia within the tissue. (B) Avidin-Alexa 488, which can specifically bind to biotin containing carboxylases in the mitochondria, was used to localize these structures. (C) Transmission image. (D) The merge image of EF5/Cy3 and Avidin-Alexa 488 staining.



**Figure 14: Density analysis of EF5 staining (Pecten oculi and retina of Embryonic nineteen days).**

(A) The image of EF5 staining. The squares on the image show the regions which are analyzed. (B) The density comparison of EF5 staining between the pecten oculi and retina. (Embryonic nineteen days). The first peak is the retina, the others are pecten oculi. (If the gray value is below 20, it is nontissue.)

### Discussion

In view of the lack of a blood supply to the inner retina of birds, we have initiated a study of the spatial oxidative state distribution

in the multi-layer avascular chick retina. In order to test the oxidative state of different layers in avascular retina, EF5/Cy3 immunohistochemical staining was used.

Our results show that the oxidative state varies in different layers of the avascular chick retina as development of the retina proceeds. The inner layers are usually more hypoxic. In general, the reaction product appears diffuse within layers of the retina with little precise cellular localization.

There are two primary means of energy production for cells. One is glycolytic metabolism. Glycolysis is a set of reactions that take place in the cytoplasm of the cells. Two net ATP can be generated from one glucose molecule through the glycolytic pathway. Lactate is produced in the presence of the enzyme lactate dehydrogenase (LDH). Glycolytic metabolism is usually inhibited by oxygen although there is an oxygen tolerant form of LDH.

Another means of energy production for the cells is oxidative metabolism, which is oxygen dependent. About thirty-six net ATP per glucose molecule can be generated through oxidative metabolism. Under aerobic condition, organic molecules can enter the citric acid cycle and can be broken down to carbon dioxide and water in the mitochondria. Lactate is not generated in oxidative metabolism.<sup>20</sup>

In vertebrate eyes, there is a blood supply between retina and choroid, the chorio-capillaris, which is thought to supply the oxygen for the outer retinal layers. In mammals including humans, additional blood vessels enter the retina from the vitreal side and ramify in the plexiform layers. However, in birds including chicks, such blood vessels are absent.

Studies *in vitro* have shown the avian retina to have a lower respiratory rate than that of most other species and an extremely high glycolytic rate.<sup>21, 22</sup> The distinctive metabolic properties are thought to be due to the relatively hypoxic environment of the avian retina. Our results provide direct evidence for the low oxygen level in regions of the avian retina.

As part of this study, the distribution of mitochondria was examined. Our results confirm the earlier studies of Buono and Sheffield, 1991, and Ruggerio and Sheffield, 1998 and indicate that mitochondria segregate to distinct regions of the chicken retina as it develops.<sup>3,4,15</sup> At about embryonic fourteen days, the mitochondria reach their final positions in the retina (in the budding photoreceptor cells, border of the plexiform layers, the inner nuclear layer which border the plexiform regions and in the ganglion cells). Ultimately, as the chicken retina develops, the inner layers lose mitochondria. In most cases, the regions of high oxygen level colocalized with the positions that have high mitochondrial density. Segregation of mitochondria somewhat reflects the distribution of oxygen level in different regions of the chicken retina as development proceeds. This is consistent with the thought that retinal vascularization influences oxygen availability to the tissue and affects the distribution of mitochondria in the chicken retina. An interesting exception is the photoreceptor layer, which is closest to the choroidal circulation, and appears to have relatively low oxygen availability in E11 and E14. It is possible that this results from the balance of oxygen supply and consumption in this area. Although there is more oxygen available for the photoreceptor cells, the mitochondrial activity of the photoreceptor inner segments could reduce the actual level significantly. Lowry O.H. et al., (1956) showed that the photoreceptor layer in monkey contained about 1/3 of the total mitochondrial activity, while in the rabbit, which has few internal blood vessels, the ratio is about 1/2, since there are fewer inner layer mitochondria.<sup>22</sup> Buono R.J. and Sheffield J.B. (1991) showed that in the chick, the ratio is 1/2. This high local density of mitochondria might deplete the local oxygen level.<sup>3,4</sup>

The characteristics of avian retina may be related to significant physiological

functions. Usually, birds have a greater visual acuity than most of other species. It is possible that the avascularity of retina aids in visual acuity. In the mammalian retina, the macula, which is the point of the highest acuity, also lacks internal blood vessels. The mammalian fovea, however, is thinner than the rest of the retina, and contains fewer inner neurons that might require oxygen.

Most avian eyes contain a unique structure, pecten oculi, which is absent in other vertebrate eyes. The pecten oculi contains blood vessels and is embedded in the vitreous body of the avian eye. Since the major difference between the eyes of many other vertebrates and those of a chicken is the complex and intricate system of blood vessels. We hypothesize that the pecten oculi may be a structure providing oxygen supply which can somewhat compensate the relatively low oxygen level in the chicken retina. The results show that pecten oculi has much higher oxygen level than the retina tissue nearby. The position, where the pecten oculi enters the retina and where the choroid layer has a blood vessel going through, has a much higher oxygen level than the retina tissue nearby which does not contain any blood vessels. It appears, however, that the oxygen that may be provided by the pecten oculi is not sufficient to support full oxidative metabolism in the inner retina. It may be enough to support the few mitochondria that we observed in the ganglion cells.<sup>3, 4</sup>

Others have reported that the cells of the pecten oculi contain a highly efficient glucose transporter. This would provide an enriched source of glucose for the retina, which would be necessary to support extended glycolysis.<sup>18,23</sup> In addition, the intracellular glucose concentration is closely associated with glucose transport, which is an important factor in the development of diabetic retinopathy.<sup>2, 24, 25</sup>

## Conclusions

The spatial oxidative state distribution in multi-layer avascular chick retina was studied. As development of the chick retina proceeds, different oxidation states in different layers were found.

For E5 chick retina, the staining was almost evenly distributed throughout the tissues. For E7 chick retina, the hypoxic stain was concentrated in a subset of ganglion cells. For E11 chick retina, the hypoxia is observed in the inner portion of the inner nuclear layer and ganglion cell layer. For E14 chick retina, hypoxia was observed at the ganglion cell layer, but additional EF5 reaction was seen in the inner portion of the inner nuclear layer and the outer plexiform layer. There was also some EF5 reaction in the region of budding photoreceptors. For E19 chick retina, the Ganglion cell layer, and the Inner Nuclear Layer were more hypoxic. In most cases, the regions of hypoxia did not colocalize with the positions that have high mitochondrial density.

We found that pecten oculi, a corrugated flap of pigmented tissue that extends along the back of the eye and is embedded in the vitreous body of the avian eye, is much less hypoxic than retina tissue nearby. We also found that there are more mitochondrial densities in pecten oculi than in retina.

## Acknowledgements

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