Detection of melanin in fisheyes iris and ocular fundus (retina and choroid) by light-emitting diode photoacoustic imaging

LED 光源を用いた光超音波画像による
魚眼球の虹彩と眼底内のメラニンの描出

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東京都立大学大学院 人間健康科学研究科 博士後期課程

人間健康科学専攻　放射線科学域

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論文要旨　Abstract

Introduction: Photoacoustic imaging (PAI) is a new technology, that combines the high-contrast, spectroscopic-based specificity of optical imaging with high-resolution ultrasonography (US). The principle of PAI is that a substance absorbs light energy according to the substance-specific absorption coefficient determined by wavelengths. The absorbed energy products ultrasound vibration. At first, the device emits light to the substance, the substance absorbs energy, the energy produces ultrasonic vibration the device receives ultrasound and constructs images. The purpose was to detect melanin in the iris and retina in whole fisheye in the fish face, not a purified single substance melanin, and to display fisheye melanin separately from deoxyhemoglobin by photoacoustic imaging (PAI).

Materials and Methods: We scanned the whole fish eye using Acoustic X and displayed melanin. The Acoustic X photoacoustic imager (Cyberdyne Co., Tsukuba, Japan) can record PAI and grayscale US simultaneously. Using 820/940nm LED light sources, it pulses light of each wavelength alternately at 4 kHz and records induced ultrasonic waves with a linear grayscale ultrasonic probe with a center frequency of 7MHz. Melanin was able to be detected separately from deoxyhemoglobin by using a dual-wavelength analysis, using the following physiological properties the difference between the absorption coefficients of the two wavelengths of melanin is greater than that of deoxyhemoglobin. Melanin's ratio of photoacoustic signals induced at 820 and 940nm is less than that of deoxyhemoglobin. A jet colormap array was used in MATLAB software

(MathWorks Inc. Natick, MA, USA) to color-code the ratio from blue (small ratio) to red (large ratio). The ratio image should display melanin blue and deoxyhemoglobin red.

Results: The photoacoustic signals revealed the iris and ocular fundus by a combination of PAI and the grayscale US. In combination with the ratio image of the photoacoustic signal ratio and grayscale US image, melanin displayed blue in the iris and ocular fundus. It was impossible to display melanin in the retina and choroid separately because they are close to each other.

Conclusions: Photoacoustic imaging displayed melanin in the iris and ocular fundus (retina and choroid) in the fisheye. Combining PAI ratio image and grayscale US can accurate positional information on the melanin in the organs not only purified single substance melanin and display melanin separately from deoxyhemoglobin. Such information will be essential for the creation of eyes from induced pluripotent stem (iPS) cells. Non-destructive and minimally invasive identification of melanin by photoacoustic imaging can contribute to regenerative medicine.

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CHAPTER 1

I. Introduction

Photoacoustic imaging (PAI) is a new technology, that combines the high-contrast, spectroscopic-based specificity of optical imaging with high-resolution ultrasonography (US). The medical application of this new technology is a hot topic due to its advantages, molecular base discrimination, high spatial resolution, and, noninvasive.

The principle of PAI is that a substance absorbs light energy according to the substance-specific absorption coefficient determined by wavelengths. The absorbed energy products ultrasound vibration. At first, the device emits light to the substance, the substance absorbs energy, the energy produces ultrasonic vibration, and the device receives ultrasound and constructs images.

PAI has the feature of spectroscopy, it can analyze the molecule. Changing the light source wavelength can create spectroscopic-based molecular images of various substances. It depends on the optical properties and optical absorption of hemoglobin, lipids, water, melanin, and other molecules [1]. PAI can image a target substance using a frequency corresponding to the target substance. At first, the device emits light to the substance, the substance absorbs energy, the energy produces ultrasonic vibration, and the device receives ultrasound and constructs images.

PAI uses infrared for transmission and ultrasound for receipt. Since the wavelength of light is shorter than that of sound waves, the spatial resolution of PAI is better than the usual ultrasound.

PAI uses infrared and ultrasound. They are non-destructive and non-invasive. PAI involves no exposure to X-rays, high magnetic field, or harmful energies, does not require

facilities that shield radiation and magnetism, and does not require restricted areas [3][4][6]. Therefore, PAI can be used repeatedly.

In recent years, PAI light sources using light-emitting diode (LED)'s have been developed. Previous light sources used laser light, which required large facilities for laser generation, and safety glasses were required for both the operator and the subject. There was technology development to generate PAI even with an LED that has $1/100^{th}$ the energy of laser light. The latest technology, the LED light source PAI is space-saving, mobile, and does not require protective glasses.

PAI is not clinically approved and is for research use only in Japan. However, clinical research has started. It can display a patient's fine blood vessel image in 3 dimensions (3D) by the spectroscopy-based drawing of hemoglobin. The use of induced pluripotent stem (iPS) cells in regenerative medicine is expected. Regenerative medicine using iPS cells has been remarkable in recent years. However, one organ has not yet been completed, but in the future, organs constructed in 3D will be created. Specially, iPS cells can differentiate into any type of cell in the body and proliferate indefinitely in culture. Ophthalmology is most advanced in regenerative medicine using iPS cells. Therefore, first, a 3D eyeball may be created from iPS cells. In this case, identification of the retina, an important element of the eye, would be a necessary technique.

PAI with high spatial resolution in 3D will be useful for molecular detection in retina. However, it has not reached the completion of one organ. In the future PAI with high spatial resolution will be useful for molecular detection.

The purpose of this study is to establish a method to identify melanin using PAI. The reason for conducting this study is to confirm that melanin is correctly produced after retinas are created using iPS cells. Then detect melanin in the iris and retina in whole

fisheye, not a purified single substance melanin, and display fisheye melanin separately from deoxyhemoglobin by PAI.

II. Outline of the thesis

There are four chapters and an appendix in the thesis.

Chapter 1

Introduction for thesis. Outline of the thesis.

Chapter 2

Photoacoustic imaging, the principles of photoacoustic imaging.

Oxyhemoglobin, deoxyhemoglobin, and melanin.

Chapter 3

Anatomy of human eyes and fisheye, structure, and features.

Chapter 4

Materials and methods.

Research results, discussion, and conclusions.

Appendix: The table of abbreviations.

Acknowledgment

CHAPTER 2

I. Photoacoustic imaging

For the diagnosis and evaluation of various disorders, photoacoustic imaging (PAI), a recently developed and promising medical imaging tool, is accessible. In Japan, PAI device is only licensed for research use and not clinically licensed.

Photoacoustic imaging (PAI) combines the high-contrast, spectroscopic-based specificity of optical imaging with high-resolution ultrasonography (US) to detect ultrasonic waves generated by infrared-irradiated molecules.

The principle of PAI is that a substance absorbs light energy according to the substance-specific absorption coefficient determined by wavelengths. The absorbed energy products ultrasound vibration. At first, the device emits infrared light on the substance, the substance absorbs energy, the energy produces ultrasonic vibration, and the device receives ultrasound and constructs images [1-6]. It has the feature of spectroscopy, so it can analyze the molecule. Changing the light source wavelength can create spectroscopic-based molecular images of various substances. It depends on the optical properties and optical absorption of oxyhemoglobin, deoxyhemoglobin lipids, water, melanin, and other molecules [1]. PAI can display images of target substances present in the tissue due to the substance-specific absorption coefficient.

PAI uses infrared for transmission and ultrasound for receipt. Since the wavelength of light is shorter than that of sound waves, the spatial resolution of PAI is better than the usual ultrasound. For example, PAI for hemoglobin can visualize fine blood vessels. The collected US data can be reconstructed in 3-dimension (3D) images with high resolution.

Photoacoustic imaging is a non-invasive, X-ray exposure-free imaging technique. It is a non-invasive technique that involves no exposure to X-rays or harmful energies,

does not require facilities that shield radiation and magnetism, and does not require restricted areas [3] [4] [6]. And PAI is non-invasive and can be repeated.

Detection of a target substance by using the physical property that a substance has a specific absorption coefficient.

The oxygen saturation of hemoglobin affects its light absorption spectrum. For instance, concentrating on the difference in light absorption between two hemoglobin wavelengths (755 nm and 795 nm), depending on the level of oxygen saturation in the hemoglobin. At 755 nm wavelength light, the absorption coefficient of deoxyhemoglobin is higher than that of oxyhemoglobin, so deoxyhemoglobin absorbs more energy and generates more ultrasound vibrations [4].

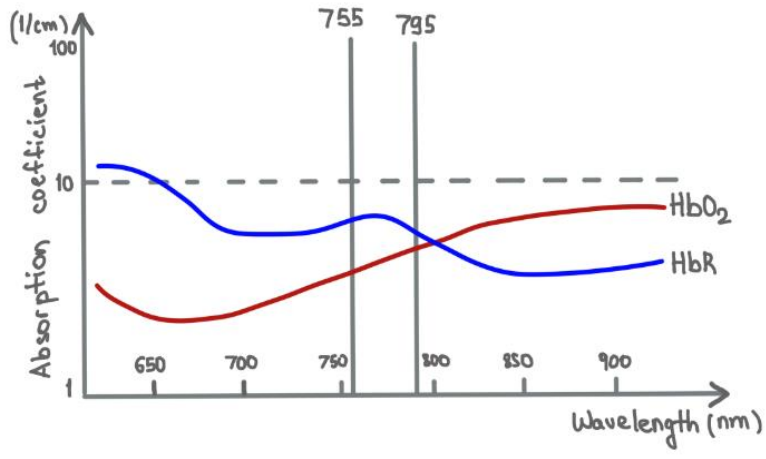

Figure 1: The difference in light absorbance between hemoglobin's 755 and 795 nm wavelengths depends on the level of oxygen saturation in the hemoglobin.

	Absorption coefficient cm^{-1}		Ratio small absorption coefficient/ large absorption coefficient
	820nm	940nm	
Deoxyhemoglobin	693.76	693.44	0.99
Melanin	470.08	292.25	0.62

Table 1: Absorption coefficient. There is a large difference between the melanin absorption coefficients of 820nm and 940nm.

II. The light source type of PAI

First, a PAI with a laser light source was developed. The light source used laser light, which required large facilities (about half the size of a school bus) for laser generation, and safety glasses was required for both the operator and the subject. The laser generators are expensive.

In recent years, PAI light sources using light-emitting diodes (LED)'s have been developed. There was new technology development to generate PAI even with an LED that has $1/100^{th}$ the energy of laser light. It is now possible to capture small vibrations and display images. The latest technology, the LED light source PAI is space-saving, mobile, and does not require protective glasses. And the wavelength of the light source can be easily changed with the LED light. LED light is cheaper than a laser generator.

III. Oxyhemoglobin, deoxyhemoglobin, and melanin

There are four possible arrangements for hemoglobin molecules:

1. Oxyhemoglobin (HbO2), symbolizes the hemoglobin molecules in their oxygen-carrying form.

2. A kind of hemoglobin called deoxyhemoglobin (deoxy-Hb), which contains no extra molecules

3. The carbon monoxide structure that is linked to the hemoglobin molecule is represented by carboxyhemoglobin (Hb CO).

4. Glycated hemoglobin (HbA1C) is the term for hemoglobin molecules that have glucose and fructose molecules bound to one another [7].

Oxyhemoglobin is a form of hemoglobin that is loosely linked with oxygen, whereas deoxyhemoglobin is a form of hemoglobin that has released its bound oxygen. This is the major distinction between oxyhemoglobin and deoxyhemoglobin. Deoxyhemoglobin is purplish in color, while oxyhemoglobin is bright red. The two types of hemoglobin produced inside the blood arteries are oxyhemoglobin and deoxyhemoglobin. Hemoglobin's primary job is to transport oxygen to the vertebrate body's metabolizing tissue [7] [8].

	Oxyhemoglobin	**Deoxyhemoglobin**
Combination	A vibrant red material is created when hemoglobin and oxygen combine	Oxygen not coupled with hemoglobin
Oxygen molecules	Carries four molecules of oxygen when it is saturated.	Carries no molecules of oxygen
State	The hemoglobin in its relaxed form	Tense hemoglobin condition
Absorption	Has a much lower absorption that happens at 660 nm.	Has a greater absorption that happens at 940 nm
Magnetic susceptibility	Weakly repelled by the magnetic field and diamagnetic	Weakly attracted by the magnetic field and non-magnetic
Occur	Occurs in blood with oxygen.	Occurs in the anemic blood.
Color	Bright red	Purplish blue
Transport	Removed from the heart via transportation	Brought close to the heart

Table 2: Comparison of oxyhemoglobin and deoxyhemoglobin

Two types of saturation:

1. Oxygen molecules can saturate hemoglobin (oxyhemoglobin)
2. Molecular oxygen desaturated (deoxyhemoglobin)

The similarity between oxyhemoglobin and deoxyhemoglobin: The two types of hemoglobin, oxyhemoglobin, and deoxyhemoglobin, are categorized according to their oxygen-bound state. Red blood cells and hemoglobin come in two different forms.

1. Oxyhemoglobin

When oxygen meets the heme portion of the hemoglobin molecule in red blood cells during respiration, oxyhemoglobin is created. This process takes place close to the lungs' alveoli in the pulmonary capillaries. After passing through the bloodstream, the oxygen is delivered to the cells where it is used as a terminal electron acceptor in the oxidative phosphorylation process to produce ATP. A drop in blood pH cannot be reversed; it is ineffective. By removing carbon dioxide through breathing or ventilation, a change in pH can be brought about, reversing the condition [8].

There are two types of hemoglobin: a tense form (T) and a relaxed form (R). Different elements in the tight form, which has a low oxygen affinity and releases oxygen in the tissues, are favored by low pH, high CO2, and high 2, and 3 BPG at the level of the tissues. In contrast, a high pH, low CO2, or low 2, 3 BPG promotes the relaxed form because it can connect oxygen more effectively. O2 affinity is also influenced by the system's partial pressure; at high oxygen partial pressures, the relaxed state is preferred. In contrast, the tense condition is preferred at low partial pressures. Additionally, the oxygen-iron heme bonding pulls the iron into the plane of the porphyrin ring, resulting in a tiny conformational shift. This shift makes it easier for oxygen to connect to the three other heme units in hemoglobin [8].

2. Deoxyhemoglobin

The form of hemoglobin without oxygen is called deoxygenated hemoglobin (deoxyhemoglobin). Oxyhemoglobin and deoxyhemoglobin have different absorption spectra; oxyhemoglobin absorbs at a wavelength of 660nm much less than deoxyhemoglobin does, whereas deoxyhemoglobin absorbs at a wavelength of 940 nm slightly more. The amount of oxygen in a patient's blood is measured using a tool called a pulse oximeter. This distinction also explains how cyanosis, or the blue-to-purple coloration of tissues under hypoxia, manifests itself.

Hemoglobin that has lost oxygen is paramagnetic. Magnetic fields only have a weak attraction for them. Oxygenated hemoglobin, on the other hand, demonstrates diamagnetism. It is a weak magnetic field's repulsion [8].

3. Melanin

Specialized cells called melanocytes, which are mostly found in the epidermal-dermal junction, produce melanin. Then extends to the keratinocytes, the majority of which are found in the epidermis. Tyrosine is an amino acid that is the source of the chemical polymer known as melanin. The pigment melanin, which ranges in color from black and brown to yellow and red, is found in the skin of both humans and animals in variable amounts [8][9].

Function: Melanin shields skin, eyes, and hair from the sun’s ultraviolet causing use oxidative stress [9].

Melanocytes, which are cells found in various parts, create melanin, which includes:

• Hair.

• Skin's deepest layer.

• Iris and pupil.

• The locus coeruleus and substantia nigra in the brain.

• The zona reticularis and medulla in the adrenal gland.

• The cochlear duct's stria vascularis in the inner ear [9].

Kind of melanin

• **Eumelanin.** Black and brown eumelanin are the two varieties. People with brown or black hair has variable quantities of the pigments brown and black eumelanin, which are responsible for the dark colors in their skin, eyes, and hair [9].

• **Pheomelanin.** lips, nipples, and other pinkish areas are pigmented by this type of melanin, and persons with red hair have an equal amount of eumelanin and pheomelanin in their bodies [9].

• **Neuromelanin.** The hues of skin, hair, and eyes are governed by eumelanin and pheomelanin, whereas the hue of neurons and the pigment in the brain are determined by neuromelanin [9].

• **Allomelanin.** Black to brown in color. It belongs to a group of nitrogen-free melanin that is frequently seen in fungi and plants [9] [10].

• **Pyomelanin.** A dark-colored pigment is found in fungi. It is a structure like that of eumelanin [11].

CHAPTER 3

I. Anatomy of human eyes

This is one of the camera eyes, the basic structure consists of one lens, one screen (retina), and one iris. One of the most significant organs in the human body, the eye is a slightly asymmetrical globe with a diameter of about an inch. The eye is shielded from mechanical harm by being enclosed in an orbit, which is a socket formed by fragments of several skull bones that form a four-sided pyramid with the top pointing back into the head. The orbit's top is composed of the orbital plate of the frontal bone and, behind it, the lesser wing of the sphenoid. The orbit's bottom is composed of fragments of the maxilla, zygomatic, and palatine bones. The great ophthalmic artery enters the orbit through the optic foramen, which is located at the nasal side of the eye. The superior orbital fissure is a larger entrance through which huge veins and nerves travel. These nerves may transmit pain and nonvisual sensory information, or they may function as motor nerves that regulate the eye muscles. Nerves and blood vessels are transmitted through cracks and canals. A layer of orbital fat that works as a ring around the eyeball and its active muscles. It enables a smooth rotation of the eyeball about the rotational center, which is practically stationary [12] [13].

The lens, which aids in focusing light on the back of the eye, is located immediately behind the iris and pupil (lens diameter is 10 mm with a maximum thickness of 3–4 mm). The majority of the eye's vitreous is made up of a clear gel. Special light-sensing cells serve to filter the lining of the eye's interior. This is referred to as the retina. Light is changed into electrical impulses by it. These impulses are sent from the retina to the brain via the optic nerve. Humans have central vision through the macula, a tiny, exceptionally sensitive region

of the retina [12] [13]. The quantity and kind of pigment in the iris determines the color of the eyes which is genetically determined. [12].

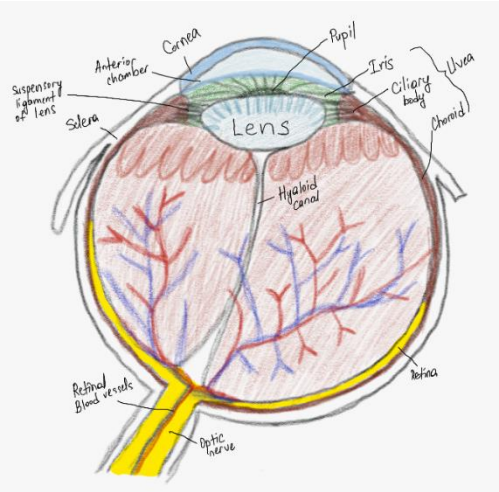

Figure 2: Human eye layers and vessels detail view.

Three levels can be identified by the human eye:

1. The outer region: (Cornea and Sclera)

1.1. Cornea

The transparent window of the eye, a transparent dome over the iris, refracts and transmits light to the lens and the retina while guarding the eye against structural damage and infection in the deeper portions. It has five distinct layers: Bowman's membrane, the stroma, the supporting structure, Descemet's membrane, and the endothelium, or inner lining. The epithelium, or outer covering, is one of these layers [12] [14].

The epithelium is composed of roughly six layers of cells and is a continuation of the conjunctiva's epithelium. The outermost layer, or epidermis, is continuously shed, while the interior layers, or basal layers, are replenished through cell division. The corneal corpuscles, cells that synthesize new collagen necessary for mending and maintaining this layer, are located between the lamellae, which are plates that make up the stroma and run

parallel to the superficial layer and are overlaid on one another like book leaves. The sheets of microscopically visible fibers that make up the lamellae are arranged in successive layers at a sharp angle to one another. Additionally, Bowman's membrane, which is between 8 and 15 microns thick, is located directly above the stroma and next to the epithelium [12] [14].

Descemet's membrane and the endothelium are located below the stromata. The former is produced by the endothelium cells, a single layer of flat cells, and is between 5 and 10 microns thick. It is formed of a different type of collagen than that found in the stroma [12] [14].

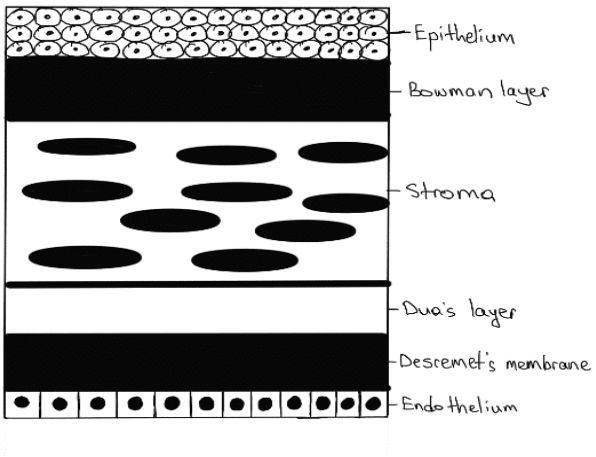

Figure 3: Cornea layer detail.

1.2. Sclera

The connective tissue covering keeps the shaped eye and shields it from internal and external stimuli [12]. The limbus is connected to the cornea and the sclera. As a result, the conjunctiva, a transparent mucous membrane, covers the visible portion of the sclera. The iris, ciliary body, and choroid make comprise the central layer of the eye, however, the iris

regulates pupil size. As a result, the amount of light that reaches the retina is controlled by the ciliary body, which also produces aqueous and regulates the lens's power and shape. The vascular layer known as the choroid provides the outer layers of the retina with oxygen and nutrients [12] [15].

2. The inner region: (Retina)

2.1 Retina

A thin, somewhat transparent, multilayered film of neural tissue makes up the retina. A sophisticated, layered arrangement of neurons designed to capture and process light. The ocular layers enclose the three transparent structures. These are referred to as the aqueous, vitreous, and lens. The component of the eye's retina that receives light and transforms it into chemical energy is responsible for sending messages from the eye into the higher regions of the brain. Since the retina is essentially an extension of the forebrain, it is a sophisticated neurological system [12] [16].

There are ten layers of cells in the retina that can be seen under a microscope. The pigmented layer, the rods, and cones layer, the membrana limitans externa, the outer nuclear layer, the outer plexiform layer, the ganglionic layer, the stratum opticum, and the membrana limitans interna. There are four main layers. (1) The pigment epithelium, which is located next to the choroid, (2) has a layer of rods and cones, which are light-sensitive cells, on its upper surface. (3) A layer of neurons known as the bipolar cells receive the modifications that light causes in the rods and cones. These bipolar cells communicate with (4) the ganglion cells, which make up the innermost layer of neurons. The sent signals travel along the projections, or axons, which make up the fibers of the optic nerve as they leave the eye. The layer of bipolar cells and the cells of the lateral

geniculate body are two parts of the nervous system that are connected by the optic nerve, which is a central tract rather than a nerve [12] [16].

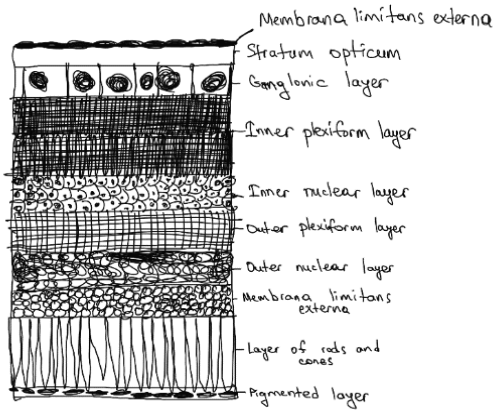

Figure 4: Retina 10 layers in microscopy

3. The middle region: (Pupil, Uveal tract, and Lens)

3.1 Pupil

The pupil, an aperture in the iris, controls how much light enters the eye through the black, circular opening in the iris. The size of a person's pupils is huge in a dark environment—possibly 8 mm or larger. The light response, which occurs as soon as the room is lit and is bilateral, causes the pupils to immediately contract, even if only one eye is exposed to the light. However, the amount of contraction in both pupils is roughly the same. Even though the strong light is kept on for a while, the pupils eventually dilate, however, the dilation is not significant. The real level of illumination determines the final condition. If this is high, the ultimate state may only have a diameter of 3 to 4 mm. If this is not as high, the end state may have a diameter of 4 to 5 mm. If this is inflated, it is known as a hippus [12].

As a result, accommodation and pupillary constriction happen simultaneously with reflex and are stimulated by the same input when a person stares at a close object. The pupil's job is to regulate how much light gets into the eye, which affects the light reaction. Constriction during close-up vision suggests additional functions and in the dark, aberrations are of little importance, thus one merely must get as much light into the eye as possible. In bright light, however, one typically needs good visual acuity, which entails minimizing aberrations [12].

Strong psychical stimuli and when any sensory nerve is triggered by dilation both cause pupil dilation. When in great pain and anxiety, this happens. The main characteristics of the iris muscles show that dilation is caused by the shortening of the radially oriented fibers, whereas constriction is caused by the shortening of the circular ring of fibers in the sphincter [12] [15].

3.2 The uveal tract

The iris, the ciliary body, and the choroid make up the uveal tract.

3.2.1 Iris

In terms of anatomy, the colored portion is the pigmented muscular curtain that is punctured by the pupil and is located close to the front of the eye, between the cornea and the lens. The iris is situated behind the cornea, in front of the lens, and ciliary body. A fluid known as humor is bathing it from the front and the back. The iris is made up of two smooth muscle sheets that work in opposition to one another. It is a contraction and enlargement. These muscles regulate the pupil's size, which affects how much light reaches the retina's sensory cells. In shiny light, the pupil is constricted by the iris's sphincter muscle. However, when the

iris' dilator muscle contracts, the aperture widens. The color of the eyes is determined by how much iris pigment is present. The eye turns blue if there is very little pigment present.

As the pigment content rises, the color changes from deep brown to black [12] [15].

3.2.2 Ciliary body

The choroid's progression forward. It has a triangular shape with a muscular ring. The area known as the ora Serrata is where the horizontal part starts. Finish up front, representing the iris's root. The flimsy is pushed into folds. It is known as the ciliary process, and the ciliary epithelium, a double layer of cells, protects the entire structure.

The outer layer, which is continuous with the pigment epithelium of the retina, is heavily pigmented, and the inner layer, which is near the vitreous body, is clear. In terms of embryology, these two layers are to be viewed as the retina's forward continuation, which ends at the ora Serrata. Secrete aqueous humor is the purpose [12] [15].

3.2.3 Choroid

Between the retina and the sclera in the back of the uveal tract is the choroid. The choroidal blood arteries are divided into three layers: large, medium, and tiny. The interior part of the choroid vessels, known as the choriocapillaris, is wider the deeper the vessels are positioned in the choroid. The sclera and Bruch's membrane serve as the choroid's exterior and internal boundaries, respectively. The choroid and sclera are where the suprachoroidal space is located. The ciliary body and choroid are joined anteriorly by the choroid, which is securely linked posteriorly to the borders of the optic nerve [12] [16].

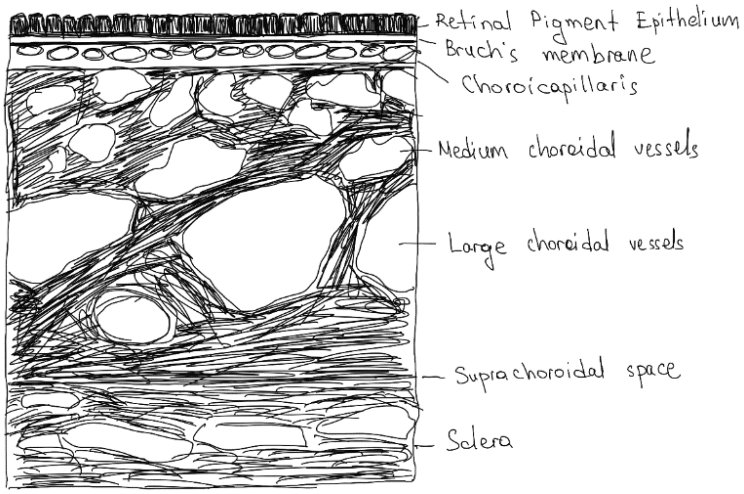

Figure 5: Cross section of Choroid.

3.3 Lens

The lens is a biconvex, avascular, colorless, almost entirely transparent structure that is flatter on its anterior surface than on its posterior surface. It is suspended within the eye by zonular fibers of Zinn that are attached to its equator; its anterior surface is bathed in aqueous humor, and its posterior surface is covered by the vitreous body. The lens is made up of a mass of densely clustered transparent fibrous cells, or lens fibers, which are encased in an elastic collagenous capsule (about 4 mm thick and 9 mm in diameter). The lens's fibers are arranged in sheets that create consecutive layers and run from pole to pole, with each fiber's midpoint located around the equator. An equatorial section would cut all the fibers crosswise, giving the appearance of a honeycomb, as opposed to a meridional (horizontal) section, which would cut the fibers longitudinally to create the appearance of onion scales. The lens fibers originate in the epithelium, which covers the anterior surface of the lens behind the capsule. The lens continues to expand through the laying down of new fibers throughout life, both during embryonic and fetal development and infant and adult life [12] [16] [17].

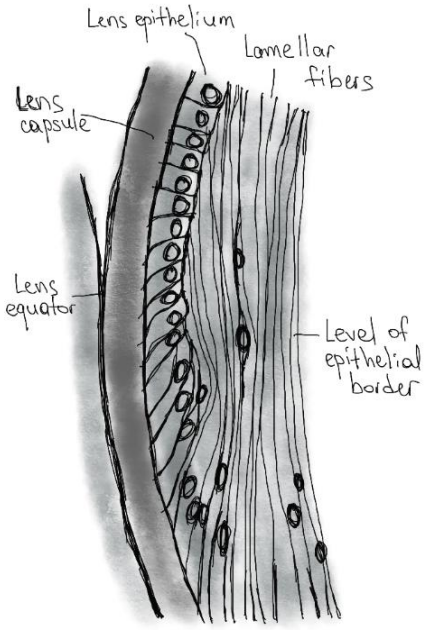

Figure 6: Lens detail view. An enlarged image of the lens in the vertical part reveals the termination of the subcapsular epithelium.

4. The conjunctiva

The conjunctiva and cornea are the only parts of the front that are not covered by this thin layer of tissue, which extends from the lining of eyelids back over the surface of the eyeball, acting as an outer covering for the anterior portion of this, and ending at the cornea. The palpebral section of the conjunctiva is the area that lines the eyelids, while the bulbar conjunctiva is the area that covers the white of the eye. Two additional, slack pieces that form breaks between the bulbar and palpebral conjunctiva extend back toward the equator of the globe. The conjunctiva at these breaks, also known as the upper and lower fornix or conjunctival sacs, is free, allowing for the movement of the eyelids and eyeballs [12].

5. The eyelid

The front surface of the eyeball must stay moist. The cornea is the part of the eyeball that faces outward. Maintaining moisture is essential. Achieved by the eyelids, which periodically brush the surface of the eye during awake hours with secretions from the glands and lacrimal apparatus. Then, to stop evaporation as you sleep, cover our eyes. Through the action of the blink reflex, the lids serve the purpose of protecting damage from foreign bodies. When the eye is open, the lids—which are essentially tissue folds covering the front of the orbit—leave an almond-shaped aperture. Canthi is the name for the almond's points. The inner canthus is the one closest to the nose, and the other is the outer canthus. The lid is made up of four layers: (1) the skin, which contains glands that open onto the lid rim and the eyelashes; (2) a muscular layer, which is primarily made up of the orbicularis oculi muscle, which is responsible for closing the lids; (3) a fibrous layer, which gives the lid its mechanical stability and is primarily made up of the tarsal plates, which border on the palpebral aperture, the opening between the lids; and (4) the innermost nonetheless, allows for a sizable amount of orbital eyeball rotation [12].

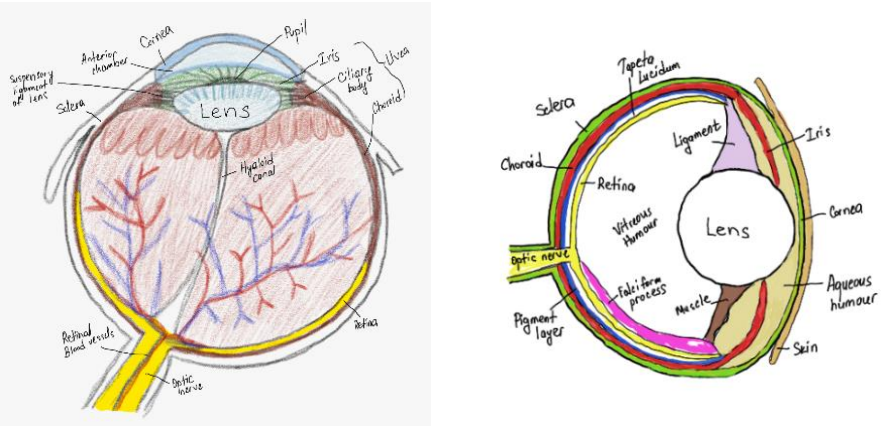

Figure 7: Human eye and fisheye comparison. The human lens has biconvex, but the fish lens has spherical.

II. Anatomy of fisheyes

Both fisheyes and human eyes are the same kinds, the camera eye. The basic structure of the camera eye is one lens, one iris, and one screen (retina). Fish lenses are spherical, while human lenses are convex. This is suitable for underwater vision. Fish retinas have rod cells and cone cells, the same as human retinas [18].

All fish have a sensory system that is crucial: vision. Although they have a larger spherical lens, fisheyes resemble those of terrestrial animals like birds and mammals. Fish often shift the focus by moving the lens closer to or away from the retina, in contrast to birds, mammals, and humans, who typically do it by changing the shape of their lenses.

Most fish species have color-producing rod and cone cells in their retinas. While some fish can see in the ultraviolet, others may respond to polarized light. Fisheyes are generally like those of other vertebrates, especially mammals, birds, amphibians, and reptiles. The cornea serves as the eye's entrance point for light, which travels through the pupil and lens. Unlike elasmobranchs (like sharks and rays), which have a muscular iris that enables pupil diameter adjustment, most fish species appear to have a fixed pupil size. Pupil shapes vary and might be slit-like or round [12].

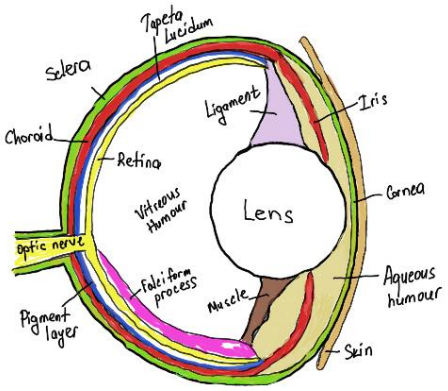

Figure 8: Fisheye anatomy. Fisheye is a spherical lens. By shifting the lens's distance from or distance from the retina, fish can change the focus.

1. Cornea Structure

Although fisheyes share many of the same components as human eyes, they have different structures and applications. Fish have an optic nerve for transmitting images to the brain, a retina with light-sensitive cells, a lens for taking pictures, an iris for focusing light, and a cornea, or outer covering. Since the cornea is nearly round, the fish's eyes can virtually fill with images of its surroundings. Like a 360-degree circle. Fish corneas virtually have the same density as water to make up for the fact that water has a strong capacity to refract, or bend images. This causes tiny curving as images are transported from water into fisheyes [12].

2. Retina structure

Rod cells and cone cells, which oversee the scotopic and photopic vision, are almost equally present in a fish's retina. Fish of most species can see color. While certain species are sensitive to polarized light, others can perceive UV.

Rod cells, which provide excellent visual sensitivity in low light settings, and cone cells make up the fish retina. Additionally, it offers greater temporal and spatial resolution than rod cells do. They make it possible for color vision since different types of cones have similar absorption properties [19] [20].

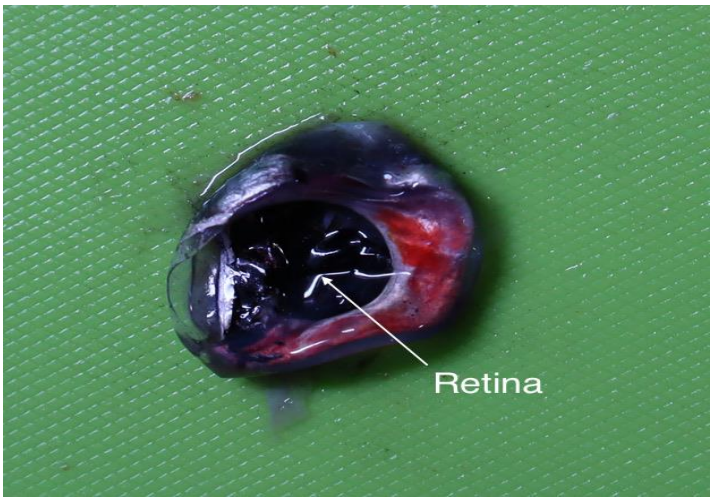

Figure 9: Fish retina. Front view of the fish eyeball with the lens removed. The black thing in the center is the retina. Like humans, fish have both rod cells and cone cells in their retina.

3. Lens Structure

Fisheye lenses have the propensity to scatter a sizable amount of light. They have spherical lenses, which are better for use in water. Fish live in light settings because water absorbs light, therefore when looking at an image straight on, they get a clear picture in the middle, but their vision becomes increasingly blurry and distorted at the edges. Fish must point directly at something to concentrate. It means that even if their field of view is nearly 360 degrees, it is transparently located in the center of the scene [12] [21].

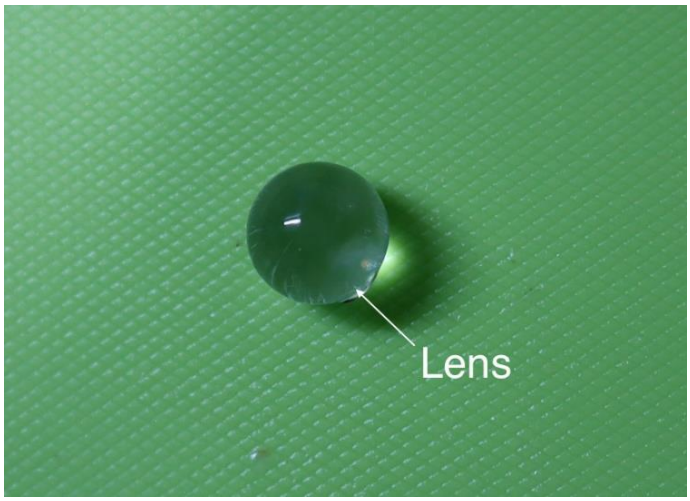

Figure 10: Fish spherical lens. The lens was removed from the fish eyeball.

4. Iris Structure

Land animals have an iris that directly controls the pupil's access to light. Except for a few shark species, fish do not possess this. But instead regulates light levels by adjusting their iris. They sometimes struggle to acclimatize to varying light levels in about 15 or 20 minutes because this takes longer. Fish have trouble blocking off intense light because they lack an outer eyelid. They must therefore seek cover or plunge far into the ocean. This frequently causes fish in aquariums to hide for several minutes after you switch on their light [12].

5. The eyelid

Fish have no outer eyelids at all. This translates to many fish having no eyelids at all. However, some sharks have nictitating membranes. This can be drawn across the eye as protection because it is a thin, translucent film. Other eyelid replacements have also evolved in fish. Some types of bonefish can draw a covering of fat over their eyes. Some fish have light-activated pigments in their eyes that make them darker to block out bright

lights, even though this is not an eyelid and only partially shades off the light and only enables it to pass via a tiny hole above the pupil [12] [22].

CHAPTER 4

I. Materials and methods

The equipment used was Acoustic X™ (CYBERDYNE Co., Tsukuba Japan), a photoacoustic imaging research machine (Figure 11). It can scan combined PAI and grayscale ultrasonography at the same time. A linear array ultrasonic scanner is in the center, with a light-emitting diode (LED) light source of different frequencies on either side. Using an 820/940nm combination LED light source, two different light wavelengths were pulsed alternately at 4 kHz and a linear grayscale ultrasonic probe with a center frequency of 7MHz (Figure 13). A sea-bream eyeball purchased from a food store was used (Figure 12). Sea bream eye was scanned 2 wavelength photoacoustic and grayscale ultrasonography signals were acquired simultaneously (Figure 14).

Melanin was visualized using a dual-wavelength analysis function that can calculate the ratio of each photoacoustic signal at 820nm and 940nm. The ratio was the small value of photoacoustic signal amplitude divided by the large value. Since more than 24 hours have passed since the death, hemoglobin in blood vessels was considered deoxyhemoglobin. Since the melanin ratio is small than the oxyhemoglobin ratio, we thought that it could be displayed separately. A jet colormap array was used in MATLAB software (MathWorks Inc. Natick, MA, USA) to color-code the ratio. The small ratio was set to be displayed in blue, and the large ratio area was set to be displayed in red [23].

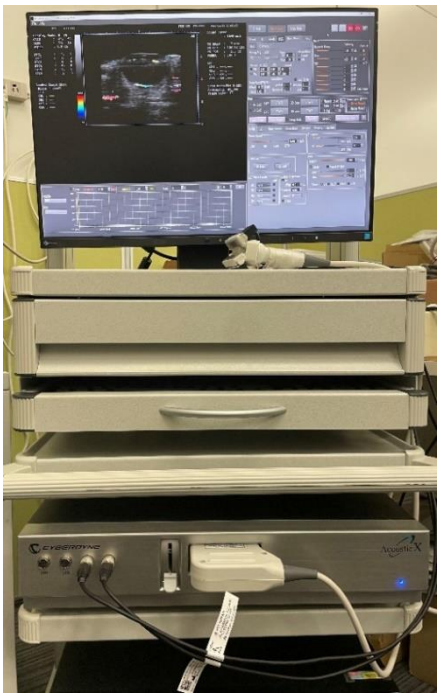

Figure 11: Acoustic X™

Acoustic X™

Combination: Grayscale US + PAI (photoacoustic imaging)

Grayscale mode: 7MHz linear scanner

LED light source: High Power High-Density LED array x 2

LED drive pulse frequency: 4 kHz

LED wavelength:

(Single) 470, 520, 620, 660, 690, 750, 820, 850, 940, 980nm

(Double) 850/690, 850/750, 820/940nm

We used: 820nm / 940nm

Figure 12: Sea bream in 0.9% saline bath.

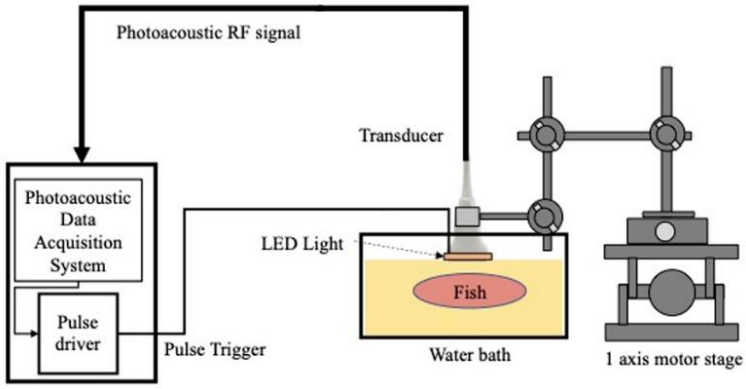

Figure 13: Data collection. Using an 820/940nm combination LED light source, two different light wavelengths were pulsed alternately at 4 kHz, and a linear probe with a center frequency of 7MHz was used to scan the eyeball of a sea bream.

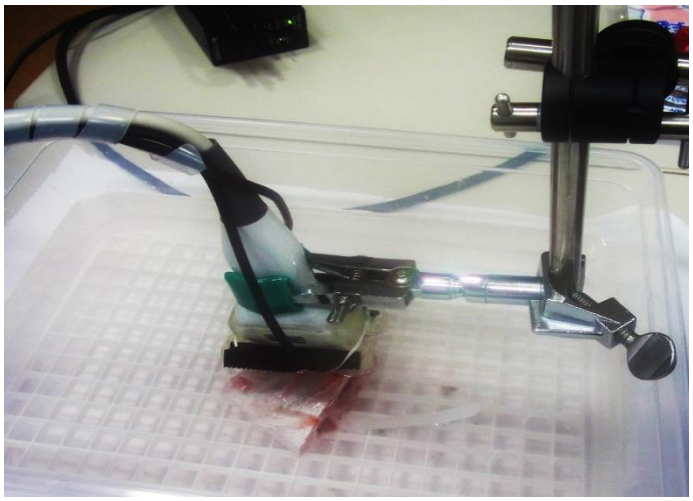

Figure 14: The photoacoustic imaging research machine, can scan the combined photoacoustic image and grayscale ultrasonography at the same time.

II. Results

In grayscale ultrasonography, they can be seen of the iris, lens, and retina anatomy of a fisheye (Figure 15). In PA mode with grayscale ultrasonography, the photoacoustic signal was presented on the iris and retina. A combination of photoacoustic and grayscale ultrasonography at 820nm was stronger than at 940nm on the iris and retina (940nm was weaker than 820nm). But only the photoacoustic signal at 820nm, was brighter than at 940nm (Figure 16, 17).

In combination with ratio image and grayscale ultrasonography, a blue area was presented on the iris and ocular fundus (retina and choroid). A small value of the ratio, the value obtained by dividing small PA intensity by large PA intensity is displayed in blue. The ratio of melanin is small. Melanin was shown blue ratio image (Figure 18). There is a large difference between the melanin absorption coefficients of 820nm and 940nm, but that of deoxyhemoglobin is small (Table 1).

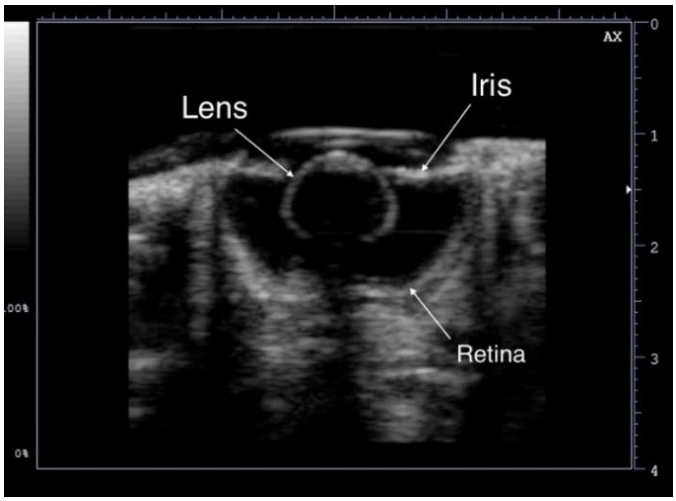

Figure 15: Grayscale ultrasonography in the fish eyeball. In grayscale ultrasonography, they can be seen of the iris, lens, and retina anatomy of a fisheye.

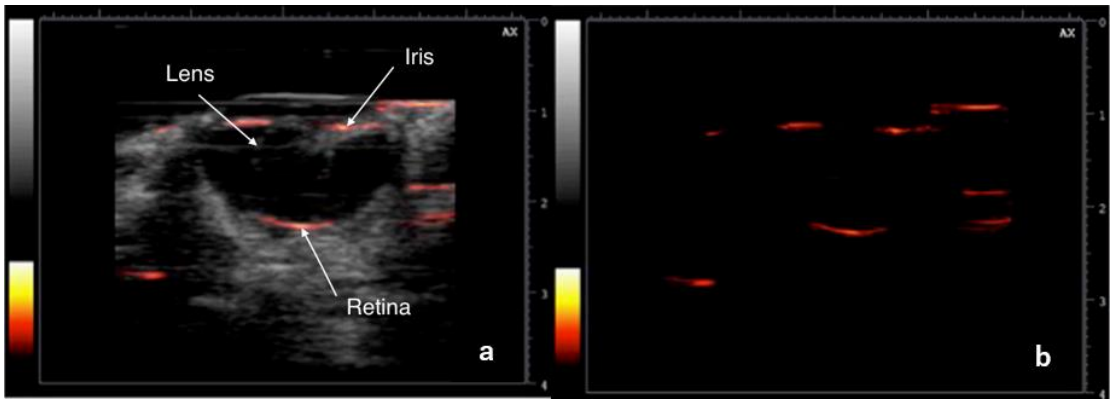

Figure 16: (a) Combination of photoacoustic and grayscale ultrasonography at 820nm. It was on the iris and retina. It was stronger than at 940nm.

(b) Only photoacoustic signal at 820nm. It was brighter than at 940nm.

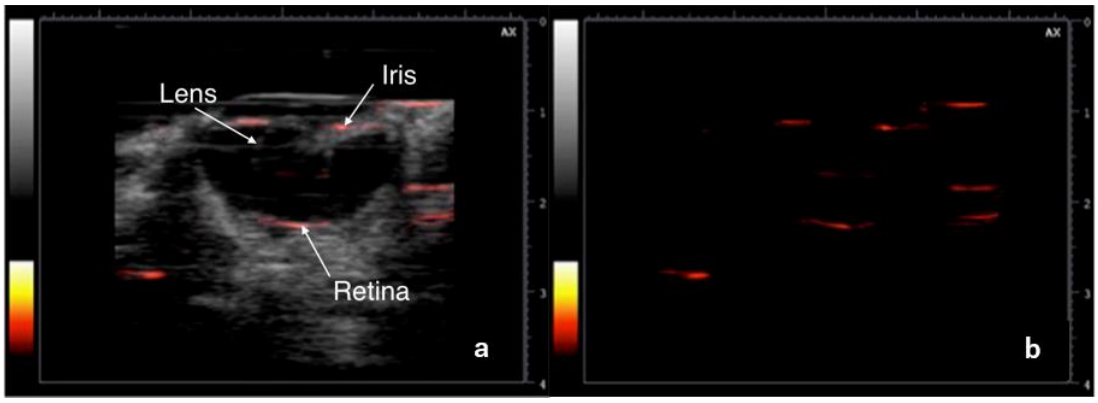

Figure 17: (a) Combination of photoacoustic and grayscale ultrasonography at 940nm. The photoacoustic signal was on the iris and retina. It was weaker than at 820nm. (b) Only photoacoustic signal at 940nm. It was darker than at 820nm.

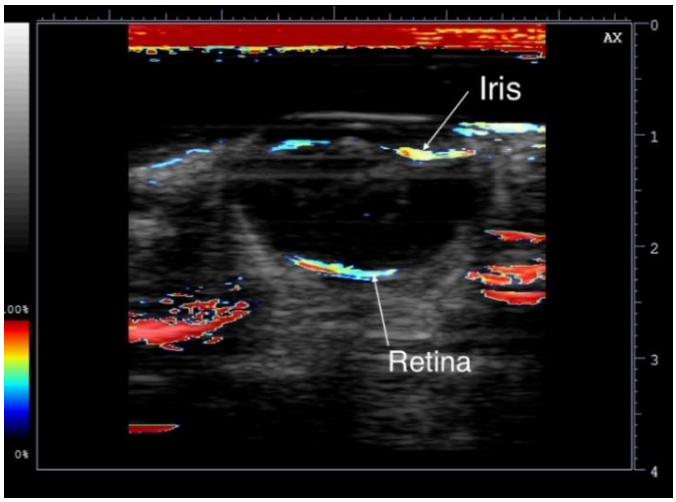

Figure 18: Combination ratio image and grayscale ultrasonography. Low ratio values were displayed in blue on the iris and retina.

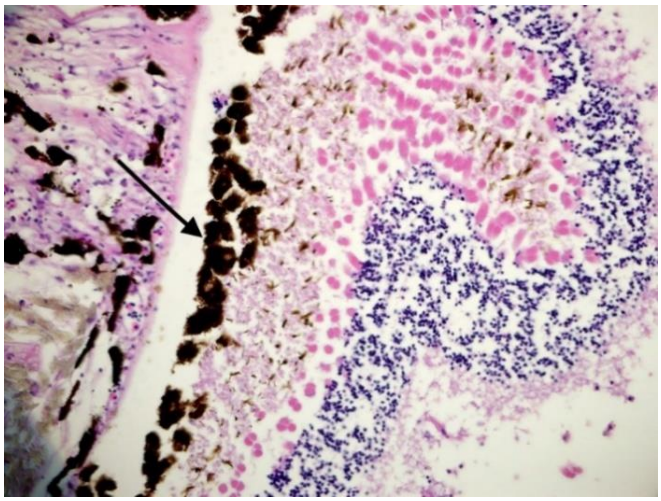

Figure 19: Histology of fisheye retina and choroid. The pigmented layer (arrow) is the outermost of the 10 layers of the retina.

The scattered brown spots on the left side of the figure are melanin in the choroid.

III. Discussion

We displayed melanin in the iris and retina in the whole fisheye, not purified single substance melanin using PAI. Our result displayed a blue area on the iris and retina in combination with ratio image and grayscale ultrasonography. The blue display shows the small ratio and displays melanin. And we display fisheye melanin separately from deoxyhemoglobin. Combining PAI ratio image and grayscale US can accurate positional information on the melanin in the organs.

We used Acoustic X^{TM}, combining PAI and grayscale ultrasound. Its light source is LED light. It is space-saving, mobile, and does not require protective glasses. And the wavelength of the light source can be easily changed with the LED light. We used 820 and 940 nm wavelength light sources and distinguished the target substance, melanin, from deoxyhemoglobin more clearly by the ratio of the absorption energies (US vibration signal intensity) of the two wavelengths. Ratio image utilizes the difference in magnitude

of the photoacoustic signal of two wavelengths and according to the size of the ratio value [5]. All hemoglobin in the blood of fish more than 24 hours after death was deoxyhemoglobin. There is a large difference between the absorption coefficients of the two wavelengths, 820 and 940 nm of melanin. On the other hand, that of deoxyhemoglobin is small. The ratio of melanin is smaller than the ratio of deoxyhemoglobin. In this study, melanin and deoxyhemoglobin could be displayed color code separately in ratio image. We had confirmed in previous experiments that these two wavelengths were suitable. Ratio images were color-coded using Jet Colormap Array and attached above the grayscale US image. Since grayscale US was transmitting and receiving at the same time as PAI, it was possible to obtain accurate positional information on melanin-containing tissues. We were able to display melanin in the iris and retina in the fisheye in the fish face. The purpose of this study was to detect melanin in the iris and retina. However, it was impossible to display melanin in the retina and choroid separately because the pigment epithelium of the retina and choroid are so close to each other.

PAI, which can non-invasively detect target molecules, is expected to use in regenerative medicine. Currently, research on regenerative medicine using iPS cells is progressing rapidly. However, it is not currently possible to create organs perfectly. The transplanted organ has not been completed from iPS cells. Shortly, humans will be able to make organs from iPS cells. For the creation of the eyeball, the formation of the melanin-lined retina and iris is essential. In addition, melanin production can be observed in skin regeneration. Such knowledge will be crucial for the production of eyes from induced pluripotent stem (iPS) cells. Photoacoustic imaging's non-destructive and minimally intrusive detection of melanin can help with regenerative medicine.

IV. Limitation

The human eye and fisheye comparison are that the human lens has biconvex, but the fish lens has spherical. This study aimed to detect melanin in the iris and retina. However, it was impossible to display melanin in the retina and choroid separately because the retina and choroid pigment epithelium are so close.

V. Conclusion

PAI displayed melanin in the iris and ocular fundus (retina and choroid) in the whole fisheye in the fish face, not a purified single substance melanin, and separately from deoxyhemoglobin. Combining PAI and grayscale ultrasonography can obtain accurate positional information on the melanin in the organs.

Non-destructive and minimally invasive identification of melanin by photoacoustic imaging can contribute to regenerative medicine.

Appendix

LED Light-emitting diode

PA Photoacoustic

PAI Photoacoustic imaging

US Ultrasound

MHz Megahertz

kHz Kilohertz

CT Computer tomography

BPG Bi Phosphor Glyceric acid

ATP Adenosine triphosphate

UV Ultraviolet

Hb Hemoglobin

HbO2 Oxyhemoglobin

Hb CO Carboxyhemoglobin

HbA1C Glycated hemoglobin

CO_2 Carbon dioxide

O_2 Dioxygen

iPS Induced pluripotent stem cells

3D Three dimensional

nm Nanometer

References

1. Kubelick KP, Snider EJ, Ethier DR, et al.: Photoacoustic properties of anterior ocular tissues, J. Biomed, 24: 056004, 1-11, 2019.

2. Shu X, Liu W, Zhang HF: Monte Carlo investigation on quantifying the retinal pigment epithelium melanin concentration by photoacoustic ophthalmoscopy, J. Biomed, 20: 106005, 1-8, 2015.

3. Beard P: Biomedical photoacoustic imaging, Interface Focus, 1: 602-631, 2011.

4. Takayuki Yagi: Photoacoustic imaging. ImPACT Innovative Visualization Technology to Lead to the Creation of a New Growth Industry, 2-4, Takayuki Yagi, Tokyo, 2018.

5. Ohta T, Shirakawa T, Okada S, *et al.*: In vitro, demonstration of melanoma metastasis in lymph nodes of prepared specimens using a light-emitting diode-based multispectral photoacoustic ultrasound imaging system, J Med Ultrasound, 29: 50-52, 2020.

6. Wonseok Choi, Eun-Yeong Park, Seungwan Jeon, et al.: Clinical photoacoustic imaging platforms, Biomedical Engineering Letters, 8:139–155, 2018.

7. Sumith Yesudasan, Xianqiao Wang, Rodney D. Averett.: Molecular Dynamics Simulations Indicate that Deoxyhemoglobin, Oxyhemoglobin, Carboxyhemoglobin, and Glycated Hemoglobin under Compression and Shear Exhibit an Anisotropic Mechanical Behavior, J Biomol Struct Dyn, 36(6): 1417–1429, 2018.

8. Mitchell R.H. Weigand, Jenifer Go´mez-Pastora, James Kim *et al.*: Magnetophoretic and spectral characterization of oxyhemoglobin and deoxyhemoglobin: Chemical versus enzymatic processes, PloS one, 16(9): 1-14, 2021.

9. Radames J.B. Cordero, Arturo Casadevall.: Melanin, Current Biology Magazine Quick guide, 30(4): 142-143, 2020.

10. Naneki C. McCallum, Florencia A. Son.: Allomelanin A Biopolymer of Intrinsic Microporosity, J. Am. Chem. Soc, 143: 4005−4016, 2021.

11. L.K.Povlich, K.E.Feldman, B.S.Shim, et al.: Electroactive Polymeric Biomaterials, Comprehensive Biomaterials, 1: 547-561, 2011.

12. Edward S. Perkins.: https://www.britannica.com/science/human-eye, 2022.12.08

13. Sorin M. Dudea.: Ultrasonography of the eye and orbit, Medical Ultrasonography, 13(2): 171-174, 2011.

14. Mittanamalli S Sridhar.: Anatomy of the cornea and ocular surface, Indian Journal of Ophthalmology, 66(2): 190-194, 2018.

15. Colin E Willoughby MD, Diego Ponzin MD.: Anatomy, and physiology of the human eye: effects of mucopolysaccharidoses disease on structure and function – a review, Clinical and Experimental Ophthalmology, 38: 2–11, 2010.

16. Paul Riordan Eva.: Anatomy & Embryology of the Eye, General Ophthalmology Chapter 1, Paul Riordan Eva, USA, 2011.

17. Eric C. Swindell, Chaomei Liu, Rina Shah, et al.: Eye formation in the absence of retina, Dev Biol, 322(1): 56–64, 2008.

18. Helfman GS, Collette BB, Douglas EF, Bowen BW.: The diversity of fishes Biology, Evolution, and Ecology, Wiley-Blackwell 2nd Edition, 84-87, Helfman GS, UK, 2009.

19. Iñigo Novales Flamarique.: Swimming behaviour tunes fish polarization vision to double prey sighting distance, Scientific Report, 9(944): 1-8, 2019.

20. Norianne T. Ingram, Alapakkam P. Sampath, and Gordon L. Fain.: Why are rods more sensitive than cones, J Physiol, 594(190): 5415–5426, 2016.

21. Yutong Zhang, Jianmei Song, Yan Ding, et al.: FSD-BRIEF: A Distorted BRIEF Descriptor for Fisheye Image Based on Spherical Perspective Model, Sensors, 21(1839): 1-26, 2021.

22. D. Rowat, K. S. Brooks.: A review of the biology, fisheries, and conservation of the whale shark Rhincodon typus, Journal of Fish Biology, 80: 1019–1056, 2012.

23. Xiao Shu, Wenzhong Liu, and Hao F. Zhang.: Monte Carlo investigation on quantifying the retinal pigment epithelium melanin concentration by photoacoustic ophthalmoscopy, Journal of Biomedical Optics, 20(10): 1-1, October 2015.

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