**TITLE:**

Using Retinal Imaging to Study Dementia

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**KEYWORDS:**

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**SHORT ABSTRACT:**

The retina shares prominent homologies with the brain and thus represents a unique window to study vasculature and neuronal structure in the brain non-invasively. This protocol describes a method to study dementia using retinal imaging techniques. This method can potentially aid in the diagnosis and risk assessment of dementia.

**LONG ABSTRACT:**

The retina potentially offers a unique “window” to study pathophysiological processes of dementia in the brain, as it is an anatomical and physiological extension of the central nervous system and shares prominent similarities with the brain in terms of vascular and neuronal structure. The vascular and neuronal structure in the retina can now be visualized non-invasively using widely available retinal imaging techniques, including fundus photography and optical coherence tomography (OCT), and quantified using computer-assisted analysis programs. Studying the association between vascular and neuronal changes in the retina and dementia could improve our understanding of dementia and, potentially, aid in diagnosis and risk assessment. This protocol aims to describe a method of quantifying and analyzing retinal vasculature and neuronal structure using retinal imaging techniques. This protocol also provides examples of retinal changes in subjects with dementia and discusses the technical issues of retinal imaging.

**INTRODUCTION:**

Due to increases in life expectancy, dementia has become a major medical problem, contributing to significant social and economic health burden globally1–5. Today, a person in the United States develops Alzheimer’s Disease (AD), the most common form of dementia, every 66 s6. It has been estimated that by the year 2050, 115 million people will be affected by AD7.

The retina offers a unique “window” to study dementia due to its similar anatomical and physiological properties with the brain. In terms of vasculature, the retinal arterioles and venules, measuring 100 to 300 μm in diameter, share similar features with cerebral small vessels, such as end arterioles without anastomoses, barrier function, and auto-regulation8,9. In terms of the neuronal structure, retinal ganglionic cells (RGCs) also share typical properties with neurons in the central nervous system (CNS)10. The RGCs are prominently connected with the brain as they form the optic nerve and project visual signals from the retina to the lateral geniculate nuclei and the superior colliculus. The optic nerve, similar to many neuronal fibers in the CNS, is myelinated by oligodendrocytes and is ensheathed in meningeal layers. Notably, an insult to the optic nerve can result in similar responses observed in other CNS axons, such as retrograde and anterograde degeneration of the axon, scar formation, myelin destruction, secondary degeneration, and an abnormal level of neurotrophic factors and neurotransmitters11–14. The association of visual symptoms with AD may also be manifested by the robust connection between the retina and the brain15,16. Given these associations between the retina and the brain, it has been suggested that the retina may reflect the subclinical pathophysiological process of dementia in the brain and thus can be used to study dementia.

The retinal vasculature and retinal neuronal structure can now be visualized and quantified non-invasively using retinal imaging techniques. For instance, retinal fundus photographs can be captured using retinal fundus cameras, and characteristics of the retinal vasculature (*e.g.,* vessel caliber, tortuosity, and fractal dimensions) can then be quantified using computer-assisted analysis programs. In addition, parameters of retinal neuronal structure (such as the thickness of ganglion cell-inner plexiform layer [GC-IPL] and retinal nerve fiber layer [RNFL]) can also be measured using optic coherence tomography (OCT) and quantified using the built-in analysis program.

In view of the importance of retinal imaging to studying dementia, this protocol aims to describe a method of quantifying and analyzing retinal vasculature and neuronal structure *in vivo* using widely available retinal imaging techniques*.* This protocol also provides examples of retinal changes in subjects with dementia and discusses technical issues related to retinal imaging.

**PROTOCOL:**

All methods described here have been approved by a local clinical research ethics committee in Hong Kong.

**Note:** For simplicity, the equipment listed in the **Table of Materials** is used to illustrate the procedures of retinal imaging and subsequent analysis. Quantification of retinal vasculature is performed with the Singapore I Vessel Assessment program (version 4.0)17 (National University of Singapore, Singapore). This protocol can be performed using a different set of equipment and although the operation of different equipment may vary, the underlying principles remain similar.

**1. Prepare Subjects for Retinal Imaging**

1.1 Dilate the pupil of the subject using mydriatic agents. Wait for at least 15 min to establish sufficient pupil dilation.

**2. Acquire Fundus Photographs of Retinal Vasculature**

[Place Figure 1 Here]

2.1. Capture an ocular fundus photograph using the fundus camera.

2.1.1 Start the fundus camera and launch the image capturing program on the computer. Rest the chin of the subject properly on the chinrest with the forehead against the head strap. Move the control lever to align the light beam properly to the subject’s pupil. Ensure the distance between the subject’s eyes and the main body is no longer than the proper working distance.

2.1.2 Align the illumination points until they appear smallest on both sides in the viewfinder. Move the external fixation target to guide the subject’s eyes until the optic disc is at the center of the viewfinder and the regions of interest (ROI) are well within the limits of the image. Adjust the focusing knob to focus on the retina.

2.1.3 Have the subject firmly look at the external fixation target and ensure the subject’s eyes are not filled with tears.

2.1.4 Depress the shutter-release button to capture an image (**Figure 1A**).

2.1.5 Check the quality of the fundus photograph captured, using **Figure 2A** as a standard. Discard the image and repeat the image acquisition process (*i.e.*, **Step 2.1.1 to 2.1.4**) if the pupil is poorly dilated (**Figure 2B**), the optic disc is not at the center of the photograph (**Figure 2C**), or the focus is not accurate (**Figure 2D**).

[Place Figure 2 Here]

2.1.6 Save all images in TIFF format with gradable resolution (*i.e.*, approximately 3,000 pixels x 2,000 pixels, at more than 150 dpi).

**Note:** The protocol can be paused here.

2.2. Open the images in the computer-assisted analysis program for automatic tracing.

**Note**: The SIVA program is used for illustration purposes only and can be substituted by other available measurement programs.

2.2.1 Select a random sample of 10% of the images and measure the height of the optic discs in these images (see **Figure 3**). Calculate the image conversion factor (ICF) using the formula:

ICF = 1800 μm/ (Average pixel height of optic discs of the images sampled).

2.2.2 Upload the captured fundus photographs to the cloud-based server and enter relevant study details, including the image conversion factor (ICF) (**Figure 1B**).

**Note:** The protocol can be paused here. Other computer-assisted analysis programs may use other non-cloud-based methods to organize the images.

[Place Figure 3 Here]

2.2.3 Open the images with the computer-assisted analysis program (**Figure 1C**). Observe that the program automatically detects the optic disc, places a measurement grid, and traces the vessel paths (**Figure 1D to 1F**).

2.3. Adjust the auto-tracing results of the retinal vasculature. Begin the inspection from the 12 o’clock position in a clockwise manner to ensure that all vessel tracings on the image are verified.

2.3.1. Check that the optic disc is accurately detected and the measurement grid is correctly placed. Adjust the measurement grid manually following steps 2.3.1.1 to 2.3.1.4, if the innermost circle does not accurately outline the optic disc rim (**Figure 4A**).

2.3.1.1 Click the “OD Center” button on the left function panel; the mouse cursor will be replaced by a green circle.

2.3.1.2 Move the green circle to the center of the optic disc (OD), and left-click to fix the circle.

2.3.1.3 Click the “Find OD” button to prompt the software to detect the OD rim and place a new measurement grid based on the new position of the OD center.

2.3.1.4 Click the “Process” button to initiate the auto-tracing process of the vessels.

2.3.2 Left click to select the vessels with incorrect vessel labels (arterioles versus venules) and click the “Vessel (T)ype” button to change the vessel type.

**Note:** Arterioles can be distinguished from venules based on their physiological differences. For example, venules are generally darker in color and wider than arterioles. Vessels with the same vessel type do not cross each other. Arterioles are labelled in red and venules are labelled in blue.

2.3.3 Extend the incomplete vessel tracings following steps 2.3.3.1 to 2.3.3.2 (**Figure 4B**).

2.3.3.1 Use the cursor to click at the distal end of the incomplete vessel tracing. Left click at points along the vessel to extend the vessel tracing.

2.3.3.2 Press “Esc” to end the tracing process when the end of the vessel is reached. Stop the tracing at the outermost white circle if the distal part of the vessel falls outside the measurement grid (see **Figure 4B**).

2.3.4 Adjust the vessel tracings if the vessel path is inaccurately traced at the crossover site (**Figure 4C**).

2.3.4.1 Click the “Select” button and then click at the point on the vessel tracing to be disconnected. Click the “Brea(k) Seg” button to disconnect the vessel tracing at the point selected. Select the disconnected segment and click the “(Del) Seg” button to delete it.

2.3.4.2 Repeat steps 2.3.3.1 and 2.3.3.2 to re-construct the remaining segment.

2.4 Lay vessel covers on all vessel segments and adjust the covers manually.

2.4.1 Click the “Find Covers” button to lay vessel covers on all vessel segments automatically.

**Note:** Vessel covers are measurement lines that estimate the approximate width of the internal lumens of the vessels.

2.4.2 Check if all vessel covers are correctly placed for all vessel segments. Left-click and drag the cursor over vessel covers to deactivate them if the vessel covers are not laid perpendicular to the vessel walls (**Figure 5A**), the vessel is obscured under another vessel (**Figure 5B**), or the vessel covers overestimate or underestimate the width of the internal lumen (**Figure 5C**).

2.5. Close the grading windows and click “send” in the pop-up dialog to upload the graded image to the cloud-based server for automatic measurement.

[Place Figure 4 Here]

[Place Figure 5 Here]

**3. Measure the Retinal Vascular Parameters Using a Computer-Assisted Analysis Program.**

**Note:** Steps 3.1 to 3.7 can be completed automatically by a computer-assisted analysis program.

3.1 Open the fundus photograph to be measured.

3.2 Place four concentric circles as measurement grids using the center of optic disc as a reference (**Figure 6A).** Label the area 0.5-1.0 disc diameters away from the disc margin as zone B, and the area 0.5-2.0 disc diameters away from the disc margin as zone C18 (**Figure 6A**), according to the modified protocol of Atherosclerosis Risk in Communities (ARIC) study19.

3.3 Measure retinal vascular caliber from both zone B and zone C, using a method modified from the ARIC study19 (**Figure 6B**). This has been widely adopted in many large population studies20–26.

3.3.1 Measure the lengths of vessel covers in the six largest arterioles and the six largest venules to estimate retinal vessel calibers.

3.3.2 Summarize the retinal arteriolar and venular calibers as central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) respectively17, using the revised Knudtson–Parr-Hubbard formula18,19.

[Place Figure 6 Here]

3.4 Identify all vessels in zone C with a width >40 μm. Calculate the retinal arteriolar and venular tortuosity from the integral of the total squared curvature along the vessel paths and normalize the value with the total arc length, bowing, and points of inflection27,28.

3.5 Compute the total, arteriolar, and venular fractal dimensions from zone C, using the established “box-counting method”29–31.

3.5.1 Divide the retinal image into a series of equally sized square boxes.

3.5.2 Count the number of boxes containing a section of the skeletonized line tracing.

3.5.3 Repeat the process with a series of different sized boxes.

3.5.4 Plot the logarithm of the number of boxes containing the line tracing against the logarithm of the size of the boxes, and calculate the slope of the resulting line; this is the fractal dimension.

3.6 Identify vessels with first bifurcation in zone C and calculate the angles (θ) subtended between the first two daughter vessels32 (**Figure 6C**). Compute the mean value to obtain the average branching angle.

3.7 Calculate the branching coefficient from zone C using the formula:

(d12 + d22)/d02, where d0 is the mean trunk caliber, and d1 and d2 are the mean branch calibers (**Figure 6C**).

**4. Assess the Thickness of GC-IPL and RNFL**

[Place Figure 7 Here]

4.1. Perform image acquisition using optical coherence tomography (OCT).

4.1.1 Open the OCT program and select the “Macular Cube” scanning protocol to start a new macular scan (**Figure 7A**).

4.1.2 Locate the pupil in the iris viewport by adjusting the chinrest. Lower the illumination if the pupil size is too small.

4.1.3 Click the “Auto Focus” button and then the “Optimize” button to improve the image quality.

4.1.4 Instruct the subject to blink a few times immediately before starting the scan.

4.1.5 Click the “Capture” button to start the scan when the border surrounding the button becomes green. Instruct the subject to focus on the visual fixation target to avoid motion artifacts.

4.1.6 Perform an optic nerve head scan with the “Optic Disc Cube” scanning protocol using steps 4.1.2 to 4.1.5 (**Figure 7B**).

4.1.7 Review the quality of the captured scan using **Figure 7C** and **7D** as a standard. Discard the image and retake the scan if the signal strength is smaller than 6 (**Figure 8A**), or motion artefacts are detected (indicated by discontinuity of blood vessels) (**Figure 8B**).

4.1.8 Save the scanning results.

[Place Figure 8 Here]

4.2. Generate the analysis printout of the macular GC-IPL thickness.

4.2.1 Select the “Macular Cube” scan records of both eyes in the analysis interface.

4.2.2 Click the “Ganglion Cell OU Analysis” to initiate the automatic analysis algorithm to assess the GC-IPL thickness of the captured image (**Figure 7E**).

**Note:** Steps 4.2.2.1 to 4.2.2.4 can be automatically completed by the analysis algorithm.

4.2.2.1 Generate a 14.13 mm2 fovea-centered elliptical annulus that has horizontal inner and outer radiuses of 0.6 mm and 2.4 mm, respectively, and vertical inner and outer radiuses of 0.5 mm and 2.0 mm, respectively.

**Note:** The size and shape of the elliptical annulus conform closely to the macular anatomy and thus correspond to the area where the RGCs are thickest in normal eyes33,34. The area within the inner ring of the annulus is not measured, since the macular GC-IPL in this area is very thin and difficult to detect accurately.

4.2.2.2 Segment the outer boundary of the RNFL and the outer boundary of the inner plexiform layer (IPL) to locate the GC-IPL (**Figure 9**).

4.2.2.3 Measure the average, minimum, and six sectorial (superotemporal, superior, superonasal, inferonasal, inferior, inferotemporal) thickness of macular GC-IPL within the fovea-centered elliptical annulus.

4.2.2.4 Report the measurement results on an analysis printout.

4.2.3 Save the analysis printout in the .pdf format.

[Place Figure 9 Here]

4.3. Generate the analysis printout of the RNFL thickness (**Figure 7F**).

4.3.1 Select the “Optic Disc Cube” scan records of both eyes in the analysis interface.

4.3.2 Click the “ONH and RNFL OU Analysis” to initiate the automatic analysis algorithm to assess the RNFL thickness of the captured image.

**Note:** Steps 4.3.2.1 to 4.3.2.6 can be automatically completed by the analysis algorithm.

4.3.2.1 Measure the RNFL thickness at each scan point and generate an RNFL thickness map.

4.3.2.2 Identify the optic disc by detecting a dark spot near the center of the scan that has a size and shape consistent with the range of an optic disc.

4.3.2.3 Position a calculation circle of 3.46 mm in diameter around the optic disc on the RNFL thickness map.

4.3.2.4 Measure and calculate the global, four-quadrants (temporal, superior, nasal and inferior), and twelve-clock-hour parapapillary RNFL thickness, using the calculation circle as the region of interest (ROI).

4.3.2.5 Compare the measured RNFL thickness to the device’s internal normative age-matched database and generate a deviation map and a significance map.

4.3.2.6 Report the measurement results on an analysis printout.

4.3.3 Save the analysis printout in the .pdf format.

**REPRESENTATIVE RESULTS:**

[Place Figure 10 Here]

**Interpretation of the Retinal Vascular Parameters:** Through the protocol described, several quantitative retinal vascular parameters can be measured from the fundus photographs. These parameters indicate the status of the retinal vasculature, which may in turn reflect similar changes in the cerebral vasculature. **Figure 10** shows the fundus photographs obtained from an AD subject and a healthy subject and their retinal vascular parameters are reported in **Table 1**. These two fundus photographs were analyzed using the method described in this protocol and are used as an example to demonstrate the interpretation of the retinal vascular parameters.

[Place Table 1 Here]

**Retinal Vessel Caliber**: The fundus photograph of the AD subject shown in **Figure 10** showed a decrease in CRAE and CRVE of Zone C (138.47 μm and 206.61 μm, respectively), when compared to the healthy subject (165.82 μm and 232.22 μm, respectively). The CRAE and CRVE summarize the retinal vessel calibers, which approximates the average width of the internal lumen in retinal arterioles and venules, respectively. Reduction in CRAE or CRVE thus indicates generalized retinal vessel narrowing, and may suggest subtle microvascular dysfunction35.

**Retinal Vascular Network Parameters:** Firstly, the retinal arteriolar and venular tortuosity of the AD subject (0.613 x 10-4 and 1.41 x 10-4, respectively) were higher than that of the normal subject (0.476 x 10-4 and 0.501 x 10-4, respectively). The higher vascular tortuosity value indicates that the retinal vessels are generally straighter in the AD subject.

Secondly, the AD subject also had reduced retinal fractal dimension (total fractal dimension, 1.472; arteriolar fractal dimension, 1.246; venular fractal dimension, 1.253) when compared to the healthy subject (total fractal dimension, 1.517; arteriolar fractal dimension, 1.316; venular fractal dimension, 1.273). Since fractal dimension represents a “global” measure that summarizes the branching complexity of the retinal vascular network30, a smaller value in the AD subject indicates a less complex branching pattern.

Thirdly, most retinal bifurcation parameters of the AD subject were deviated from the optimal value. Specifically, the arteriolar and venular branching angle of the AD subject (67.17o and 60.109o, respectively) were further away from optimal values, which are approximately 75o, when compared with the normal subject (81.16o and 73.19o,respectively)36. Furthermore, the arteriolar branching coefficient of the AD subject (2.432) was also severely deviated from the optimal value, which is approximately 1.2636. This represents an increase in the total cross-sectional area across the bifurcations37.

**Interpretation of Retinal Neuronal Parameters:** Through the protocol described, one should obtain two analysis printouts showing the parameters of RNFL thickness and the GC-IPL thickness (illustrated in **Figure 11A and 11B**, respectively). While the RNFL measurements reflect the health of the unmyelinated axons of RGCs, the GC-IPL measurements indicate the health of the cell bodies and dendrites of RGCs. Since the size of RGC cell body is 10-20 times the diameter of their axons, GC-IPL thickness has been shown to be more strongly related to cognitive impairment38. In both reports, three maps were shown to aid the subsequent analysis, namely (a) thickness maps, (b) deviation maps, and (c) significance maps.

In the thickness map, warmer colors represent higher thickness values and cooler colors represent lower thickness values; in other words, the denser the orange/yellow ring, the thicker the retinal layer. In the deviation maps of both reports, a super-pixel is shown in red or yellow if the thickness value falls outside the 99% or within 95%–99% centile range, respectively. In addition, the analysis algorithm also compares the measurement results with the normative database and reports the findings as a significance map. The significance map of the GC-IPL thickness is reported in the form of six sectors, while that of the RNFL thickness is reported in a form of four quadrants and 12 clock-hours. All types of significance maps are color-coded to match the thickness, with values within the normal range in green (p=5%–95%), borderline values in yellow (1%<p<5%), and values outside the normal range in red (p<1%). Numeric values inside or near sectors represent average thickness in the corresponding sectors.

**Figure 11A** and **Figure 11B** are the analysis printouts obtained from an AD subject. The fading of warm colors and the appearance of light blue areas in the thickness maps indicates the thinning of the GC-IPL and the RNFL in the AD subject. While the former change suggests RGC loss, the latter change suggests a loss of RGC axons. In addition, several sectors of the significance maps are labelled in red or yellow, suggesting that the RNFL and GC-IPL thickness of these sectors are reduced in the AD subject. The exact location of thickness reduction is also visualized by the red or yellow super-pixels in the deviation maps.

[Place Figure 11 Here]

**Unanalyzable or Ungradable Retinal Images**: Unanalyzable retinal images may be obtained for several reasons. For instance, GC-IPL or RNFL segmentation failure may occur due to retinal pathologies, including age-related macular degeneration, diabetic retinopathy, and epiretinal membrane. **Figure 12A** demonstrates an example of segmentation failure due to diabetic macular edema. OCT studies have also shown that OCT scan quality and thickness measures can be affected by dry eyes39, cataracts40–43, floaters and other vitreous opacities44,45. In addition, the retinal vasculature analysis may also be hindered by media opacity (such as a cataract), which affects the visibility of the retinal vasculature (**Figure 12B**).

[Place Figure 12 Here]

**FIGURES and TABLE LEGENDS**

**Figure 1. Schematic diagram showing the procedures of retinal vasculature measurement.**

(**A**) Ocular fundus photography is used to obtain an optic-disc-centered fundus photography. **Figure 1A** is identical to **Figure 2A**, both of which represent an optimal quality of fundus photograph. (**B**) Upload the captured images to the cloud-based server and enter relevant study details, including the image conversion factor (ICF). (**C**) Download and open the fundus photographs from the cloud-based server in the program. (**D, E, F**) The program automatically detects the optic disc, places a measurement grid, and traces the vessel paths. (**G**) The graders are required to check the accuracy of the auto-tracings and, if necessary, perform manual adjustments. (**H**) After confirming the accuracy of all tracings, a spectrum of retinal vascular parameters, including vessel calibers, tortuosity, fractal dimension and bifurcation, will be measured automatically. Step (**D**) to Step (**F**) can be done automatically by the computer-assisted analysis program.

**Figure 2. Optimal and suboptimal fundus photographs.** The image quality of fundus photographs directly affects the measurement and analysis of the retinal microvasculature. Hence, the quality of fundus photographs must be checked immediately after image acquisition. The image should be discarded if one of these artefacts is observed. These images were captured using a 50-degree fundus camera.

**Figure 3. A schematic diagram illustrating the calculation of the image conversion factor (ICF)**. To calculate the ICF, randomly select a 10% sample of images from the total number of images expected from the study (Step 1). Then, measure optic disc height in pixels from sample images (Step 2). Calculate the ICF using the formula: ICF= 1800 μm / (Average pixel height of optic discs from sample images), where 1800 μm is the estimated height of a normal optic disc (Step 3). As magnification effect and image resolution differ from camera to camera, it is necessary to calculate an accurate ICF for each camera used for every study.

**Figure 4. Common errors of the auto-tracing**. The auto-tracing of the program is not completely accurate and manual adjustments are required to ensure the accuracy of the measurement. This figure shows common errors of the auto-tracing and demonstrates optimal results after manual adjustments. (**A**) Incorrect detection of the optic disc could lead to misplacement of the measurement grid and affect the subsequent measurements. Ideally, the innermost circle should outline the optic disc rim. (**B**) Incomplete vessel tracing could lead to the incorrect measurement of fractal dimension, tortuosity, *etc*. The vessel path should be traced until the end of the vessel. If the distal part of the vessel falls outside the measurement grid, the tracing can be stopped at the outermost white circle. (**C**) Vessel tracings at the crossover sites are subject to a higher tendency for error and thus require special attention.

**Figure 5. Incorrect vessel covers.** This figure shows examples of incorrect vessel covers that should be deactivated and excluded from the measurement. (**A**) Vessel covers should be deactivated if they are irregular and not perpendicular to vessel walls. (**B**) Vessel covers should be deactivated if the vessel being traced is obscured under another vessel (**C**) Vessel covers should be deactivated if they cannot represent the approximate width of the vessel.

**Figure 6. Quantification of retinal vasculature.** (**A**) Zone B (defined as 0.5-1.0 disc diameters away from the disc margin) is used to measure vessel calibers of zone B according to the Atherosclerosis Risk in Communities Study. Zone C (defined as 0.5-2.0 disc diameters away from the disc margin) is used to measure vessel calibers of zone C and a spectrum of retinal vascular network parameters (such as tortuosity, fractal dimension, and bifurcation). (**B**) Vessel covers are measurement lines used to estimate the retinal vascular calibers (or retinal vessel diameters). Incorrect vessel covers can be manually excluded from the measurement. (**C**) At each bifurcation, the program automatically detects the branching angles (θ) of all vessels that have their first bifurcation within zone C. In addition, the branching coefficient is also calculated using the formula: Branching coefficient = (d12 + d22)/d02, where d0 is the trunk caliber, and d1 and d2 are the branch calibers.

**Figure 7. Image acquisition and optimal results of retinal neuronal structure analysis.** Optical coherence tomography (OCT) is used to measure the thickness of the ganglion cell-inner plexiform layer (GC-IPL) and retinal nerve fiber layer (RNFL). (**A, B**) The GC-IPL and RNFL can be imaged using the built-in “macular cube” and “optic disc cube” scanning protocols respectively. (**C, D**) It is critical to check the image quality immediately after image acquisition. Discard the image and retake the scan if the signal strength is smaller than 6, or motion artefacts are detected. (**E, F**) After finishing the image acquisition, the built-in algorithm can automatically analyze the image and generate a report for interpretation.

**Figure 8. Sub-optimal results of optical coherence tomography.** Common sub-optimal results of the optical coherence tomography (OCT) include (**A**) poor signal strength (strength value <6), and (**B**) motion artefacts. The quality of OCT images should be checked immediately after image acquisition, and the scan protocol should be repeated if these artefacts are observed.

**Figure 9. Retinal layers used for the assessment of the retinal neuronal structure.** The ganglion cell analysis (GCA) algorithm detects the outer boundary of the retinal nerve fiber layer (RNFL) and the inner plexiform layer (IPL) to yield the combined thickness of the GCL and the IPL. The thicknesses of the GCL and the IPL are measured together, as the boundary between the GCL layer and the IPL is anatomically indistinct, but the combined thickness is indicative of the health of RGCs. The measurement of the RNFL is done using the HD-OCT optic nerve head (ONH) algorithm, while the measurement of the GC-IPL is done using the HD-OCT ganglion cell analysis (GCA) algorithm.

**Figure 10.** **An example to show the differences in retinal vasculature between a normal subject and an AD subject.** (**A**) Fundus photographs of a healthy subject showing wider vessel caliber (CRAE of Zone B, 156.45 μm; CRVE of Zone B, 207.54 μm; CRAE of Zone C, 165.82 μm; CRVE of Zone C, 232.22 μm), higher retinal vascular fractal dimension (total fractal dimension, 1.517; arteriolar fractal dimension, 1.316; venular fractal dimension, 1.273) and smaller retinal vascular tortuosity (arteriolar tortuosity [104], 0.476; venular tortuosity [104], 0.501). (**B**) Fundus photographs of an AD subject showing narrower vessel caliber (CRAE of Zone B, 116.38 μm; CRVE of Zone B, 186.93 μm; CRAE of Zone C, 138.47 μm; CRVE of Zone C, 206.61 μm), smaller retinal vascular fractal dimension (total fractal dimension, 1.472; arteriolar fractal dimension, 1.246; venular fractal dimension, 1.253) and higher retinal vascular tortuosity (arteriolar tortuosity [104], 0.613; venular tortuosity [104], 1.41). These images were captured using 50-degree fundus camera and were analyzed using the method described in the protocol.

**Figure 11. Analysis printout of the retinal neuronal structure from a subject with AD.** The analysis printouts of both GC-IPL and RNFL thickness include three types of thickness map, namely the thickness map, the deviation map, and the significance map. (**A**) The red and yellow super-pixels in the right deviation map (red arrows) indicate the RNFL thickness of these parts of the retina fell outside the 99% or within 95%–99% percentile range, respectively. Consistently, the decreased RNFL thickness of these regions is also indicated by the red and yellow sectors in the two significance maps, namely “RNFL quadrants” and “RNFL clock hours”. While the red sectors indicate the RNFL thickness fell outside the normal range (p<1%), the yellow sector indicates borderline values (1%<p< 5%). All together, these maps suggest that the RNFL thickness was reduced in the AD subject. (**B**) The interpretation of the GC-IPL thickness is similar to that of the RNFL thickness. In the AD subject, the thickness map shows light blue areas (red arrows), which visualize the thinning of the GC-IPL. In line with this finding, the red and yellow super-pixels in the deviation maps (red arrows) also indicate the thickness of these regions fell outside the 99% or within 95%–99% percentile range, respectively. Reduction of GC-IPL thickness in these regions is also illustrated by the red sectors in the significance map.

**Figure 12. Unanalyzable or ungradable images.** Most suboptimal results could be corrected using the methods described in the protocol. However, several types of retinal images are unanalyzable and should be discarded. (**A**) In the OCT, segmentation failure may occur due to retinal pathologies, including age-related macular degeneration, diabetic retinopathy, and epiretinal membrane. This figure demonstrates a segmentation failure due to diabetic macular edema. (**B**) The visibility of the retinal microvasculature is reduced by media opacity, such as opacity due to a cataract. This figure demonstrates that severe media opacity can render the image unanalyzable.

**Figure 13. Retinal imaging is a potentially valuable tool to study vascular and neuronal changes associated with dementia.** It has been proposed that dementia is associated with neuronal injury and small vessel disease in the brain. Given the retina, an extension of the central nervous system, shares prominent similarities with the brain, these pathophysiological changes may be reflected in the retina as retinal neuronal and vascular damage. With the methods described in this protocol, the retinal neuronal changes can be quantified as the thickness of RNFL and GC-IPL using optical coherence tomography (OCT), while the retinal vascular changes can be quantified as vessel calibers and vascular network parameters using fundus photography and computer-assisted analysis program. Studying the associations between the retinal changes and dementia may provide new insights into the pathophysiology of dementia and, potentially, aid in diagnosis and risk assessment.

**Table 1. Retinal Vascular Parameters of an AD subject and a normal subject.** The fundus photographs of these two subjects are shown in **Figure 10**. The AD subject showed reductions in CRAE and CRVE, fractal dimension, and an increase in tortuosity. In addition, the branching angles and coefficients of the AD subject also deviated from optimal values.

**Table 2. Interpretations of major retinal vascular parameters.** Vessel calibers and retinal vascular network parameters are two main categories of parameters reported by the computer-assisted analysis program. CRAE and CRVE document generalized retinal vessel narrowing or widening, reflecting subtle retinal microvascular dysfunction. Retinal vascular network parameters capture the “optimality” and “efficiency” of blood distribution in the retinal network, which in turn reflect the integrity of the cerebral microcirculation.

**DISCUSSION:**

This protocol describes retinal imaging methods to study retinal neuronal and microvascular structures *in vivo.* As retinal changes reflect different aspects of the vasculature and neuronal structure in the brain, this method can be used to study dementia and, potentially, aid in diagnosis and risk assessment.

In the illustrated example shown in **Figure 10** and **Table 1**, the AD subject showed decreased vessel caliber. Consistently, it has been reported that increased CRVE is associated with incident dementia46 and vascular dementia46, and decreased CRVE and CRAE are associated with Alzheimer’s Disease47,48.

Furthermore, the AD subject also showed decreased fractal dimension, increased vascular tortuosity, and a suboptimal branching coefficient and branching angle when compared to the normal subject. These changes indicate the alterations in global geometrical patterns of the retinal vascular network49–56. Since the branching pattern of the vascular network, according to Murray’s principle, is structurally developed to minimize the energy required to maintain blood flow57, these parameters capture the “optimality” and “efficiency” of blood distribution in the retinal vascular network, which may in turn reflect the integrity of the cerebral microcirculation58. It has been reported that reduced retinal fractal dimension is associated with dementia47,48,59 and cognitive function60, because retinal hypoxia resulted from retinal vessel rarefaction and collapse may reduce the complexity of the retinal vascular network61. Increased venular and arteriolar tortuosity are also shown to be associated with AD62. Regarding the retinal bifurcation parameters, sub-optimality of the branching angle indicates that the retinal vascular network in the AD subject has decreased efficiency of tissue perfusion and increased energy loss37. Alteration in branching angle may also indicate changes in blood flow63,64, endothelial dysfunction65,66, and attenuation of oxygen saturation67. In addition, deviation from the optimal value of the branching coefficient may also increase energy cost, reducing the efficiency of circulation, and metabolic transport37. The reported associations of major retinal parameters with AD are summarized in **Table 2** and have also been discussed in details previously8,58,68,69.

As illustrated by **Figure 11**, the AD subject also showed decreased thickness of RNFL and GC-IPL. Although the thickness of the GCL and the IPL can only be reported together owing to their ambiguous anatomical boundaries, the combined thickness is indicative of the health of RGCs38. It is increasingly evident that thinning of GC-IPL38 and/ or RNFL70–78 is associated with AD. Recently, a large-scale population study also reported that thinner macular RNFL is associated with poorer cognitive function, such as poorer prospective memory and poorer performance of numeric and verbal reasoning79. In addition, retinal RGC axonal loss, as indicated by thinning of RNFL, is also reported in non-AD dementias80–82.

**Critical Steps of Retinal Imaging:** To achieve optimal results, several steps in the protocol require special attention. Regarding the procedure of image acquisition, it is important to master the imaging procedure of OCT and ocular fundus photography, since a prolonged imaging time may induce eye fatigue and thus increase the likelihood of motion artefacts. In addition, the contrast and saturation of the images should be standardized during image acquisition to avoid coarse adjustment in the later steps of image processing. The contrast and the saturation can be varied with different study cohorts and camera types.

Regarding the measurement of retinal vasculature, it is important to calculate the ICF for every camera used in the study to adjust for the magnification effect and the difference in image resolution. Adjustment by ICF is important for the accurate measurement of dimensional parameters, including retinal vessel calibers. In addition, when grading the fundus photographs with the program, graders should be masked to the participant’s characteristics as the program involves a certain amount of manual correction. Also, the graders should receive proper training and their reliability of measurement should be evaluated first, before grading the images.

It is also important to report retinal vessel caliber in both zone B and zone C. It is believed that the vessel caliber of zone C is more sensitive and more precise with smaller standard error17, possibly due to inclusion of more peripheral small vessels in the retina, which are anatomically and physiologically similar to the small vessels in the brain. Hence, the caliber of zone C is used in this protocol to study dementia. However, the caliber of zone B should also be reported as the caliber measurement within zone B has been widely used in numerous epidemiological studies.

It should be emphasized that the equipment and the measurement program used in this protocol are for illustration purposes only and similar results can be obtained using other retinal imaging techniques. However, in most cases the numerical parameters reported by different measurement systems should not be interpreted interchangeably83. Yip *et al.* have developed an algorithm for conversion between three commonly used retinal vessel calibers measurement software, which may be useful to compare results from different studies83.

**Significance of Retinal Imaging:** Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) imaging are two common *in vivo* imaging methods to study the CNS. However, the application of MRI is limited by its spatial resolution to detect subtle degenerative changes of less than 500 μm. The use of PET imaging is also limited by its high cost and the availability of PET facilities. In addition, although cerebral small vessel disease has been linked to dementia84–90, the limitations of current neuroimaging technologies hinder the direct assessment of the changes in the cerebral small vessels, such as cerebral arteriolar narrowing, changes in vascular tortuosity, and capillary micro-aneurysm. In contrast, retinal imaging demonstrates several features that make it a valuable tool to study dementia.

Firstly, the retina, when compared with other parts of the CNS, is highly accessible for non-invasive imaging in live individuals. As the pupil allows bidirectional passage for the illuminating and imaging light rays, the retina can be imaged directly and rapidly using a fundus camera, which is a classic retinal imaging technique based on the principle of monocular indirect ophthalmoscopy. Fundus photography has demonstrated high sensitivity, specificity, and inter-examination and intra-examination agreement91. Furthermore, *in vivo* cross-sectional retinal images can now be captured by OCT with high resolution, based on the principle of low-coherence interferometry92–96. After image acquisition, a three-dimensional retinal image can also be constructed by combining cross sections. As a result, retinal imaging allows longitudinal *in vivo* monitoring in all age groups to observe dementia-related changes.

Secondly, the retinal neuronal structure is organized as distinguishable layers and each layer represents different elements of the neuronal architecture. For instance, the GC-IPL represents the retinal ganglionic cells and their dendrites, while the RNFL represents the axons of the retinal ganglionic cells. Notably, accurate demarcation of retinal neuronal layers, such as GC-IPL and RNFL, can now be achieved with advanced segmentation algorithms33,97, and damage that manifests as a distortion of the normal architecture can be easily detectable. Since the retinal neurons share prominent similarities with the CNS neurons, dementia-related changes in the retinal neuronal structure can be used to study the pathological processes of dementia and might be used as prognostic factors for dementia.

Thirdly, objective, semi-automated, and standardized assessment of retinal images is now possible using computer-assisted analysis programs. As illustrated by this protocol, the computer-assisted analysis programs can automatically trace the retinal vasculature captured by the fundus photograph and, based on the tracing results, measure a spectrum of retinal vascular parameters, such as vessel calibers, tortuosity, fractal dimensions, and branching angle. During the measurement process, the graders are only required to verify the results of auto-tracing and, if necessary, adjust incorrect vessel tracings manually. Previous studies have reported that intragrader and intergrader reliability were moderate to high49. Similarly, the OCT built-in analysis program can also automatically measure parameters of retinal neuronal structure and compare the results with the normative database, which is not race specific and consists of RNFL measurements of 328 healthy individuals from 5 different ethnic groups98. This feature of retinal imaging helps to improve measurement efficiency and consistency by reducing the amount of work required from each grader.

Lastly, retinal imaging technologies can now image the retina at resolutions of several microns, which is at least an order of magnitude than can be achieved with conventional brain imaging techniques. For instance, the spectral domain-OCT can now image the retina in three-dimensional volume at a high scan speed, high axial resolution and a high degree of reproducibility99–105. This allows direct visualization and quantification of subtle changes in the retina.

Taken together, retinal imaging can collect unique information on the cerebral vasculature and neuronal structure that is distinct from current brain imaging techniques, suggesting that retinal imaging may provide a complementary approach to study the pathophysiology of dementia 9,35,58,68,106–108.

**Limitations of the Method:** Retinal imaging is an increasingly popular method to visualize and quantify the microvasculature and neuronal structure in the retina8,109. However, users of this protocol should be aware of its potential limitations for critical interpretation of the results.

First, the quality of ocular fundus photographs and OCT images are affected by a range of ocular factors. For instance, variations in refractive error and axial length may affect the magnification and apparent dimensions of retinal vascular caliber and structures110. Differences in retinal pigmentation, presence of media opacities, photographic technique, camera type (*e.g.,* mydriatic, non-mydriatic, hand-held), and image quality (*e.g.,* brightness, focus, and contrast) may also introduce additional sources of variation and affect measurements111–116. In addition, motion artefacts may be common in an old subject if the image acquisition process is prolonged.

Second, the retinal vascular and neuronal architecture can be affected by many systemic and local pathological processes, and thus some retinal manifestations are not specific to a particular disease. For instance, retinal arteriolar narrowing has been correlated to systemic peripheral vasoconstriction and hypertension, while retinal venular widening has been correlated to endothelial dysfunction, inflammation, and microvascular hypoxia117, and diseases such as cardiovascular disease118 and diabetic retinopathy119. RNFL thinning is also observed in other neurodegenerative diseases, including glaucoma, Parkinson’s Disease, and multiple sclerosis10. It is also noteworthy that age-related reduction in RGCs and RGC axons may occur in the normal retina99,120. These factors limit the potential of retinal imaging as a screening tool.

Third, the associations between retinal changes and dementia remain inconclusive. For instance, the associations of smaller vessel calibers with AD were not replicated by Williams *et al.*59, and the association of narrower arteriolar caliber with AD found by the Singapore Epidemiology of Eye Disease program study was also lost after adjusting for confounding cardiovascular factors48. Furthermore, increased venular and arteriolar tortuosity in AD has also not been consistently observed47,59. It is also noted that in a large-scale study, the association of arteriolar fractal dimension with dementia was lost in the fully adjusted model59.

Fourth, the computer-assisted analysis programs are only semi-automated and require manual adjustment by trained graders49,121. Manual input, even following a standardized protocol, may introduce additional variability in the retinal measurements.

**Future applications of the method:** In terms of scientific research, given the accessibility of the retina and its similarities with the brain, the retina appears to offer a promising means of studying AD-related changes in the cerebral microvasculature and neuronal structure. Since dementia is now thought to involve vascular processes84–90,122, retinal imaging may provide new insights into the microvascular etiology (versus macrovascular etiology) of dementia10,35,58,106,108,123 and facilitate our understanding on different dementia subtypes. In addition, as the association between retinal changes and dementia remains inconclusive, this protocol can also be used to further examine the utility of retinal imaging in population screening or diagnosis of dementia.

In terms of clinical research, retinal imaging might potentially be used in clinical settings to facilitate the preclinical diagnosis or risk assessment of dementia, to confirm clinically diagnosed AD, and to monitor the disease progression or response to therapy. The application of retinal imaging in population screening is particularly intriguing as neuronal and microvascular changes, which might be reflected by similar retinal changes, occur much earlier than the appearance of cortical atrophy and cognitive decline124,125. However, the strength of associations between retinal imaging measures and dementia is only modest and several retinal correlates of AD have not been consistently observed8,47,48,59,109. More prospective clinical studies with large cohorts are required to evaluate the clinical utility of retinal imaging in the diagnosis and monitoring of AD.

In terms of the technical aspects of our method, recent advances in retinal imaging techniques, such as the ultra-wide field retinal imaging and the OCT-angiography, may allow us to obtain more information from the retina. The ultra-wide field retinal imaging technology, based on the principle of confocal laser scanning microscopy combined with a concave elliptical mirror, can capture up to 200o of the retina in a single image without pupil dilation126,127 and thus allow more extensive assessment of peripheral retinal lesions. It is possible that assessment of more peripheral vessels may provide an even better representation of the overall retinal vasculature17. It has been reported that the ultra-wide field retinal imaging can achieve satisfactory performance in both vessel segmentation and width estimation. In addition, the invention of OCT-angiography also allows non-dye-based mapping of the retinal capillary network, which might provide more information on microvascular changes related to dementia. In view of image analysis methods, more research is required to explore other state-of-the-art image processing and quantitative methods, such as tree topology estimation128, to analyze the images captured by these imaging modalities.

[Place Figure 13 Here]

This protocol describes a non-invasive, quantitative and semi-automated method to study dementia using retinal imaging techniques (**Figure 13**). Considering the accessibility of retinal imaging and its robust associations with the brain, imaging the retina may provide new insights into pathophysiological processes of dementia occurring in the brain and, potentially, aid in the diagnosis and risk assessment of dementia. However, the associations reported remain controversial and further studies are required to assess the potential utility of retinal imaging. It should also be noted that a thorough clinical evaluation remains essential in the assessment of dementia.

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