Video Article

Quantitative Fundus Autofluorescence for the Evaluation of Retinal Diseases

Stephen T. Armenti[1], Jonathan P. Greenberg[2], R. Theodore Smith[1]

[1]Department of Ophthalmology, NYU School of Medicine

[2]Harkness Eye Institute, Columbia University

Correspondence to: R. Theodore Smith at roland.smith@med.nyu.edu

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Abstract

The retinal pigment epithelium (RPE) is juxtaposed to the overlying sensory retina, and supports the function of the visual system. Among the tasks performed by the RPE are phagocytosis and processing of outer photoreceptor segments through lysosome-derived organelles. These degradation products, stored and referred to as lipofuscin granules, are composed partially of bisretinoids, which have broad fluorescence absorption and emission spectra that can be detected clinically as fundus autofluorescence with confocal scanning laser ophthalmoscopy (cSLO). Lipofuscin accumulation is associated with increasing age, but is also found in various patterns in both acquired and inherited degenerative diseases of the retina. Thus, studying its pattern of accumulation and correlating such patterns with changes in the overlying sensory retina are essential to understanding the pathophysiology and progression of retinal disease. Here, we describe a technique employed by our lab and others that uses cSLO in order to quantify the level of RPE lipofuscin in both healthy and diseased eyes.

Video Link

The video component of this article can be found at https://www.jove.com/video/53577/

Introduction

The retinal pigment epithelium (RPE) supports the function of the sensory retina through numerous processes [1]. Age-related macular degeneration (AMD) is the most important cause of untreatable blindness in industrialized countries and is characterized by changes in the RPE, including loss of pigment, loss of function and atrophy. In AMD and in normal aging, the RPE accumulates fluorescent, lysosome-derived organelles containing phagocytosed photoreceptor fragments, referred to as lipofuscin granules. The accumulation of RPE lipofuscin has been thought to indicate oxidative dysfunction [1], but recent studies have shown that the RPE morphology remains normal in aged eyes with high lipofuscin levels [2]. However, abnormal patterns of lipofuscin distribution, in particular loss of lipofuscin, are documented markers for AMD and AMD progression, both histologically and clinically [3,4]

Defective processing of RPE lipofuscin has also been shown to occur in certain inherited retinal degenerations. Patients suffering from Stargardt disease (STGD) accumulate lipofuscin in the RPE at a young age, eventually developing vision loss similar to that seen in AMD [5]. These findings suggested that lipofuscin accumulation may itself be toxic and drive RPE dysfunction [6,7]. However, a detailed imaging study of subjects with STGD over time did not confirm that focal lipofuscin accumulation led to subsequent RPE loss [8]. Hence, although lipofuscin abnormalities are markers for retinal degenerations, a role for direct toxicity of lipofuscin remains unproven.

The RPE is the most posterior cell layer of the retina, but generates the majority of fluorescent signal from the ocular fundus. Generation and detection of autofluorescence (AF) derived from the RPE can be performed using confocal scanning laser ophthalmoscopy (cSLO), which allows for visualization of the spatial distribution of fundus AF. Certain retinal degenerations demonstrate distinctive patterns of fundus AF, and AF imaging aids in the diagnosis and monitoring of these conditions. Although standard AF imaging is clinically important, quantitative AF (qAF) has become an important means of assessing RPE health. We and others have developed a standardized approach that can reliably determine qAF levels at specific retinal locations [9]. qAF has potential applications in the diagnosis and monitoring of retinal conditions, and may also have utility in prognosis and risk stratification. In addition, the diagnostic capabilities of qAF have also been described for certain retinal disorders [10-12]. Here, we provide step-wise details for performing our technique accompanied by a visual demonstration of its application in the evaluation of healthy and diseased eyes.

Protocol

Ethics Statement: All patients enrolled in these studies were done so in accordance with approved institutional review board oversight at New York University School of Medicine.

1. Patient Selection and Initial Preparation for Imaging

Note: The following materials are required: 0.5% tropicamide ophthalmic solution, 2.5% phenylephrine ophthalmic solution, cSLO equipped with spectral domain optical coherence tomography (SD-OCT), and internal fluorescence reference.

1. Prior to imaging, appropriately set up cSLO for data acquisition according to manufacturer's instructions.
2. Mount an internal fluorescent reference in the cSLO. Note: The internal fluorescent reference, as purchased from the manufacturer, is housed in a metal ring and is placed directly behind the camera lens. If a cSLO from another manufacturer is used, there may be a different configuration than that which is described in this protocol.
    1. In order to insert the reference into the cSLO, twist the lens to remove it, unscrew the machine's metal ring, and replace it with the new metal ring containing the reference.
    Note: the internal fluorescent reference is essential to the qAF technique, as it allows correction for variation in laser power and detector sensitivity/gain.
3. Have patients recruited for imaging undergo a routine dilated eye examination and obtain background information regarding past ocular history and underlying medical conditions that may manifest with ocular findings.
4. Dilate pupils with 0.5% tropicamide and 2.5% phenylephrine. CRITICAL STEP: Dilate pupils to at least 6 mm. Note: This is essential for uninterrupted passage of light, and thus for optimal visualization and measurement of the fundus.
5. Prior to imaging, position the patient properly on the cSLO, with chin resting on the chinrest, forehead placed against the forehead rest, and lateral canthi properly aligned with the indicators.

2. Baseline Imaging of the Ocular Fundus

1. First, image the fundus with near infrared reflectance (IR) light (wavelength of 820 nm) in order to centralize the camera over the macula and obtain rough focus.
    1. With patient properly positioned, switch the hardware setting on the control panel to IR imaging mode, position camera manually until the fundus is in full focus, and take an image
    2. Adjust the setting on the control panel to "IR-OCT", which uses spectral-domain optical coherence tomography (SD-OCT) in conjunction with IR imaging to evaluate the macula for underlying disease.
    3. Use the guides present in the imaging window to correctly orient the OCT to the IR image of the fundus. To achieve optimal SD-OCT quality, position the camera such that the OCT image is in the top third of its imaging window. Acquire at least one horizontal line scan through the fovea and spanning the entire imaging field.

3. Setting up qAF Imaging

1. Use "High speed" image acquisition. Note: This setting allows for faster image acquisition, which decreases risk of signal loss due to patient movement and resulting blockage of light by the iris or eyelids.
2. Use "Mean of 9" frames. Note: This setting allows for the rapid, sequential capture of 9 image frames, which can be subsequently "averaged" to reduce noise and artifact (see below).
3. Use "30 x 30 degree" field. Note: This refers to the degrees of retinal area captured during image acquisition.
4. Prior to imaging, warn patient about the blue light, as this can be alarming at first.
5. Turn on the AF mode and align camera axis such that the screen is maximally "filled" with fundus AF (minimal darkening of the sides and corners of the image).
    1. If patients have difficulty tolerating the bright blue light, start imaging with the camera further away from the eye and then bring the camera slowly toward the patient until the fundus is in full view.
    CRITICAL STEP: If light entering into or exiting from the eye is obstructed, decreased signal will result. Localized decrease is visible as asymmetric darkening of an image side or corner. Generalized loss of signal is seen when eye movements alone obstruct light passage.
6. Align the camera such that the AF signal is at its highest level throughout the field. Aim for maximal signal rather than sharpest image, however they roughly correlate. Adjust the camera focus by moving the cSLO to reposition the camera either manually or with the ophthalmoscope joystick.
7. Adjust the sensitivity/gain such that fundus AF is easily visible, but avoid over-saturation. During image acquisition, colored pixels visible in either the internal reference (located at the top of the image) or the fundus indicate over-saturation and thus loss of signal.

4. Image Acquisition

Note: CRITICAL STEP: The goal in image acquisition should be to obtain 2 high quality 9-frame image stacks per session to control for variability between images within a session. After repositioning the patient and camera, obtain a second session of two images to assess and control for variability. All images will ultimately be calibrated to the internal reference (described below).

1. Acquire images only after at least 20 sec of "bleaching" (full exposure of retina to AF light) to minimize absorption of light by rhodopsin in the sensory retina [9].
    1. Use this period to optimize camera alignment, focus and sensitivity.
2. Have the subjects blink before each image acquisition as a fresh tear film improves signal quality.
3. Avoid eyelids in the plane of acquisition.

Note: It may be helpful to manually open eyelids for more challenging patients. The authors recommend that an assistant performs this task.

4. Optimize alignment before each image acquisition to ensure that light is not obstructed by the iris resulting in decreased signal.
   Note: As at least minor movement is very common, the authors recommend fine realignment as necessary before each image acquisition. Guiding and encouraging the patient while imaging helps decrease movement. The authors also recommend using the machine's foot-pedal as the "acquisition button" so as to minimize operator distraction.
5. Perform post-image processing by computing the "mean" of the 9-frame stack to increase signal-to-noise ratio. Calculate the mean of a stack using the cSLO software by selecting the option to calculate the mean.
   Note: There may be stacks where some frames are not of optimal quality (*i.e.*, have localized or generalized decreased signal relative to the optimal frames), therefore carefully inspect each stack and deselect suboptimal frames before computation of the mean.
   Note: Mean images with acceptable signal-to-noise ratios can be processed from at least 3 frames. It is normal for the sides and corners of each image to have lower signal than the central 20 degrees due to limitations within the cSLO itself.
   1. If the software asks whether the operator would like to normalize the gray levels (*i.e.*, stretch the histogram) between 0 and 255, select "No". This maintains unaltered gray levels for analysis.

5. Image Analysis

1. Analyze AF images as previously described using image analysis software developed for the qAF technique (IGOR; [9]). A brief description of a typical image analysis using this software is included below.
   1. Load the qAF program in IGOR and import images (in .bmp format) exported from the cSLO software with pixel aspect ratio 768 x 768 (default output parameter).
   2. Select the image in the drop-down menu and initiate analysis.
   3. Reposition calibration indicators on the image such that the "crosshairs" overlie the fovea and the "bracket" abuts the optic disc (these indicators are used to scale and position the ROIs). When properly positioned, use software prompts then populate the image with circumferential ROIs surrounding the fovea (see **Figures 1, 2**).
   4. Select the option in IGOR to calibrate the overall gray levels of each image to those levels present in the internal fluorescent reference.
      Note: This step allows for calibration of machine-related factors, including the electronic zero level of each image and the internal reference calibration factor of each machine, as well as patient-related factors, including age, refraction and corneal curvature.
      Note: The analysis software then displays the fixed regions on the resultant image and a qAF value is demonstrated within each region. qAF values are also automatically entered into a spreadsheet format in a separate window."
   5. Generate qAF "heat maps" by selecting the option within the command prompts. All images and data can be exported from the IGOR software in an excel spreadsheet by selecting the appropriate options.

Representative Results

This technique was used to study qAF in both healthy [13] and disease states [10-12]. In healthy eyes (**Figure 1**), AF emitted from the RPE is distributed relatively uniformly throughout the fundus (**Figure 1A**). Reduced intensity is seen in the central macular region due to blocking of light by macular pigment, and at the sides and corners of the image due to the optics of the eye and camera. Vessels appear dark and should be in clear focus in well-acquired images. **Figure 1B** demonstrates a corresponding heat map representation of the qAF levels from **Figure 1A**. Cooler colors correspond to areas of lower intensity while warmer colors correspond to areas of higher intensity. Maximum intensity is generally seen in the second concentric 8-segment ring (indicated in **Figure 1B**). This region is also less subject to imaging related variability than regions closer to the image borders, and is outside of the central region where macula pigment has a large impact on qAF levels. Thus, the mean intensities of this ring are used for most data analyses [13]. **Figure 2** presents a representative analysis in an eye with AMD demonstrating geographic atrophy (GA), an advanced form of AMD. This form of AMD results in localized areas of RPE loss, evidenced by markedly reduced or absent AF, and causes progressive central vision loss.

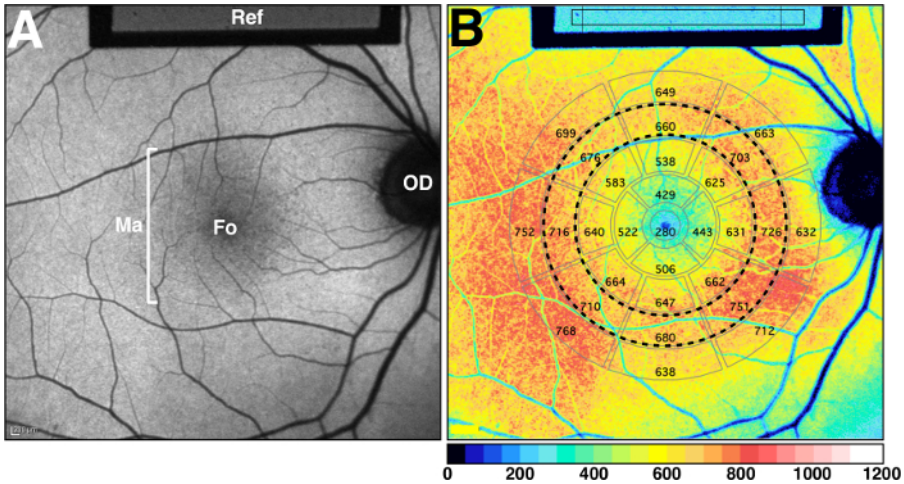

Figure 1. Autofluorescence in healthy eye. (**A**) Autofluorescence (AF) image of the right eye of a normal patient. OD: optic disk, Fo: fovea, Ma: macula, Ref: internal reference. (**B**) Post-processing qAF map of AF image from (**A**). Warmer colors correlate to higher AF intensity. Fixed regions are shown, and qAF values of each region are indicated. 8-segment perifoveal ring used in data analysis is indicated by the dashed line. Please click here to view a larger version of this figure.

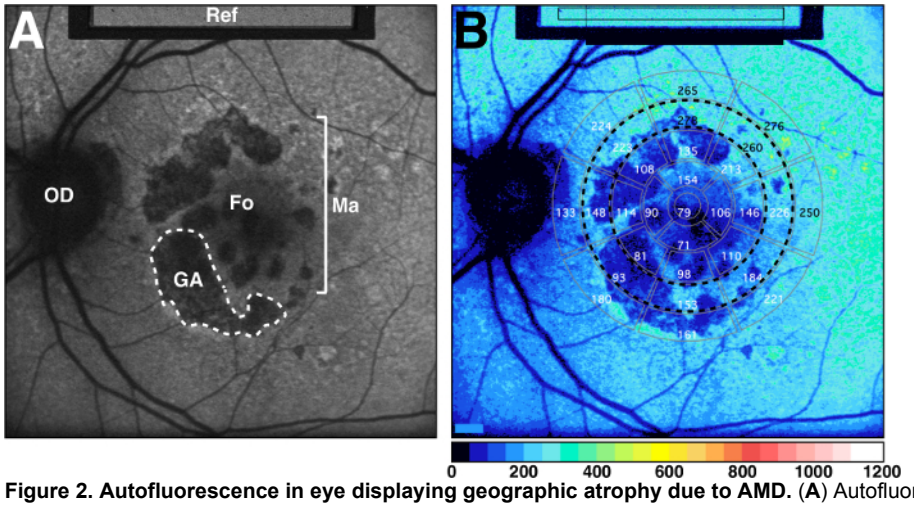

Figure 2. Autofluorescence in eye displaying geographic atrophy due to AMD. (**A**) Autofluorescence (AF) image of the left eye of a patient with advanced AMD showing geographic atrophy (GA) of the RPE (representative region circumscribed by dashed line). OD: optic disk, Fo: fovea, Ma: macula, Ref: internal reference. Note markedly reduced and absent AF in regions corresponding to GA in the macula. (**B**) Post-processing qAF map of AF image from (**A**). Fixed regions are shown, and qAF values of each region are indicated. 8-segment perifoveal ring used in data analysis is indicated by the dashed line. Please click here to view a larger version of this figure.

Discussion

Abnormal RPE lipofuscin distribution, whether increased or decreased, is a sensitive marker of retinal disease and is generally associated with loss of sensory retina function. Here, we describe application of qAF for the evaluation of RPE lipofuscin. Incorporation of an internal fluorescent reference to correct for variable laser power and detector sensitivity[9] alongside our standardized imaging technique allows for reliable quantitation of AF levels. It is our goal that this method will aid in the diagnosis and monitoring of retinal disease, and eventually in assessing the efficacy of therapeutic interventions, such as drug or gene therapy. qAF may also assist in stratification of individuals at-risk for conditions such as AMD.

We have generated a large normative database of qAF data to be used as a reference tool for the interpretation of retinal pathology [13], and have also described qAF in several disease states, including Stargardt disease [10], bull's eye maculopathy [12] and Best disease [11]. In healthy retina, there are different qAF levels between ethnic groups, with significantly higher qAF in whites than blacks and Asians, and demonstrated that women have higher qAF levels than men. Perhaps most strikingly, qAF increases as patients age, which corresponds with RPE lipofuscin levels, as previously measured by spectrofluorometry [14]. Although current normative data only extend up to age 60, it appears that patients have a measured decrease in RPE lipofuscin after age 70 [14]. Interestingly, it does not appear that changes in RPE cell number occur as patients age [2]and so the observed decreases in AF may be due to a redistribution or reduction in RPE lipofuscin [3]. It will be interesting to determine whether these decreases in AF in old age correlate with impaired RPE function and increased risk of AMD.

This protocol allows for reliable quantitative results that can be compared longitudinally, between patients, and between cSLO machines. One limitation is that qAF cannot accurately measure AF in older patients with lens opacities, which limits the technique in a subset of this high-risk population. One possible solution is to use a reflectometry method to individually estimate light losses in the media. Fortunately, older patients

who are pseudophakic with clear posterior capsules and known intraocular lens transmission characteristics are good candidates for qAF. However, the pupils of elderly patients and those with prior cataract surgery often do not dilate to more than 6mm, which limits light passage and prevents accurate data collection. In order to image such patients, an aperture may be inserted into the cSLO by the manufacturer in order to decrease the diameter of the laser. This modification has been successfully utilized to perform qAF imaging in mice [15]. Another limitation is that the technique is challenging to perform and is highly operator dependent. It is essential to consistently obtain high quality images to ensure accurate measurements of AF levels. In order to achieve this, the authors recommend strict adherence to the imaging protocol as well as adequate practice. Although some patients poorly tolerate the bright flashes of light required for AF imaging, assuring patients that exposure levels are well within the safe limits is helpful [1]. To allow for optimal imaging, it is critical to dilate pupils to at least 6 mm and to obtain multiple images with unobstructed light transmission (see above). Achieving optimal focus and avoiding oversaturation of pixels are also essential. Helpful practices include communication with the patient while imaging, use of an assistant if the eyelid needs to be lifted and use the foot pedal for triggering the camera, as described above.

In summary, understanding the pathophysiology of the RPE in retinal degenerations remains an area of active research, and of potentially important therapeutic impact. As qAF allows for direct comparison of AF levels in images obtained longitudinally, between patients and between centers, it is a valuable tool that can contribute to this understanding as well as provide useful clinical information. The detailed protocol outlined here will assist others in the acquisition of reliable qAF data, and that the important clinical applications of qAF will encourage research centers and clinical retina specialists to make use of the qAF technique.

Disclosures

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