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Exploring photoreceptor reflectivity via multimodal imaging of outer retinal tubulation in advanced age-related macular degeneration

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Abstract

Purpose—To investigate the microscopic structure of outer retinal tubulation (ORT) and optical properties of cone photoreceptors in vivo, we studied ORT appearance by multimodal imaging, including spectral domain optical coherence tomography (SD-OCT) and adaptive optics scanning laser ophthalmoscopy (AOSLO).

Methods—Four eyes of 4 subjects with advanced AMD underwent color fundus photography, infrared reflectance imaging, SD-OCT, and AOSLO with a high-resolution research instrument. ORT was identified in closely spaced (11 μ m) SD-OCT volume scans.

Results—ORT in cross-sectional and en face SD-OCT was a hyporeflective area representing a lumen surrounded by a hyperreflective border consisting of cone photoreceptor mitochondria and external limiting membrane, per previous histology. In contrast, ORT by AOSLO was a hyporeflective structure of the same shape as in en face SD-OCT but lacking visualizable cone photoreceptors.

Conclusion—Lack of ORT cone reflectivity by AOSLO indicates that cones have lost their normal directionality and waveguiding property due to loss of outer segments and subsequent retinal remodeling. Reflective ORT cones by SD-OCT, in contrast, may depend partly on mitochondria as light scatterers within inner segments of these degenerating cells, a phenomenon enhanced by coherent imaging. Multimodal imaging of ORT provides insight into cone degeneration and reflectivity sources in OCT.

Keywords

Age-related macular degeneration. cones; ellipsoid; Müller cells; outer retinal tubulation; spectral domain optical coherence tomography; photoreceptors

Introduction

Advances in high-resolution imaging such as spectral-domain optical coherence tomography (SD-OCT) and adaptive optics (AO)-assisted imaging¹ are revealing new insights into photoreceptor optics and cone degeneration in living human retinas with age-related macular degeneration (AMD) and other retinal disorders.

SD-OCT reveals four hyperreflective bands in healthy outer retina. These have been termed External Limiting Membrane (ELM), Ellipsoid Zone (EZ), Interdigitation Zone (IZ), and Retinal Pigment Epithelium (RPE)/Bruch's Complex.² These bands are visible due to the precise vertical compartmentalization and horizontal alignment of outer retinal cells and tissues (Müller cells, photoreceptors, RPE, and Bruch's membrane).^{3,4} SD-OCT terminology remains under active discussion, principally because AO-assisted OCT (AO-OCT) imaging shows thin reflective planes attributed to interfaces between cone inner segments (IS) and outer segments (OS).⁵⁻⁷ This appearance seemingly supports the name for this band (IS/OS junction) originally assigned during earlier OCT development.⁸⁻¹¹

For high-resolution imaging to achieve maximal utility in clarifying disease progression, it is important to understand how reflectivity from subcellular sources is detected by various imaging modalities. Two important physical principles contributing to tissue reflectivity in OCT are scattering and waveguiding. For biological structures larger than the wavelength of light, Mie scattering is independent of wavelength, and it is anisotropic in the forward direction.¹² In cultured cells, mitochondria and lysosomes are strong light scatterers consistent with Mie theory.¹³⁻¹⁵ In OCT, the backscatter interferes with light from a reference path and forms bands of varying reflectivity that are displayed in a B-scan.¹⁶ For waveguiding, an optical fiber with a high index of refraction relative to the surrounding medium allows light to propagate by total internal reflection in patterns of energy distribution called modes.^{17,18} By AO-assisted en face imaging, waveguiding photoreceptors appear as hyperreflective spots in an array representing the cone mosaic and their modal waveguiding property.^{18,19} Due to the high refractive index and internal packing geometry of IS mitochondria, they are thought to contribute to both scattering and waveguiding.²⁰ IS volume is 75% occupied by tightly packed bundles of highly elongated mitochondria, resembling dry spaghetti.²¹

The relative contributions of scattering and waveguiding in photoreceptors can be tested with outer retinal tubulation (ORT), a neurodegeneration and gliosis commonly found in macula of advanced AMD²²⁻²⁶ and some inherited retinal diseases.^{22,27-29} ORT contains degenerating long- and middle-wavelength-sensitive cones³⁰ pointing into a lumen encircled by the ELM, which is formed with Müller cells.^{26,31} ORT cones degenerate by OS loss, IS shrinkage, and mitochondrial fission and migration within the IS, leaving an ELM circle lacking photoreceptors at the end-stage.^{26,32} On SD-OCT B-scan, ORT is identified as a

hyperreflective circular or ovoid profile with a hyporeflective lumen, located in the outer nuclear layer.^{22,26} Persisting mitochondria in degenerating cones of ORT can scatter light even when cones are severely misaligned.^{26,31} A waveguiding effect seems unlikely when degenerating cones not only lack OS, but are also radially aligned, as they are around ORT lumens.²⁶

To investigate scattering and waveguiding of degenerating cone photoreceptors in vivo, we studied ORT by multimodal imaging, including AO scanning laser ophthalmoscopy (AOSLO) and SD-OCT. We predicted that ORT would be hyporeflective on AOSLO due to a paucity of waveguiding.

Methods

The study followed the Tenets of the Declaration of Helsinki and was approved by the Institutional Review Board at the University of Alabama at Birmingham (UAB). The study complied with the Health Insurance Portability and Accountability Act of 1996. Written informed consent was obtained from participants after the nature and possible consequences of the study were explained.

Patients

Study patients were recruited from the Callahan Eye Hospital Clinic, of the UAB Department of Ophthalmology, and Retina Consultants of Alabama if they met the inclusion criteria for this study. Eligible patients were at least 60 years of age and had been previously diagnosed with AMD, had one of three ICD-9 codes for AMD (365.50, 365.51, 365.52) listed in their charts (n = 2,885), and had best-corrected visual acuity (BCVA) of 20/50 or better in either the study or non-study eye as recorded in the chart. A total of 210 patients with clinical SD-OCT scans and ORT was identified. Therefore, the prevalence of ORT associated with AMD in this patient population was 7.3%. Patients were excluded if they had cataract or other media opacities, retinal disease besides AMD, previous posterior eye surgeries and laser treatments in the study eye, or diabetes mellitus. The remaining 59 patients not excluded for the aforementioned reasons were considered for enrollment.

Eight patients agreed to participate and underwent BCVA measurement by the Electronic Visual Acuity protocol³³ on the day of imaging. Pupils were dilated with 1.0% tropicamide and 2.5% phenylephrine hydrochloride before imaging sessions. Color fundus photographs, infrared reflectance (IR) imaging, and SD-OCT (Spectralis, Heidelberg Engineering, Carlsbad, CA) high-density volume scans in the location of the ORT were acquired with 11 μ m spacing between B-scans. Incident light was nominally perpendicular to Bruch's membrane, and B-scans were an average of 15 to 19 images. En face images for each SD-OCT volume were generated using the 3D View Transverse option in the Spectralis software (Version 1.9.10.0), with a transverse plane selected to maximize the view of the ORT network in each SD-OCT volume. En face images were overlaid on IR images in the Heidelberg Spectralis software. Directional OCT³⁴ was considered for this study, but due to the lengthy imaging protocol, acquisition of interpretable directional OCT volumes was not possible within reasonable time limits.

High-resolution AOSLO image acquisition

After SD-OCT imaging, six subjects were invited to participate in AOSLO imaging. The AOSLO is a new generation research system developed specifically to accommodate imperfections of imaging eyes of older people with AMD.³⁵ It is equipped with a custom high-speed Shack-Hartmann wavefront sensor and a high-speed deformable mirror (Hi-Speed DM97-15, ALPAO SAS, France) with 97 actuators with stroke up to 30 μm that provide improved correction ability for wavefront distortion. It uses a low coherence light source (Broadlighter S840-HP, Superlum, Russia) to mitigate light interference artifact thereby producing high fidelity retinal images.³⁶ The AOSLO acquires the retinal images over a field of view about 1.2° inside the eye at a frame rate of 15 Hz. The subject's head was aligned and stabilized using a head-mount with a chin-rest. A fixation target consisting of a bright green light dot moving on a calibrated grid that was placed in front of the eye via a pellicle beam splitter to help the subject's fixation. The subject saw the dot on the grid paper through the pellicle beam splitter. Subjects who could not use the study eye to fixate on the target were asked to use the other eye for this purpose. To ensure that the ORT can be differentiated from other structures such as retinal and choroidal blood vessels, we acquired large field images of the retina around the ORT identified by SD-OCT. AOSLO videos were recorded across an area of $15^\circ \times 15^\circ$ to $20^\circ \times 20^\circ$. An AOSLO imaging session lasted approximately 1 hour. Before recording images, the gain of the AOSLO photo-detector was tested to obtain proper image brightness and contrast according to a real-time histogram of the retinal video and remained constant through the whole imaging session. Data presented were collected from one imaging session for each subject.

AOSLO image processing and analysis

Image distortions caused by nonlinearities in the resonant scanner and by eye movements were eliminated by customized software.³⁷ Registered images were averaged to enhance signal-to-noise ratio. Images of different retinal locations were manually aligned using punctate reflectivity (i.e. cones in normal tissue, other features in atrophic tissue) to create a continuous montage (Photoshop, Adobe Systems Inc., Mountain View, CA). AOSLO image pixel size was computed from an image of a precisely calibrated dot grid placed at the retinal plane of a model eye. Montaged AOSLO, IR, en face SD-OCT, and color fundus images were registered manually by aligning retinal vessels and capillaries. Brightness of some images was adjusted for illustrative clarity in the figures.

Results

Four eyes of 4 subjects with clinically diagnosed AMD and macular ORT were successfully imaged by AOSLO and included in this study. Of the original six subjects, AOSLO images from one subject with peripapillary ORT and one subject with a small area of capsule opacity could not be acquired. Study eyes included the right eye of an 82-year-old man with geographic atrophy and BCVA 20/100 (Figure 1); the right eye of a 66-year-old woman with geographic atrophy and BCVA 20/70 (Figure 2); the right eye of a 77-year-old man with neovascular AMD and BCVA 20/640 that was 20/200 three years earlier (Figures 3 and 4, two time points); and the right eye of an 83-year-old woman with geographic atrophy and BCVA 20/25 (not shown).

In each of the study eyes, ORT was identified by cross-sectional SD-OCT (Figures 1–4, panels D of each figure). Because the reflective band completely encircled the lumen in these cases, all the ORT shown in the figures are considered closed, and each contain some internal punctate reflectivity.²⁶ ORT were considered perilesional²⁴ in eyes with geographic atrophy (located along the border of atrophy, Figures 1 and 2), and considered pseudodendritic²⁴ in eyes with neovascular AMD (stretching across a scarred area, Figures 3 and 4). Accordingly, en face OCT revealed an ORT with a hyperreflective band surrounding a weakly reflective lumen in branching shape (Figure 1–4, panels C, G of each figure) that was sometimes complex. In contrast, AOSLO revealed that these ORT are hyporeflective (Figure 1–4, E) while also showing that cones elsewhere in the macula are normally reflective (Figure 1–4, insert to panels E of each figure). The hyporeflectivity of the ORT is below the level of normal variability in cone reflectivity seen in AOSLO. By IR, ORT appears hyporeflective (Figure 1–4, panels F of each figure).

Discussion

Multiple subjects with ORT associated with advanced AMD underwent multimodal imaging, including SD-OCT and AOSLO, to investigate ORT structure and optical properties. AOSLO is a challenge in older people due to factors mentioned in the Methods, which limited our sample. However, ORT could be studied in younger individuals with choroideremia,²⁷ who would likely have preserved fixation and clear ocular media, using other technologies like directional OCT. Because we checked all ORT by each modality while referring to comprehensive retinal histology (<http://projectmacula>), we confirmed the characteristic reflective band and outer nuclear layer location of each ORT, and we ruled out other sinuous structures such as vasculature as signal sources.³⁸ The axial resolution of our AOSLO is 70 μm yet we are confident that ORT hyporeflectivity is due to true optical effects and not due to inability to focus on the correct plane (insets of figures). By cross-sectional and en face SD-OCT imaging, the ORT border appears hyperreflective, which we previously attributed to a combination of IS mitochondria and ELM.^{26,31} By AOSLO, ORT in areas of GA and around active neovascularization appears hyporeflective. The within-eye comparison of intact cones and degenerate ORT cones provided an excellent system for observing waveguiding and scattering by multimodal imaging to elucidate mechanisms of reflectivity.

Our data represent another example of how the IS ellipsoid is differentially revealed by AOSLO and SD-OCT imaging. By AOSLO, cone reflectivity is reduced over drusen and at the edge of geographic atrophy even when the EZ is visible by SD-OCT.³⁹ In stage 1 and 2 subretinal drusenoid deposits in AMD, photoreceptor reflectivity by AOSLO is reduced, yet by SD-OCT, the EZ is reflective.³⁵ In retinas with macular telangiectasia type 2 or with hydroxychloroquine retinopathy, the EZ is visible by SD-OCT despite the absence of a normal cone mosaic with AO imaging.⁴⁰ One study using AOSLO to image a family with a mutation of mitochondrial DNA found widely spaced and abnormally waveguiding cones, suggesting that mitochondrial health contributes to cone optics.⁴¹ Taken together, these data suggest that the relative contribution to reflectivity of scattering and waveguiding depends on the health of cones as well as the imaging method. To this prior work, our data add value

by interpreting these effects using insights into cone degeneration provided by recent histology of human AMD macula.^{26,31,32}

The high index of refraction of photoreceptors relative to their surrounding interphotoreceptor matrix allows light to propagate in patterns known as waveguide modes.^{17,18} These properties allow photoreceptors to be imaged at high contrast using AO-assisted imaging techniques.^{19,42} An AO system minimizes aberrations due to the optics of the eye by incorporating a wavefront sensor and deformable mirror. These components allow light emitting from waveguiding photoreceptors via the optical Stiles-Crawford effect to be collected efficiently.⁴³ Waveguiding photoreceptors appearing as hyperreflective spots in an array are thought to require OS integrity and contact with the retinal pigment epithelium apical processes.^{40,43,44} The number of modes in one fiber depend on the wavelength of light and the diameter of the fiber. Thus, a cone photoreceptor from the perifoveal retina, where cross-sectional IS diameters are large (7 μm),⁴⁴ may have multiples modes.¹⁸ Non-waveguiding photoreceptors appearing hyporefective can be cones lacking OS or normal cones with normal sensitivity⁴⁵ and with diurnal cellular dynamics.^{46,47}

In Figure 5, we hypothesize that photoreceptor reflectivity is dominated by contributions resulting from waveguiding in healthy cones with OS and scattering in degenerating cones without OS. In a normal eye (Figure 5A and B), photoreceptors are oriented toward the pupil center^{48,49} and IS contain bundles of mitochondria each ~ 20 μm long.³² AOSLO illuminates cones by focusing light on the ELM and collects reflected light by a double pass through a large pupil (~ 6 mm) (Figure 5A). Because of this large pupil, the cone numerical aperture is narrower than both the Stiles-Crawford effect⁵⁰ and the AOSLO numerical aperture formed by the dilated eye. Thus, for healthy cones giving a strong directional reflection (also called a guided component),⁵⁰ all the reflected light is collected, and AOSLO renders a clear image of the IS mosaic (Figure 5B). Degenerating cones with remnant or absent OS do not waveguide but still have reflective organelles. In IS, prominent mitochondrial fission results in many small and dispersed organelles (Figure 5C).³¹ These form a diffuse reflector, scattering incident light over a wide angle. Only light within the AOSLO numerical aperture (black angle, Figure 5) is collected, resulting in low reflectance (Figure 5C). This may explain why ORT structure is hyporefective on AOSLO even with excellent lateral resolution at 2.8 μm . An OCT image is formed by interference of light collected from the retina and a reference light. Reflective structures are enhanced by the coherent nature of OCT. Both cone plasma membrane and mitochondria possibly contribute to reflectivity. Using morphometric data from our prior publication,³² we can estimate that ORT cone IS contain, on average, 30% more mitochondria by surface area than cone plasma membrane; therefore, ORT appear hyperreflective (Figure 5D). Independently, we showed that the reflectivity of a Closed ORT does not vary in cross-section.⁵¹ The reflectivity of other ORT shapes in cross-section did not reveal a consistent trend.⁵¹ However, if this explanation is correct, why are not all degenerating cones as hyperreflective on SD-OCT as ORT cones? Perhaps in cones that are only slightly deflected (e.g., over small subretinal drusenoid deposits³⁵), IS mitochondria are still relatively long and fascicular and thus increased scattering due to fission is not yet apparent. Evidently, mitochondria degeneration may represent a critical stage at which cones lose their waveguiding property. Thus, distinct

phases of mitochondrial dysfunction, a process central to neurodegeneration, may be visible in photoreceptors through multimodal retinal imaging.

SD-OCT nomenclature, testable in both human subjects⁵² and animal models,⁵³ has implications toward future studies using imaging to monitor molecular pathways and retinal diseases. Our data support the Ellipsoid Zone nomenclature proposed by an international consortium.² By SD-OCT, surviving cones, although misaligned around ORT lumens, provide a reflective signal from mitochondria and the ELM even though lacking OS or an IS/OS junction.^{31,32} The hyperreflective ORT border bears a high similarity to the EZ band seen in unaffected areas of the same eye, implying that this band is not necessarily an anatomical IS/OS junction. A refractive index boundary and/or change of the waveguiding properties between IS and OS of both cones and rods⁵⁴ does not seem likely in the absence of OS. However, the ORT lumen likely contains fluid with a low refractive index. This hypothesis is supported by our data that cones in ORT do not waveguide. AO-assisted imaging may detect less light scattered from the retina, making waveguided light less visible, yet ORT is still visible by AO-OCT.⁶ Although dependent on the imaging modality, EZ reflectivity mechanisms also include tapering of the IS ellipsoid, which is not present in stubby degenerating cones, in addition to mitochondria-mediated waveguiding and scattering.^{18,55}

We are at a new beginning of understanding photoreceptor optics, thanks to cellular level retinal imaging that is being validated through the strong independent variable of pathology.^{26,31,32,56–59} The structurally and molecularly unique IS mitochondria allow for in vivo monitoring of photoreceptor health.^{60–62} Future studies of ORT and photoreceptor optics should also use directional OCT, keeping in mind that these images may be difficult to interpret in eyes with neovascularization, as retina topography is deformed by scar. Different imaging systems incorporating SD-OCT and AO may be tapping into different parts of a larger truth about photoreceptor optics, as revealed by ORT. The competing nomenclatures of EZ versus IS/OS junction rely on results from SD-OCT and AO-assisted retinal imaging, respectively, and therefore it is important to understand how these two technologies detect scattered light versus waveguiding. These differential properties will enable clinicians to follow more detailed stages of photoreceptor degeneration in living patients.

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Summary statement

In age-related macular degeneration, outer retinal tubulation appears hyperreflective by spectral domain optical coherence tomography due to cone photoreceptor inner segments that contain light scattering mitochondria and hyporreflective by adaptive optics scanning laser ophthalmoscopy due to cones that lack outer segments and have lost their normal directionality and waveguiding property.

Figure 1.

Outer retinal tubulation (ORT) in geographic atrophy secondary to age-related macular degeneration (AMD) by multimodal imaging. This ORT is closed²⁶ and perilesional.²⁴ **A.** AOSLO montage in grayscale overlaid on color fundus photograph of right eye from an 82-year-old man (AMD-033) diagnosed with AMD. **B.** Infrared reflectance (IR) image. **C.** En face view from SD-OCT transverse reconstruction of high density B-scans through ORT overlaid on IR image. Higher magnification of ORT in blue boxes and waveguiding cones in yellow box are in E–G. **D.** SD-OCT B-scan taken through green line in A–B shows location of ORT (cyan arrowhead). Higher magnification of ORT and waveguiding cones in yellow box by AOSLO (**E**), IR (**F**), and En face (**G**). Cyan arrowheads in E–G point to level of SD-OCT B-scan in D. Side of yellow box inset is 200 μm .

Figure 2.

Outer retinal tubulation (ORT) in geographic atrophy secondary to age-related macular degeneration by multimodal imaging. This ORT is closed²⁶ and perilesional.²⁴ **A.** AOSLO montage in grayscale overlaid on color fundus photograph of right eye from a 66-year-old woman (ORT-134) diagnosed with AMD. **B.** Infrared reflectance (IR) image. **C.** En face view from SD-OCT transverse reconstruction of high density B-scans through center of ORT overlaid on IR image. Higher magnification of ORT in blue boxes and waveguiding cones in yellow box are in E–G. **D.** SD-OCT B-scan taken through green line in A–B shows location of ORT (cyan arrowhead). Higher magnification of ORT and waveguiding cones in yellow box by AOSLO (**E**), IR (**F**), and En face (**G**). Cyan arrowheads in E–G point to level of SD-OCT B-scan in D. Side of yellow box inset is 200 μm .

Figure 3.

Outer retinal tubulation (ORT) in neovascular age-related macular degeneration by multimodal imaging. This ORT is closed²⁶ and pseudodendritic.²⁴ **A.** AOSLO montage in grayscale overlaid on color fundus photograph of right eye from a 77-year-old man (AMD-ORT-041) diagnosed with AMD. **B.** Infrared reflectance (IR) image. **C.** En face view from SD-OCT transverse reconstruction of high density B-scans through center of ORT overlaid on IR image. Higher magnification of ORT in blue boxes and waveguiding cones in yellow box are in E–G. **D.** SD-OCT B-scan taken through green line in A–B panels shows location of ORT (cyan arrowhead). Higher magnification of ORT and waveguiding cones in yellow box by AOSLO (**E**), IR (**F**), En face (**G**). Cyan arrowheads follow ORT E–G. Side of yellow box inset is 200 μm .

Figure 4.

Outer retinal tubulation (ORT) in neovascular age-related macular degeneration by multimodal imaging. This ORT is closed²⁶ and pseudodendritic.²⁴ **A.** AOSLO montage in grayscale overlaid on color fundus photograph of right eye from a 74-year-old man (AMD-ORT-041, same subject as in Figure 3, three years before) diagnosed with AMD. **B.** Infrared reflectance (IR) image. **C.** En face view from SD-OCT transverse reconstruction of high density B-scans through center of ORT overlaid on IR image. Higher magnification of ORT in blue boxes and waveguiding cones in yellow box are in E–G. **D.** SD-OCT B-scan taken through green line in A–B panels shows location of an asymmetric ORT in cross-section (cyan arrowhead). Higher magnification of ORT and waveguiding cones near retinal vessel (shadow) in yellow box by AOSLO (**E**), IR (**F**), En face (**G**). Cyan arrowheads follow ORT in E–G. Pink arrowheads point to same cyst in all images. Side of yellow box inset is 200 μm.

Figure 5.

Multimodal imaging of normal and degenerating cones. **A.** In a normal eye, photoreceptors are oriented toward the pupil center, and inner segments (IS) contain bundles of long mitochondria. AOSLO illuminates cones by focusing light (as indicated by the red triangle) on the ELM and collects the reflected light, by a double pass through a large pupil. Due to waveguiding, light coupled inside the cone (within the cone's numerical aperture) traverses the outer segment and is scattered near the tip of the outer segment. Part of the scattered light is coupled back inside the outer segment and is guided back toward the pupil when it emerges from the photoreceptor IS. This part of the light is collected by either AOSLO or OCT. **B.** Because of this large pupil, the cone numerical aperture is narrower (red triangle) than AOSLO numerical aperture (angle between black lines). Reflected light is collected, and AOSLO renders a clear IS mosaic. **C.** Degenerating cones with remnant or absent OS do not waveguide but still have reflective organelles. These form a diffuse reflector, scattering incident light over a wide angle (red arrows). Only light within the AOSLO numerical aperture (angle between black lines) is collected, resulting in low reflectance. RPE is present but also degenerating. **D.** Because ORT cone IS contain many dispersed mitochondria, which reflect light (red arrows), OCT renders ORT with a hyperreflective border. Dotted circle represents ELM of ORT with the number of cones reduced for clarity. RPE is absent.

Cells: RPE, retinal pigment epithelium; C, cone photoreceptor (IS, inner segments; OS, outer segments); R, rod photoreceptor; H, horizontal cell; B, bipolar cell; M, Müller cell; Am, amacrine cell; DA, displaced amacrine cell; G, ganglion cell. Layers: ChC, choriocapillaris; BrM, Bruch's membrane; ELM, external limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer; ILM, inner limiting membrane. Drawings are not to scale.