Visualization and quantification of an active ingredient in hair fibers: an example with the penetration of Coconut oil

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Abstract

Background : Lipids play a major role in the structural integrity of the hair. The penetration of vegetable oils in the hair fiber is still the subject of studies because hair remains a complex organic tissue. This investigation aims to implement two methods to highlight the localization and the quantification of coconut oil in the hair fiber

Methods : Coconut oil applied to hair strands was detected on cryo-sections with the Nile red dye and visualized with Confocal Laser Scanning Microscope. The triglyceride concentration of coconut oil was determined after solvent extractions and after separation with High Performance Thin Layer Chromatography

Results : Our results showed that high-resolution lipid imaging with a light microscope can be obtained in hair tissue. This method allowed the detection of the penetration of exogenous lipids. The combination of selective solvent extraction with cryogenic grinding followed by HPTLC made it possible to confirm this penetration and to quantify the triglycerides of coconut oil.

Conclusion: Our work has proven that conventional methods can be optimized to show the penetration and quantification of coconut oil in hair fibers.

Key words: Hair, Lipids, High Performance Thin Layer Chromatography (HPTLC), Confocal microscopy

Introduction

Hair shaft is a complex organic tissue made up of nanoscopic elements, it is also described as “the most sophisticated biological composite material” [1]. Hair fibers contain three distinct parts that are distinguished in cross-section: the cuticle, the cortex, and the medulla. The cortical and cuticular cells are highly organized, keratinized and intimately intertwined. A Cell Membrane Complex (CMC) rich in proteins and lipids keeps all cells linked together. Although the hair shaft contains a low content of lipids, these play a major role in the structural integrity of the hair. Hair lipids are mainly made up of a mixture of cholesterol sulphate, ceramides, cholesterol, free fatty acids and cholesterol esters [2]. Amongst these lipids, particular attention

is paid to 18-methyleicosanoic acid (18-MEA) present at the surface of the cuticle. External and internal lipids provide a protective barrier that can be damaged or lost due to environmental and chemical factors [3] [4] .

To restore and protect the hair, lipids extracted from plants are very often incorporated into hair products. For instance, we can mention coconut oil. This oil is commonly used due to the presence of triglycerides made up of medium-chain fatty acids (mainly lauric acid). The size of this molecule is believed to improve the diffusion of the molecule into the hair, making it an excellent candidate for claiming deep nutrition.

The penetration of coconut oil [4] and its quantification [5] in the hair shaft were already shown. Penetration was evidenced by secondary ion mass spectrometry-SIMS while quantification was performed using tritium labeling. Nevertheless, SIMS has some limitations regarding the fine localization of coconut oil and radiolabeling techniques must be implemented in a specific and constraining environment. The optimization of methods to better define the localization of lipids, to determine the quantity likely to penetrate the hair shaft remains essential to the development of hair care.

The localization of the lipids in hair was described with light microscope using fluorescent probes [6] [7] [8]. Amongst these articles, there is great diversity of image quality. The resolution is often not sufficient to appreciate the exact localization of the lipids. This variability in resolution can be attributed to the nature of this keratinized tissue [9] [10]. Although high-resolution images of hair are not at first sight associated with light microscopy, a few articles show that it is possible to achieve it [11] [12] [13] . High resolution hair imaging is needed to clearly determine where the endogenous lipids are. This high resolution is more than ever important for visualizing the supply of exogenous lipids. Specimen preparation, sectioning process, mounting medium, and choice, as the handling of the microscope itself can contribute to better images.

The same goes for the extraction and separation of lipids. Given the nature of the hair tissue, the extraction of all the lipid remains difficult. The optimal extraction is always sought, and new protocols are very often proposed [14].

The aim of this study is to optimize two conventional methods to study lipids in hair tissue and to demonstrate the penetration of coconut oil. Particular attention was first paid to the preparation of the samples. Then, the use of Nile red dye as a lipid probe with a confocal microscope was carried out to improve the localization of endogenous and then exogenous lipids later. In a second step, in order to identify and quantify the presence of coconut oil into hair despite the presence of endogen lipids, we chose to work with a cryogenic grinding device to have better access to the material, associated to extraction solvents. Lipid separation and quantification were performed by High Performance Thin Layer Chromatography (HPTLC) with a multi-migration system.

Our results showed that high resolution lipid imaging with a light microscope can be achieved in hair tissue. This method allows to detect the penetration of exogenous lipids. The use of selective solvent extraction associated with cryo-grinding followed by HPTLC enabled us to confirm and quantify the presence of triglycerides allocated to the coconut oil.

Materials and Methods

Hair samples

Natural black hair was obtained from two healthy volunteers. The volunteers had never subjected their hair to any chemical treatments. The length of the selected hair sample was 10 cm.

Coconut oil treatment

Initially, the hair fibers were washed with standard shampoo. 1 g coconut oil/g hair was applied into each strand with massage for 2 minutes to mimic consumer application routine. Combing was done to distribute the oil over the length. The oil was left on the hair for 24 hours then the hair strands were washed with a reference shampoo. A second and third application of coconut oil followed by washing with the reference shampoo were carried out with the same protocol. Products were applied on dry hair. Untreated and coconut oil treated hair come from the same volunteer. Localization and quantification of lipids were conducted for each condition.

Sample preparation & Microscopy investigation

A hair fiber bundle was held vertically in distilled water (mounting medium) on a cryostat aluminum stub. The preparation was positioned above liquid nitrogen. After freezing the water, the sample was transferred to a cryostatic chamber (-20°C). Thin sections 6 µm thick were carefully made to avoid artifacts due to razor blade. Sections were collected on glass slides.

A first series of tests was conducted on untreated hair to improve hair imaging. A first high resolution hair imaging with a light microscope was conducted to characterize the autofluorescence common to all hair types. The autofluorescence was revealed with 488 nm laser excitation. The second high resolution hair imaging was led after staining with Nile Red dye to characterize the endogenous lipids of the hair. Nile red is a well-known dye to reveal lipids [15]. A solution of 1mg/ml of Nile red in Dimethyl sulfoxide (DMSO), is diluted ½ in distilled water. Solution is filtrated before use. The sections were incubated in the staining solution for 10min at room temperature and then rinsed gently with distilled water. Sections were mounted between the glass slide and a coverslip in a glycerin solution. Hair fiber cross sections were imaged using a Confocal Laser Scanning Microscope Leica SP5 with a 1,4 NA 63X oil immersion objective. Nile red stain was excited with the 514 nm Laser line. Image was scanned at 200 Hz and averaged at least 4 times.

Transmission electron microscopic observation

The hair samples were prepared according to the method described by Ji *et al*;2013 [17]. The TEM image of the cuticle was used to illustrate the comparison between the confocal image produced in the same area.

HPTLC analysis :

Lipids Extraction

Untreated and coconut oil treated hair samples were first extracted with cyclohexane in a Soxhlet extractor, continuously for 2 hr [17]. This first extraction was completely dried in a Büchi rotary evaporator. Hair recovered from the Soxhlet was crushed using cryogenic grinding

with ball milling. The hair powders obtained were then successively extracted with chloroform/methanol 2:1, 1:1, 1:2 and then with methanol, for 2hr each, at room temperature [2]. The different extracts of chloroform/methanol were then combined, filtered, dried and weighed to constitute our second extract. Each extract was dissolved at a known concentration in its respective extraction solvent

Lipids Analysis

Glass-backed HPTLC plate Si60 F254 was used as the stationary phase to separate lipids extractions previously obtained. In order to separate and visualize the majority of the classes of lipids present in the hair on the same plate, a multi migration [18] was carried out in 7 steps with mixtures of increasing polarity with 5 solvents: dichloromethane, ethanol, acetone, heptane and ethyl acetate. After drying, the plate was immersed in a solution of primulin before being observed at 366 nm.

Quantification of coconut oil triglycerides

Coconut oil is essentially a triglyceride consisting of glycerol groups and medium chain fatty acids (MCFAs). This oil has a very high content of MCFAs and the predominant type is lauric acid [19]. HPTLC method was performed to semi-quantify the coconut oil penetration by following triglycerides of lauric acid. In order to quantify, our choice focuses on a single molecule the trilaurin (ref T2009000_Merck). Trilaurin is a triglyceride obtained by formal acylation of the three groups of glycerol by lauric acid. The thin layer was analyzed by fluorometric and densitometric method with Scanner CAMAGÓ system and Wincat'sÓ software. Different spots of different volumes of the same trilaurin solution were deposited to determine the concentration of triglycerides present in extractions 1 and 2 for the hair of the two volunteers.

Results & discussions

High-resolution hair imaging with a light microscope

It is known that natural hair exhibits an auto-fluorescence. Our first attention was to get a better image of hair autofluorescence to assess our optimized method. We can notice that this autofluorescence is present on the whole section (Fig. A). Spots of intense fluorescence are localized in the cortex and can be attributed to nuclear remnants. Cuticle cells can also be distinguished by an alternating fluorescent and non-fluorescent bands. Although the fluorescence bands in the cuticle appear**s** quite thick, the low intensity of the signal does not allow a better definition of this area of the hair. It should be noted also that around the medulla we can guess an area where the fluorescence is less intense.

The same operation was performed after Nile red staining to image endogenous lipids (Fig. B). The fluorescence dye is only emitted after interaction with lipids. Due to a high signal-to-noise ratio, we were able to achieve a very well-defined image. The high fluorescence of Nile red forces the intensity of the laser to be lowered. As a consequence, the natural autofluorescence of the hair cannot be detected. Lipids are present in each distinct region of the hair. The signal is fairly homogeneous and evenly distributed in all the medulla. In the cortex, the signal reveals

a pattern that highlights cortical cell boundaries. The signal reveals the CMC which contains lipids. The cuticle is also well labelled. The image of the cuticle is very similar to the image obtained with autofluorescence. The cuticle CMC must be labelled as well, however fluorescence bands are larger than the Cortex CMC. The image is particularly sharp which allows us to increase the magnification (Fig. C). The shape of the fluorescence bands shows variable thicknesses. The side facing the center of the hair has a flat surface. The opposite side, facing outward shows waves. Compared to a transmission electron microscopy image of the cuticle (Fig. F), which clearly shows the sub lamellar layers present inside each cell of the cuticle, one can notice a similar shape with the endocuticle (Fig. C). The attribution of this fluorescent band as the endocuticle is consistent with different articles. This variable thickness of the endocuticle has already been reported [20]. The endocuticle has been shown as a diffusion pathway for molecules [21]. The presence of lipids at this location has been reported by AFM coupled to IR [22] [23].
At this magnification, in the cortex, it is possible to fully appreciate the convoluted pattern of CMC reflecting the shapes of cortical cells and their interlocking with each other. Note the presence of lipids in the form of bright spots in each cortical cell. The use of light microscope makes it possible to apprehend the hair section in its entirety, unlike electron or near-field microscopy which only visualizes a small region.

Lipid imaging after coconut oil treatment

The Nile red lipids revelation was done after coconut oil treatment (Fig. D) and compared with untreated hair (Fig. E) from the same volunteer. Untreated hair displays the same lipids localization as previously described (Fig. B). After coconut oil treatment the signal appears brighter in all regions. This image suggests that penetration can go all the way to the core of the fiber. The alternance of fluorescence band in the cuticle is particularly well defined. Cortical CMC and the endocuticle sublayer show irregular thickness and different intensity of signal can be distinguished in some area. However, we cannot certify that this signal increase is related to the penetration of coconut oil.
To validate our hypothesis, we supplemented these observations with a chromatographic analysis capable, of distinguishing different classes of hair lipids, of analyzing the lipids specific to coconut oil, but also of semi-quantifying the penetration of the latter.

Triglyceride identification after coconut oil treatment

Different studies have proposed protocols to separate surface lipids from internal lipids [7] [17]. The lipids at the surface level of hair fibers were mainly extracted with solvent such as ether, hexane, while the internals lipids were subsequently extracted with solvents such as chloroform, methanol.
Based on this literature, we carried out a first extraction with cyclohexane and a second one with a chloroform/methanol mixture. Note, in order to have better access to internal lipids, cryogenic grinding of the hair was carried out after the first extraction.

Figure G, shows the results of HPTLC analysis of hair with and without coconut oil treatment and the difference between the first and second extraction. First, HPTLC analysis of untreated hair (fig. G f & g) showed two types of lipid profile between the first and second extraction.

This confirms that we have on one side an extraction of non-polar lipids such as cholesterol (Fig. G.a) while on the other side we have an extraction of more polar lipids such as ceramide (Fig. G b & c).
Extractions 1 and 2 of the hair treated with coconut oil (Fig. G h & I) show the same profiles as the untreated hair, but it can be noticed that fluorescence bands appear at the same retention factor (Rf) value level as the coconut oil extract (Fig. G e). One of them is much more intense and appears from the first extraction. This difference in intensity suggests that the coconut oil triglycerides penetrated mainly to the surface. However**,** these triglycerides seem to have penetrated more deeply since they are still detected after the second extraction.
We wanted to quantify the penetration of coconut oil. A specific standard had to be found that was as equivalent as possible to coconut triglycerides. The trilaurin was selected and the molecule migrated (Fig. G d) at the same Rf level as the coconut oil extract. We confirmed that coconut oil is composed mainly of triglycerides and we demonstrated that trilaurin is the correct reference to quantify the concentration of triglycerides. Based on the calibration curve of trilaurin, the determination of coconut oil triglycerides was performed. The triglyceride concentration results for each volunteer are shown in Table 1. Successive applications of coconut oil permit the penetration of coconut oil into the hair shaft. The triglyceride concentrations for both volunteers appear in the same proportion.

Lipid imaging after solvent extraction

Considering the nature of hair, the extraction of lipids is not easy, and the extraction procedure is always subject to discussion. Considering the lipid imaging obtained previously, we decided to image the lipids after the first extraction. Figure I shows the lipids remaining after extraction with cyclohexane. The staining over the entire section is much less intense than the same sample before extraction (fig. H). The loss of lipids is particularly visible in the CMC cortex. However, the staining is still present in the medulla and in the cuticle. The result suggests that the first extraction does not seem to just extract lipids from the surface. This result also suggests that coconut oil might penetrate deeper and in greater amounts.

Conclusion

Despite the nature of hair tissue, we have shown that it is possible to obtain a high-resolution image of hair in cross section with a conventional microscope. The use a lipid probe allowed to clearly define the localization of endogenous lipids. This localization appears consistent with the literature but allows a very precise localization of the lipids over the entire section. Working on an optical section with a confocal microscope, enables us to compare in the same condition, untreated hair and hair treated with coconut oil. A clearly higher intensity “lipid” signal in all the fiber was detected after the coconut oil treatment, leading to the hypothesis of its penetration.
The relationship between this signal increase and the presence of coconut oil components into the hair fiber was confirmed after selective extraction and separation with HPTLC. The results show that the coconut oil is mainly extracted after the first extraction, which seems to mean that the coconut oil is preferentially located on the surface of the hair. Nevertheless, although present in less quantity, coconut oil was also detected after the second extraction supposed to extract the internal lipids. This detection after the second extraction is probably partly due to the

grinding device. The concentration of triglycerides was carried out and shows a relatively important quantity of triglycerids from coconut oil. The image of the lipids remaining on a cross section after extraction with cyclohexane opens a reflection on the distinction between the extraction of surface lipids and internal lipids. This work shows a good complementarity between the two optimized methods. Optimizations could still be made, with new extraction and separation protocols or with the latest generations of light microscopy. This work also paves the way for the localization and quantification of other components of plant origin.

Conflict of Interest Statement : NONE

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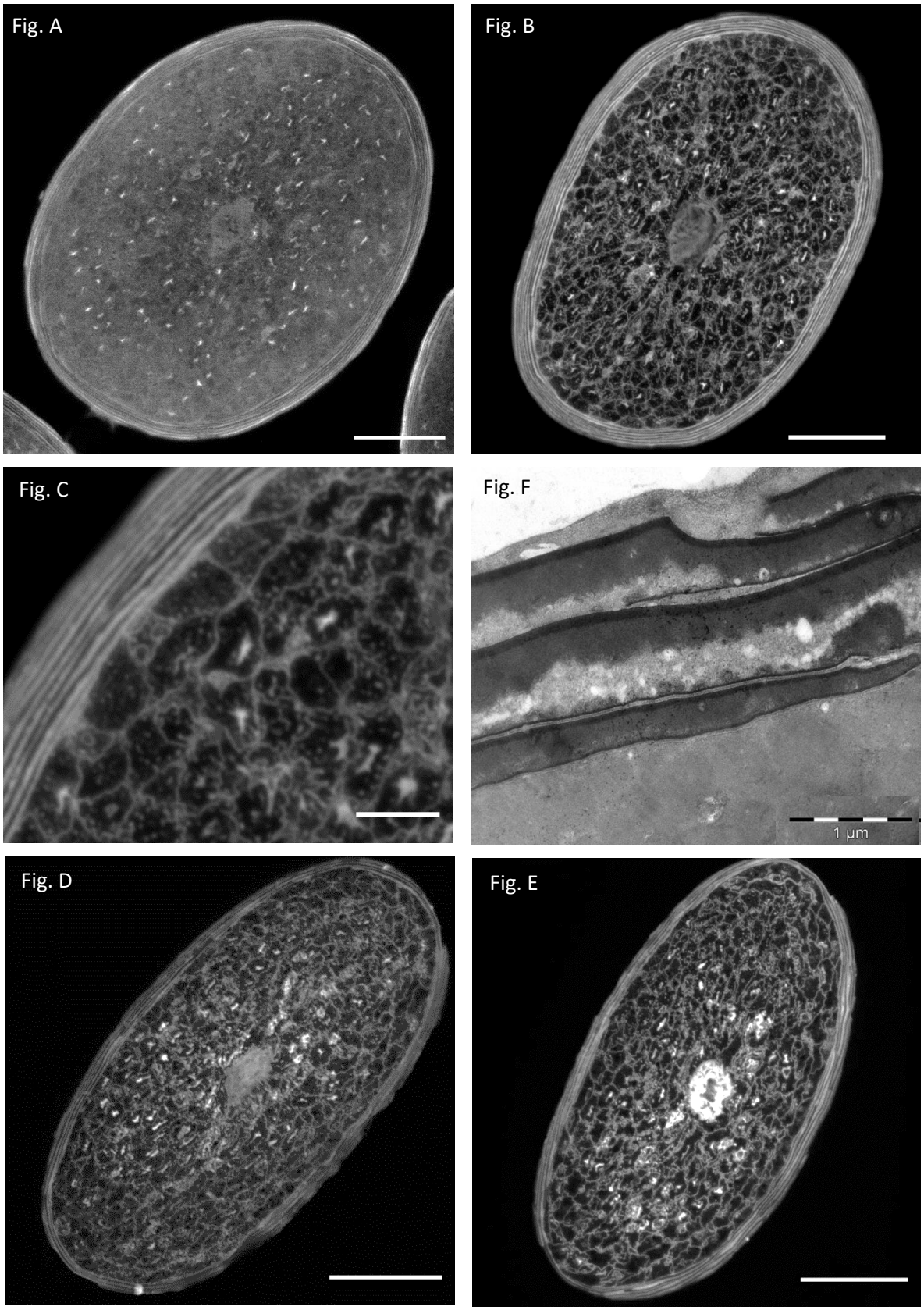

Fig. A to E - Hair sections with Confocal imaging. Fig. F - Focus on the cuticle area with Transmitted Electron Microscopy. Fig. A - Autofluorescence. Fig. B to E Fluorescence after staining with Nile Red dye. Fig. D - Before coconut oil treatment; Fig. E - After coconut oil treatment (Scale bar 20 µm).

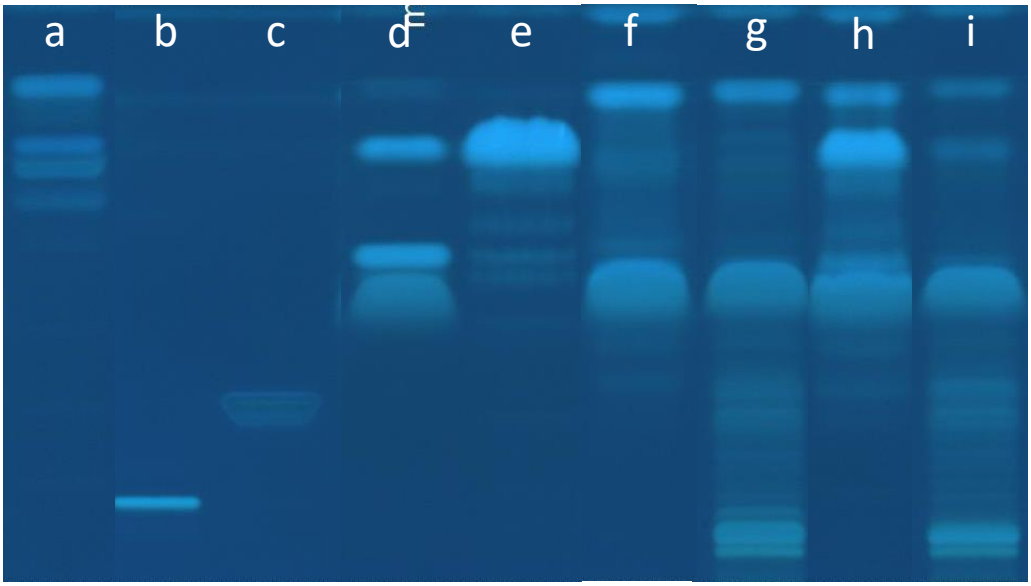

Fig. G - Lipid analysis of hair treated and non-treated with coconut oil using a High Performance Thin-Layer Chromatography. (a) Cholesterol; (b) Ceramide Gly; (c) Ceramide NdS; (d) Trilaurine; (e) Coconut oil; (f & g) Untreated hairs (f) with Cyclohexane; (g) with chloroform/methanol; (h & i) Treated hairs with coconut oil (h) with Cyclohexane; (i) with chloroform/methanol

Extraction	Untreated Hair	Treated Hair volunteer 1	Treated Hair volunteer 2
1	ND	29 mg/g +/-3.5	27 mg/g +/-0,6
2	ND	1 mg/g +/-0.25	1 mg/g +/-0.18

Table 1 - Triglyceride concentration for each volunteer after extraction 1 and 2.

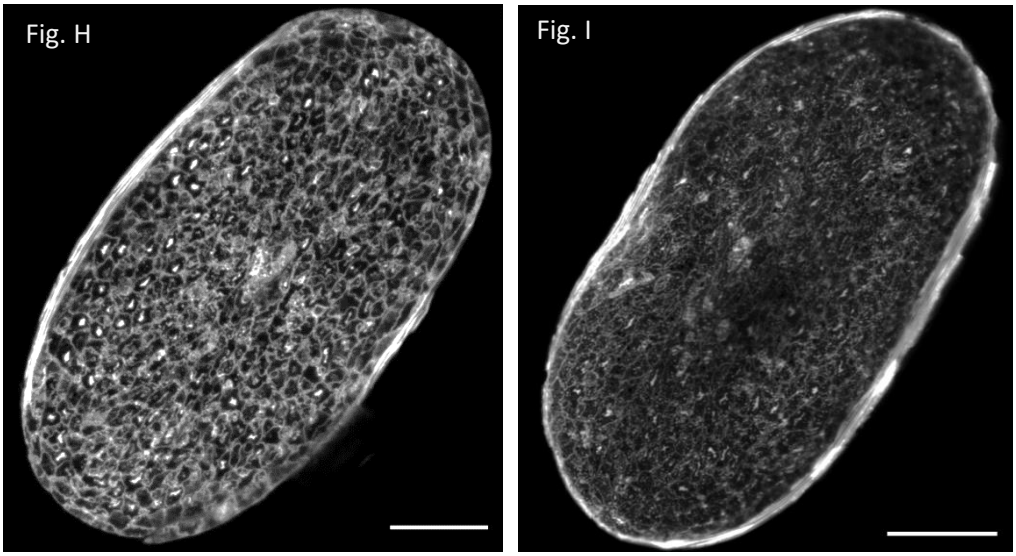

Fig. H & I. Fluorescence after staining with Nile Red dye - Confocal imaging. Fig. H untreated hair before cyclohexane extraction. Fig. I untreated hair after cyclohexane extraction. Scale bar 20 µm