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3D Reconstruction of human hair by confocal microscopy

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Synopsis

Confocal microscopy is a highly original, non-destructive method for observing objects in three dimensions and in their natural environment. As such, it is well suited to studies of human hair.

The tandem-scanning reflected-light microscope was used to study the hair surface in its natural environment, which included sweat and sebum. Micrometric measurements assessed the effects of classical treatments (permanent waving and bleaching), swelling in water and urea solution, and stretching.

A confocal laser scanning microscope was used to reconstruct a volume of hair shaft. The internal structures (cortex and medulla) were contrasted using a fluorescent marker (rhodamin) and could be observed in both longitudinal and transverse optical sections at all levels.

Our results demonstrate the value of confocal microscopy in describing hair structure and the effects of treatments on the cuticle. Methods based on classical optical and electron microscopy are far more complex and time-consuming than confocal microscopy, which represents an extremely promising technique for cosmetic research.

INTRODUCTION

The cylindrical shape of hair precludes straightforward observation of its surface by means of light microscopy, particularly at high magnifications. High-resolution images are therefore generally obtained by scanning electron microscopy (SEM), but the high vacuum and gold coating required prevent actual visualization of the cuticle (1). Internal structures (cortex and medulla) are classically studied on serial sections, which, needless to say, involve destruction of the sample. Confocal microscopy (2) provides high-resolution images with virtually no out-of-focus areas. In addition, the optical sectioning property preserves the integrity of the sample, even though it is limited by the opacity of the specimen, light-absorbing structures, surface reflections, refraction/scattering, and the design of the objective lens (numerical aperture and working distance). A series of optical sections is acquired and stored for later 3D reconstruction or computation of volume.

In this study, we used two confocal microscopes to study the surface aspect and internal structure of human hair. The tandem-scanning reflected-light microscope (TSM), invented by Petran *et al.* (3), gave high-resolution images of the surface, and was used to view and quantify the effects of cosmetic treatments, swelling and stretching on the topography of cuticular cells. Internal structures were visualized using a confocal laser scanning microscope (CLSM). Fine details of the cortex and medulla were identified by the use of a fluorochrome.

MATERIALS AND METHODS

HAIR SAMPLING

Freshly plucked hair and eyelash were simply collected on the authors at the time of the experiment. In all other cases, commercially available Caucasian virgin brown hair was used in standardized conditions.

HAIR TREATMENTS (4)

A permanent wave treatment was comprised of two steps: reduction and oxidation. Reduction was done with ammonium thioglycolate (1 M), adjusted at pH 9 with ammonia, for 30 minutes at 30°C. Hair was rinsed and fixed with a 2.5% hydrogen peroxide solution adjusted at pH 3 with hydrochloric acid. Finally, samples were rinsed with tap water.

Bleaching was performed by immersing virgin hair in hydrogen peroxide (6%)/sodium persulfate (10%) solution, adjusted to pH 10 with ammonia for 45 minutes at 35°C, and then thoroughly rinsing with tap water.

Swelling was obtained by immersing virgin hair in either deionized water or 8 M urea at room temperature for ten minutes.

STAINING WITH FLUOROCHROMES

10 mg of virgin hair were immersed for one hour in 5 ml of 0.05% solutions of rhodamin B in water or octadecyl-rhodamin in ethanol/water (9:1) at 60°C. The excess of fluorochrome was removed by dipping hairs for ten seconds in tetrachloroethylene; the hair was then dried in an oven at 105°C overnight.

BASIC PRINCIPLE OF THE CONFOCAL MICROSCOPE

Whereas the classic light microscope homogeneously illuminates a large area within the sample, the confocal microscope acts with a focused beam of light. The reflected light at the focused point does go through a pinhole in front of a detector (TV camera, photo multiplier). Out-of-focus reflections cannot pass the pinhole and consequently are not imaged. An image is generated at the focal plane by scanning the light spot in X and Y directions with either rotating mirrors or a Nipkow disc. The image does not contain any information from above and beneath the focal plane and is called an optical section.

Thick semi-transparent specimens (almost all biological tissues) can be vertically scanned. In-depth optical sections are then stacked for volume rendering by computer treatment. At the end of the procedure, the sample has not been destroyed. As no blurring spoils the image, the resolution is improved (0.25 μm) compared with that obtained by conventional light microscopy (0.6 μm).

HAIR SURFACE STUDY

The Tracor-Northern TSM is a real-time confocal microscope equipped with a Nipkow scanning disk. The sample is illuminated by a 100-watt mercury-arc lamp. Reflected light from the focal plane is captured by a monochrome video camera (COHU-0.02 lux) and rapidly digitized via 4-data flow processors (PC-Oeil) on a microcomputer. Image processing is carried out by PC-Oeil, which provides a 3D reconstruction based on up to 256 stacked optical sections using 3D+ software (MISIS-Tracor Europa).

This gives two 512 × 512 image arrays coded on 256 gray levels, which are known as the "reflection map" (Figure 1) and the "topologic map" (Figure 2). Respectively, they provide the brightness and spatial position (X, Y, and Z coordinates) of each pixel. Combining the two maps gives a "perspective view" image (Figure 3), which reproduces the cylindrical shape of the hair. Line profiles can be generated interactively and provide local surface roughness parameters (see below).

Freshly-plucked or treated hair was simply attached to a microscope slide coated with double-sided adhesive tape and observed with no further preparation. A dry 100X objective was selected, providing a resolution of approximately 0.25 μm in X, Y, and Z. 3D images containing full information were obtained by stacking 100 optical sections in 0.1-μm vertical steps. Noise reduction by frame averaging or gray processing was unnecessary.

Hair was stretched between two parallel jaws moved by an electric motor, thus avoiding manipulation during the experiment. The hair was glued to the jaws using cyanoacrylate resin. Twenty optical sections in 0.5-μm vertical steps were collected at each step of the extension for 3D reconstruction; each 3D image thus obtained was made to overlap the preceding ones by interactive shifting in X and Y coordinates in order to examine

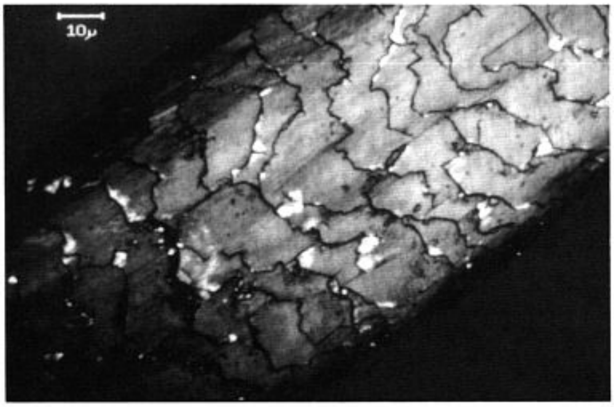

Figure 1. "Reflectance map" of a natural brown hair. Local changes in brightness can be evaluated on a 256 gray scale range.

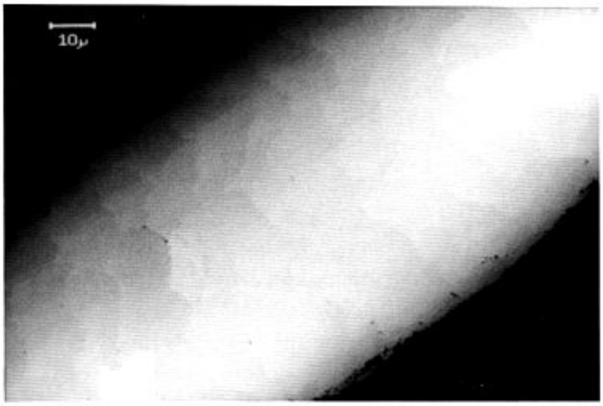

Figure 2. "Topologic map" of the same hair. This map contains the vertical position of each pixel coded on 256 gray levels from 0-black = bottom to 255 white = top.

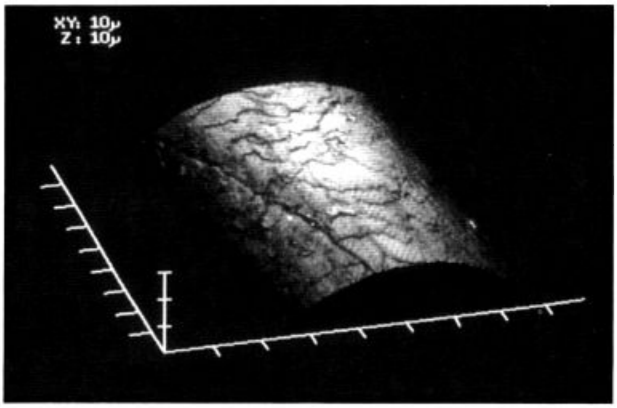

Figure 3. "Perspective view" showing the curvature of hair by pooling information on both brightness and altitude. The best angle of view can be interactively selected by rotation, tilting, and shadowing.

the same field of view at any given step. Fast scanning of the sequence of images gave movement virtually in real time, facilitating the perception of subtle changes during the elongation.

HAIR VOLUME STUDY

The Molecular Dynamics Sarastro instrument is a confocal microscope (5) based on a focused laser beam (argon-ion). Scanning is ensured by two rotating mirrors (X,Y), and image formation takes less than five seconds for a 256 × 256 pixels array. This microscope is used to acquire fluorescence signals.

We selected a 60X oil-immersion objective. The contact fluid reduced surface reflection and consequently improved the quality of in-depth optical sections. Noise was reduced by image averaging (four frames). A full cylinder of hair shaft was reconstructed in three dimensions on a Silicon Graphics workstation.

The fluorescent hairs were glued to microscope slides coated with double-sided adhesive tape. Between 40 and 100 optical sections were stored, depending on the diameter of the hair shaft, for complete volume reconstruction.

RESULTS

HAIR SURFACE EXPERIMENTS

Freshly plucked hair was imaged in its natural environment. Figure 4a shows sweat droplets attached to the free border of cuticular cells. The joints between the successive optical sections were invisible and the border lines of the cuticle were finely drawn with no interruption. The confocal image was comparable to an SEM picture (Figure 4b). Figure 5 shows natural deposits of sebum and dust particles on an eyelash. Sebum filled the spaces between adjacent cuticular cells, facilitating dust deposition. Neither sweat nor sebum could be observed in conventional SEM.

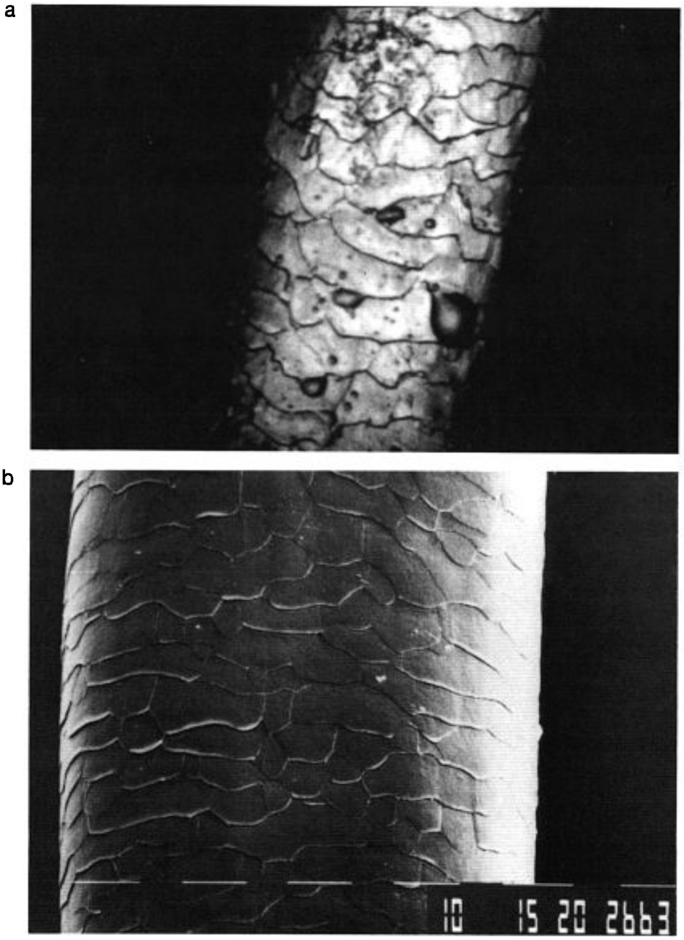

Figure 4. Freshly plucked hair: a) confocal image showing sweat droplets; b) "cleaned" SEM image.

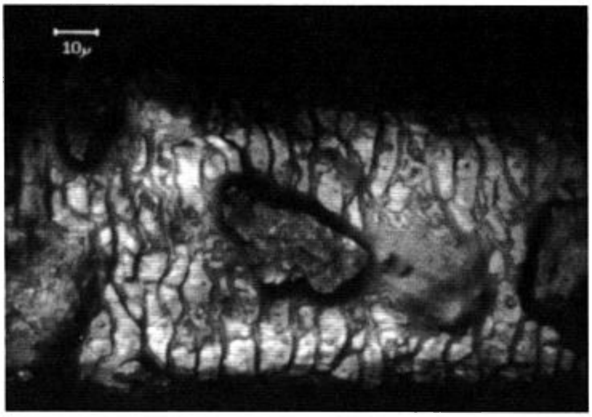

Figure 5. Plucked eyelash showing sebum and dust deposits on the cuticle.

Virgin brown hairs were used as a standard to evaluate the effects of cosmetic treatments. Figure 6 shows the surface aspect of non-treated hair. The altitude profile generated along the straight line constructed interactively showed periodic events. These reproduced the longitudinal pattern of the cuticle. Measurements gave 0.3-μm jumps from cell to cell, in accordance with the reputed values (6) for cuticle cell thickness. Comparable profiles recorded by using mechanical profilometry (7) did not draw such a periodicity.

Permanent waving (Figure 7a) lifted the free border of the cuticular cells. The altitude profile reproduced the surface aspect of each cuticular cell, with a curved appearance. The jump was about 0.6 μm, compared to 0.3 μm for untreated hair. This difference assessed the lifting.

Bleaching (Figure 7b) had a corrosive effect on the surface, as assessed by the flattened profile. The crenelated free borders of cuticle cells and the widened intercellular dis-

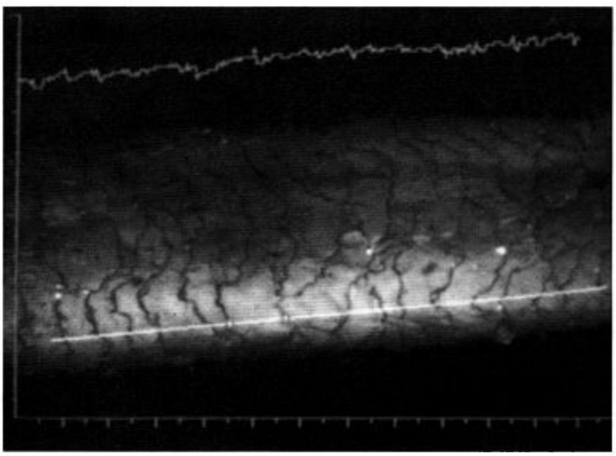

Figure 6. Virgin brown hair. Horizontal scale: 10 μm between large bars in X and Y. Vertical scale: 10 μm between large bars in Z. The upper curve reproduces the altitude profile of the hair surface along the white straight line. Differences in altitude are calculated by using the vertical scale.

a

b

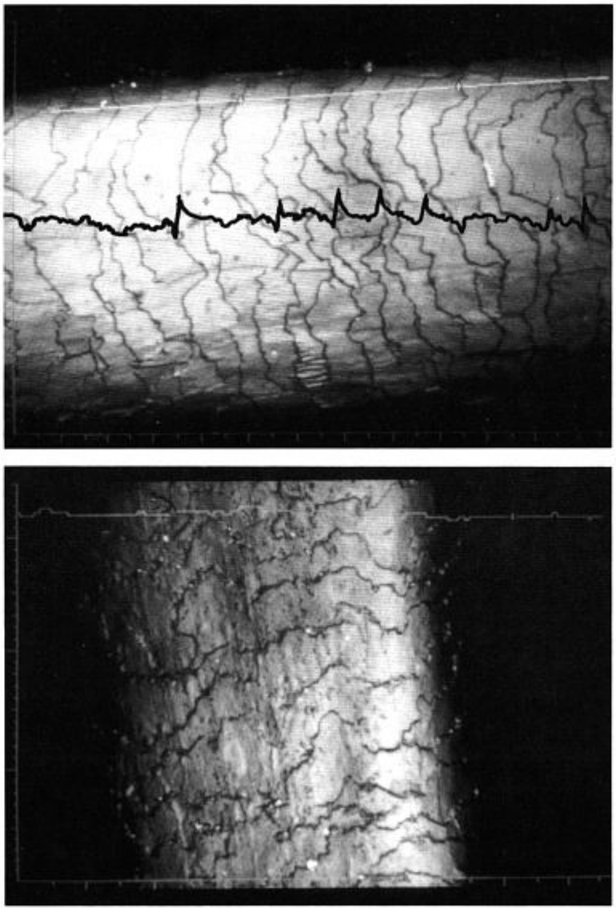

Figure 7. Cosmetic treatments: a) permanent waving; b) bleaching.

tances confirmed the loss of material. A less-uniform brightness confirmed the chemical degradation of the cuticle.

Swelling in water (Figure 8) led to bulging of the surface. The altitude profile showed large undulations with a period of about 34 μm (4–5 cells) and an amplitude of about 0.8 μm. There was no accompanying lifting of the cuticular cells. Urea (Figure 9) had a more drastic swelling effect, with a shorter period (15 μm) but a higher amplitude (2.6 μm). This periodic bulging of the surface could be explained by local resistance of the cuticle to the pressure exerted by a turgid cortex.

The effect of stretching is clearly observed in Figures 10a–d. The limited number of optical sections used in this experiment reduced the vertical (Z) resolution to 0.5 μm, and consequently the periodicity of the cuticle could not be observed. Nevertheless, the increased brightness of the cuticular cell border showed that the cells rose with an extension of as little as 10%. This was accompanied by sliding and, in the example shown, local cracks in the cuticle. When such cracks appeared, sliding and rising of neighboring cells was not seen. At 20% extension, the altitude profile indicated that cracking reached a depth of about 4 μm and involved the whole thickness of the cuticle.

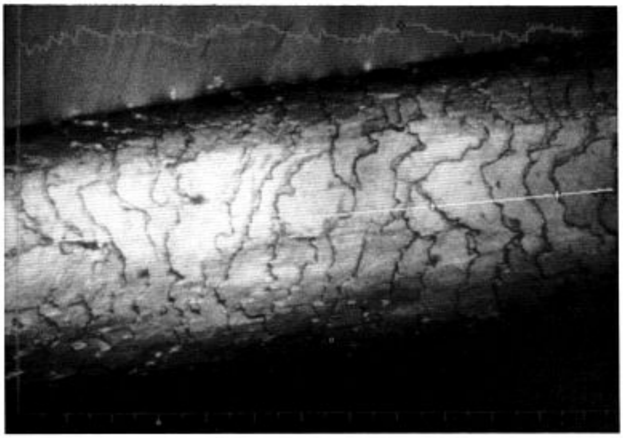

Figure 8. Swelling in water. Waviness is clearly drawn on the profile (scales as in Figure 6).

The flat bottom of the pit in the profile suggested that the crack was limited to the cuticle and did not thus involve the cortex.

HAIR VOLUME EXPERIMENTS

Staining with rhodamin B in water. The fluorochrome stained the free border of cuticular cells (Figure 11a). The vertical optical cross section (Figure 11b) showed that it did not penetrate into the cortex. No internal structures were visible. The cuticular envelope drew exactly the cross-sectional shape of the hair. A fair autofluorescence of the adhesive can be seen on each side of the hair section.

Staining with octadecyl rhodamin in ethanol:water. This fluorochrome penetrated through the cuticle into the cortex, selectively staining membrane components and giving high-definition images of the cortex. The optical section 20 μm below the surface (Figure 12a) showed cuticular cells at the periphery and highly fluorescent cortical cells with a typical fusiform shape within a network of fibrous keratin bundles. The axial optical section (Figure 12b), taken 40 μm below the surface, showed that rhodamin

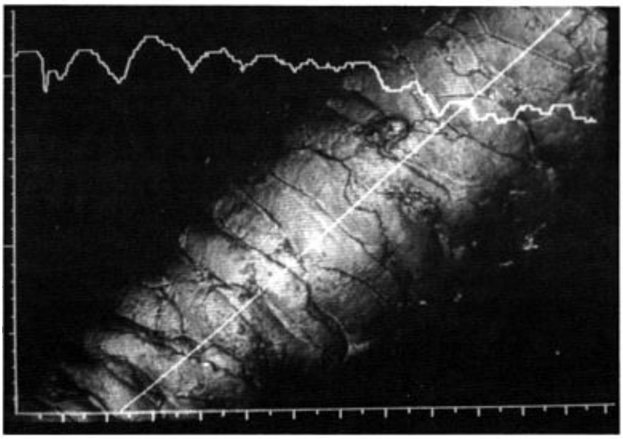

Figure 9. Swelling in urea. Note the bulged aspect of the surface (scales as in Figure 6).

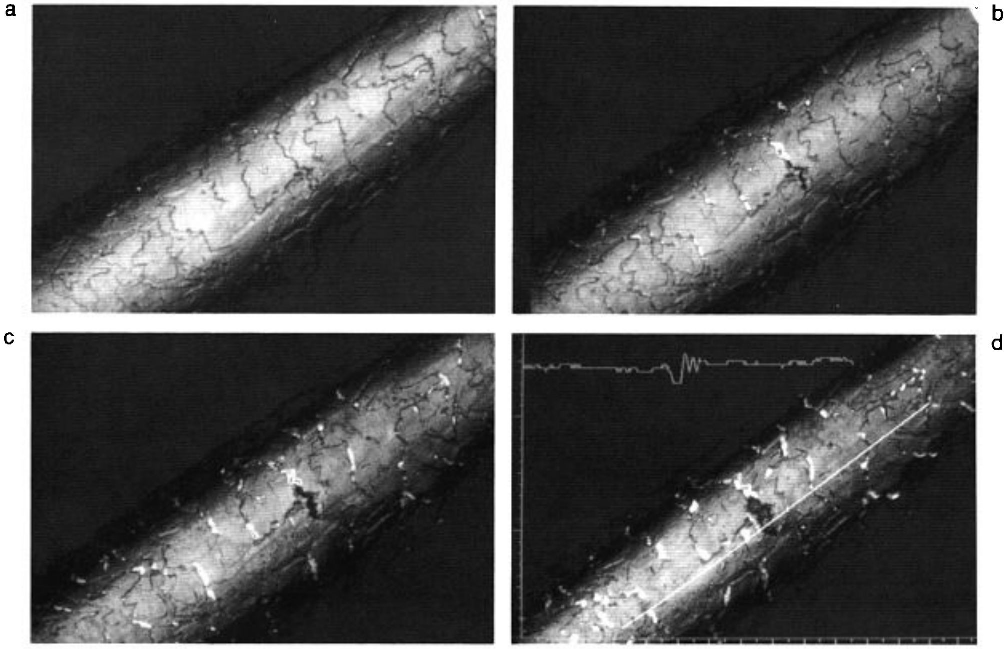

Figure 10. Stretching experiment: a) before stretching; b) 10% extension; c) 15% extension; d) 20% extension.

penetrated into the medulla. The right part of the medulla shows typical septation; the cortical arrangement was even more clearly defined.

DISCUSSION

We tested the performance of the confocal microscope for the observation of the surface and internal structure of human hair in various conditions.

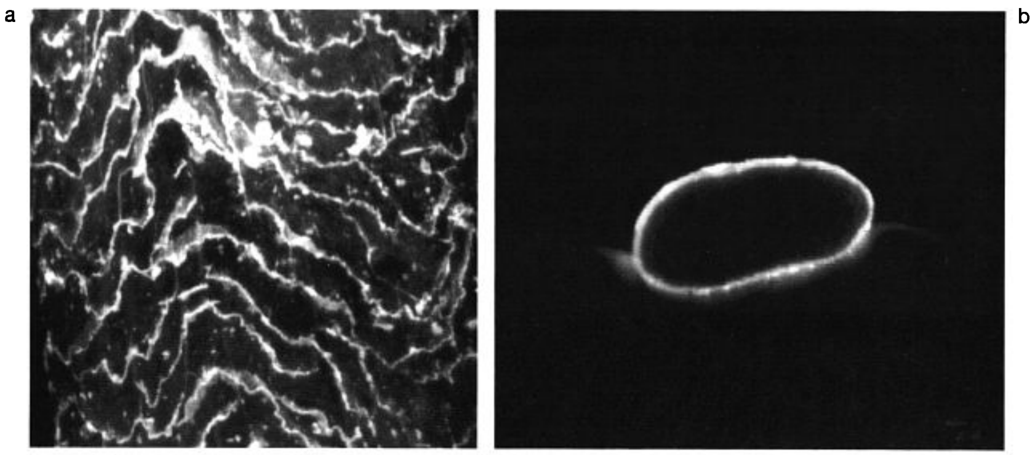

Figure 11. Rhodamin B fluorescence: a) hair surface; b) cross section showing that the fluorochrome did not penetrate into the cortex.

a b

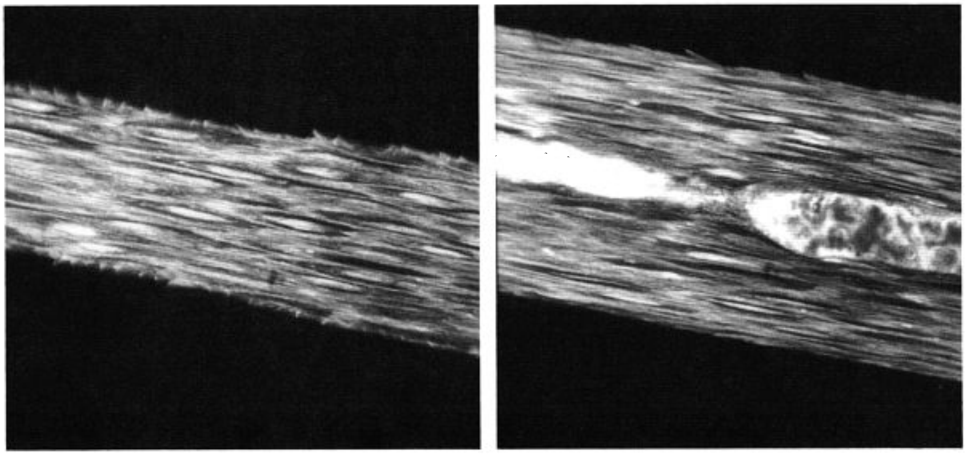

Figure 12. Octadecyl rhodamin fluorescence: a) longitudinal section from 20 μm below the surface; b) longitudinal axial section cutting the medulla.

The main advantage of confocal microscopy over conventional light microscopy is improved resolution, as confirmed by the images presented here.

This led to a remarkable similarity between the images reconstructed from optical sections acquired by the TSM and those provided by the SEM. SEM requires extensive sample preparation that can introduce artefacts. In general, exogenous deposits on the hair surface are disturbed by the coating required for SEM. In addition, the fact that electron microscopy requires samples to be observed in a vacuum rules out visualization in their natural environment.

Our results show that oil- and water-based deposits can be visualized on the hair surface by means of confocal microscopy, and this opens up new possibilities for cosmetic research, particularly in the study of protective preparations such as waxes and oils.

Another advantage of confocal microscopy is that it provides not only 3D images, but also exact spatial measurements. It is therefore possible to quantify changes in the organization of cuticular cells and to compare objectively the efficacy of cosmetic products. The periodicity, contours, orientation, thickness, and lifting of the surface scales can be measured to within about 0.1 μm, and the rapidity and simplicity of the method make it possible to apply valid statistical methods to the results. In addition to micrometric measurements, the illumination of the specimen by reflected white light provides information on brightness in the "reflection map." Quantitative evaluation of brightness needs a calibration of the TV camera. This was not done in our experiment, but we were able to evaluate local variation in brilliance on a single fiber. Such information is not provided by the SEM.

Important modifications of the hair fiber caused by permanent waving have already been reported (8), but subtle changes of the surface aspect of the cuticle had never been assessed. Images of the TSM showed that the treatment lifted the free border of cuticular cells; this effect could be objectively assessed.

Bleaching is known to cause very little damage to the hair surface, as observed by conventional SEM (8,9). Zelinski (7) recorded an increase in roughness but did not interpret his observation. With the confocal microscope, we were able to confirm the corrosive effect of the treatment, as previously suggested (8).

The simplicity of this imaging method and its non-destructive nature open the way to studies of dynamic phenomena in four dimensions. This is illustrated by our study of swelling and stretching. Although hair swelling has previously been studied by means of physical techniques, this is the first time, to our knowledge, that periodic phenomena (bulging) have been described. The same is true of the stretching studies. Although measurements of resistance to traction have classically been used to evaluate the visco-elastic properties of hair, changes in surface structure during stretching have rarely been reported because of technical difficulties inherent in conventional microscopy. The variations we observed in the brightness of the borders of cuticular cells during stretching suggest their rising as previously reported by Brown and Swift (10).

Hair specialists are well aware of the fastidious and time-consuming nature of methods for preparing longitudinal and transverse sections. Even the most skilled technicians rarely produce sections that are perfectly aligned with the hair axis. The optical sectioning property of the confocal microscope overcomes these problems, since sections can be visualized at any given level and in any given direction in a fraction of a second.

The images of the internal hair structures obtained after labeling with a fluorescent marker were also of exceptional quality, compared to those obtained by observation of fiber sections in conventional fluorescence microscopy (11). Finer details of the structure of the cortical cells were observed, both in the radial and axial directions.

This technique also provides a simple tool for monitoring the penetration of dyes throughout the hair thickness.

In conclusion, confocal microscopy is highly suited to studies of human hair and the effects of cosmetic preparations. It is particularly adapted to observations of rounded surfaces and is thus a valuable alternative to classical microscopic methods. A particular advantage is the non-destructive nature of the technique, which means that samples can be observed both before and after treatment; this opens up exciting new possibilities for cosmetic research.

ACKNOWLEDGMENTS

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