

# *In vitro* and *in vivo* study of dye diffusion into the human skin and hair follicles

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**Abstract.** We present experimental results on the *in vitro* and *in vivo* study of dye diffusion into human skin and hair follicles. We have studied some commercially available dyes for potential using in the laser selective thermolysis. The degree and the depth of hair follicle dyeing inside the skin were determined. For hairs in different stages the sebaceous gland was stated as a reservoir for a dye administration. It was found that the penetration depth of dyes is about 1.2 mm from the skin surface. We have developed the biocompatible Indocyanine Green lotions and the method for *in vivo* dyeing and dye in depth monitoring. Shift on 16–21 nm of absorption peak of Indocyanine Green to the longer wavelengths due to Indocyanine Green binding with cell proteins in the human skin was found. © 2002 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.1486247]

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## 1 Introduction

The area of application of selective laser photothermolysis includes cancer,<sup>1–6</sup> various diseases of skin and hair follicles, and hair removal.<sup>7–9</sup> The main natural absorber is the melanin concentrated in a hair shaft and follicular papilla. Unfortunately, absorption of the melanin in hair shafts is not so high at 700–850 nm. Optical density of hairs from red-haired subjects is about  $0.1 \pm 0.05$  and that from brown-haired subjects is about  $0.45 \pm 0.2$ .<sup>10</sup>

In the skin an absorber of laser energy melanin<sup>11–14</sup> or special absorbers as carbon particles<sup>15</sup> or dyes<sup>3–6</sup> can be used. For follicle diseases treatment the dyes, carbon, and melanin preparations can be administered topically.<sup>15,16–19</sup> Selective absorption of laser radiance by absorbers induces intense heat and damage of the target cells.

For effective removing of unwanted or excess hairs or for treatment of skin diseases, especially acne, designing the technology of skin appendages dyeing is very important.

It is expected that NIR dyes absorbing at the shorter wavelengths than the strong water bands should have the maximal selectivity, because the absorption of any biological component of the skin and its appendages is very low in this wavelength range. Commercially available medical and food dyes have been studied. Absorption spectra of all dyes and skin samples were measured. Pre- and postdyed epilated hair samples and biopsy hair samples were collected and analyzed. We have designed the technology of dyeing of the human skin and hair follicles.

## 2 Hair Structure

The structure of hair and its changing in different stages are of great importance for in-depth dyeing of the hair shaft and

follicle. We have studied the human body hairs from the forearm and lower leg in various stages: anagen, catagen, and telogen. The hair in the anagen stage [Figure 1(a)]<sup>20,21</sup> continues to produce a hair shaft for a period from a few months to a few years. During this period its structure remains constant. The expanded, bulbous section of the lower hair follicle, including the hair matrix and the dermal papilla, is known as the hair bulb. The isthmus of the follicle is the portion lying in between the point of attachment of the arrector pili muscle and the point of entry of the sebaceous gland duct. The infundibulum lies above the entry of the sebaceous duct and merges with the surrounding surface epidermis. Hair follicle length in different stages changes with development, however, the area of the follicle from arrector pili muscle to skin surface remains permanent.

Just external to the hair shaft are the three layers forming the inner root sheath (IRS). External to the IRS is the clear-celled outer root sheath (ORS). In the lower portion of the follicle (below the isthmus), the ORS does not keratinize. The nonkeratinized inner layer of cells lies against the fully keratinized IRS. At the level of isthmus, where the IRS disintegrates, the ORS keratinizes.<sup>20</sup> Hair follicle length in the anagen stage is about 3 mm. The hair canal, infundibulum, and the sebaceous gland are a path for in depth penetration of dye.<sup>19</sup> The sebaceous gland can serve as a reservoir for the dye.<sup>22</sup> The higher concentration of the dye within the sebaceous gland can provide the dye diffusion into the depth of the hair follicle and skin. At the level of isthmus, where the IRS is desintegrated and keratinized ORS makes only a few cells layer, the dye can diffuse in surrounding tissue and into the depth more effectively then through the stratum corneum on the skin surface.

A telogen hair is distinguished by its fully keratinized club, which is surrounded by an epithelial sac [Figure 1(c)].<sup>20,21</sup> Telogen progresses over a period of about 3 months. Hair

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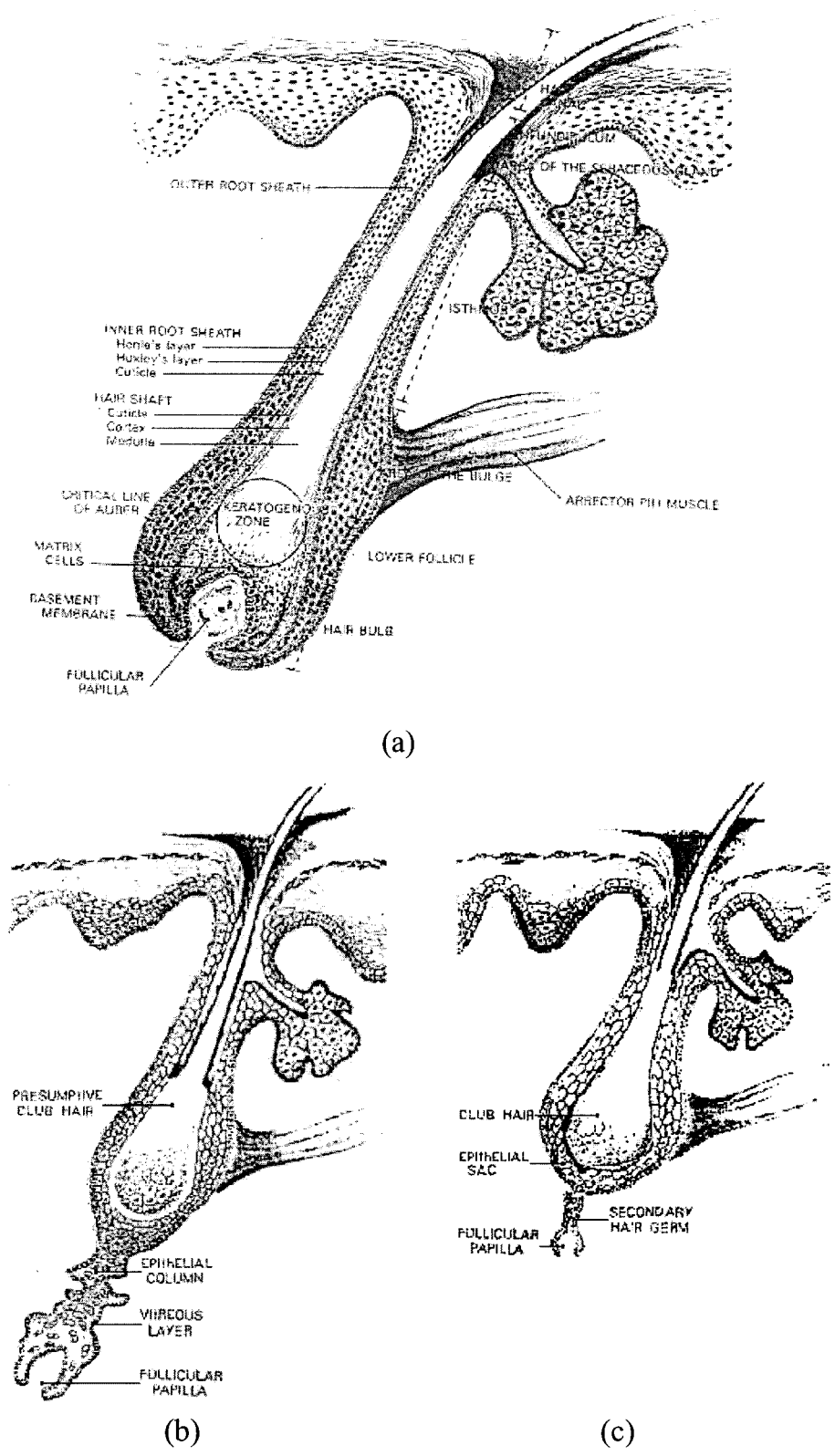


Fig. 1 Anatomy of a hair follicle: (a) anagen, (b) catagen, and (c) telogen stages (see Ref. 21).

follicle length in the telogen stage decreases to about 1 mm. The transitive stage between anagen and telogen is the catagen stage. The dramatic changes seen during catagen are condensed into a brief period of 2 or 3 weeks. Catagen fol-

licles have several anatomic features that are peculiar to this phase of the life cycle. Above the papilla, the follicular epithelium remains as a thin column of pale-staining, nonpigmented, undifferentiated cells [Figure 1(b)].<sup>20,21</sup> The length of

the hair follicle in the catagen stage varies from 1 to 1.5 mm.

Anagen hairs come in a variety of sizes ranging from the large terminal hairs found in the beard and on the scalp to the fine minute vellus hairs covering most of the glabrous skin. Both vellus and terminal hairs go through all stages of the follicular life cycle, but the length of the hair shaft and period of anagen are much shorter in vellus hairs.<sup>21</sup> Follicular structure of hairs in each stage is the same for all types of hairs.

We took an interest in vellus hairs (hairs on the leg and arm relate to them). They are involved in acne formation and usually are exposed to the laser selective thermolysis.

### 3 Methods and Materials

We used the following dyes: the Phospholipid Nanoemulsion with carbon particles, the food dye Indigo Carmine, and medical dye Indocyanine Green (Aldrich Chemical Co., USA). Some other commercially available dyes (e.g., Methylene Blue, Toluidine Blue, Erioglaucine, and Fast Green) were tested in our previous paper.<sup>22</sup>

Absorption spectra of all dyes in the 400–1500 nm wavelength range were recorded by the spectrophotometer CARY-2415 “Varian” (Australia).

Various liquid carriers, often used in cosmetic preparations, were chosen as solvents of the dyes. Concentration of Indigo Carmine (IC) was 10 mg/ml and concentration of Indocyanine Green (ICG) was in the range from 1 to 4 mg/ml. The Phospholipid Nanoemulsion (PN) was dissolved in vegetable oil up to volume fraction 0.1.

The human skin test sites were 5×5 cm rectangular in size. Preoperative preparation included the treatment of the site by ethanol or hot compress in 5–10 min. Then the warm dyeing solution was spread on the skin surface of a volunteer. For increasing of the dye diffusion into a hair follicle we have applied massage and heating (up to 45 °C). The time of the procedure was 15–20 min. Then the dyeing solution was removed by a cotton wool tampon impregnated by warm water.

Hair samples were taken from exterior side of the forearms and lower legs of six healthy volunteers with fair or brown hairs. Hairs were epilated just after dyeing and after 15, 30 min, 1, 2, and 5 h after dyeing.

For *in vivo* and *in vitro* biopsy measurements we have chosen Indocyanine Green as a dye having the greatest absorption coefficient at 800 nm. ICG has a chemical formula of  $C_{43}H_{47}N_2O_6S_2Na$  and molecular weight of 775. It is a tricyanocyanine type of dye with infrared absorbing properties. It has little absorption in the visible range. It is used as a diagnostic aid for blood volume determination, cardiac output, or hepatic function. ICG has been used extensively in laser welding because of its high absorption at wavelength 808 nm of the powerful laser diodes. The principal advantages causing the acceptance of this dye in medical practice were the presence of an absorption maximum near the isobestic point of hemoglobin and oxyhemoglobin around  $\lambda = 800$  nm, the confinement to the vascular compartment by binding to plasma proteins, the very low toxicity, and the rapid excretion, almost exclusively into the bile. ICG has a somewhat bizarre light absorption behavior as a function of concentration because it tends to aggregate in water at high concentrations. This means that the effective absorption does not increase linearly with increasing of concentration. Furthermore, ICG tends to de-

grade with exposure to daylight. The photodegradation is mitigated when ICG is bound to albumin, but it still proceeds slowly (days). In long-term experiments the optical density of Indocyanine Green solutions in plasma as well as in distilled water generally diminishes, even in the dark. On the seventh day a new absorption maximum starts to appear at  $\lambda = 900$  nm, possibly caused by further aggregate formation leading to much larger particles.<sup>23</sup> The photodegradation is also concentration dependent.<sup>24</sup>

A skin biopsy was done by a standard method with the use of a biopsy punch (Acuderm Inc., USA) from the exterior side of the forearm of two healthy volunteers with brown hairs. Skin with the growing hairs was chosen for the biopsy. Size of a tissue sample was 2 mm in diameter and 4–5 mm in depth.

For *in vitro* study we used the *post mortem* (24 h) human skin samples with hairs. The area of the samples was about 2×2 cm and the thickness was about 3–4 mm. Before the experiments the samples were saved in saline. Skin was dyed by a Palomar ICG-lotion (lotion with Indocyanine Green developed by Palomar Medical Products Inc., USA, on the base of a glycerol–ethanol–water mixture adding DMSO) for 30 min. The dyeing solution was spread only on the center part of the epidermal surface of the samples. The method of dyeing *in vitro* was the same as *in vivo*. To provide a temperature regime corresponding to *in vivo* experiments the skin samples were placed into the water bath. The temperature inside was about 37 °C. Dyed human skin biopsy samples were taken using the same procedure as for an *in vivo* biopsy.

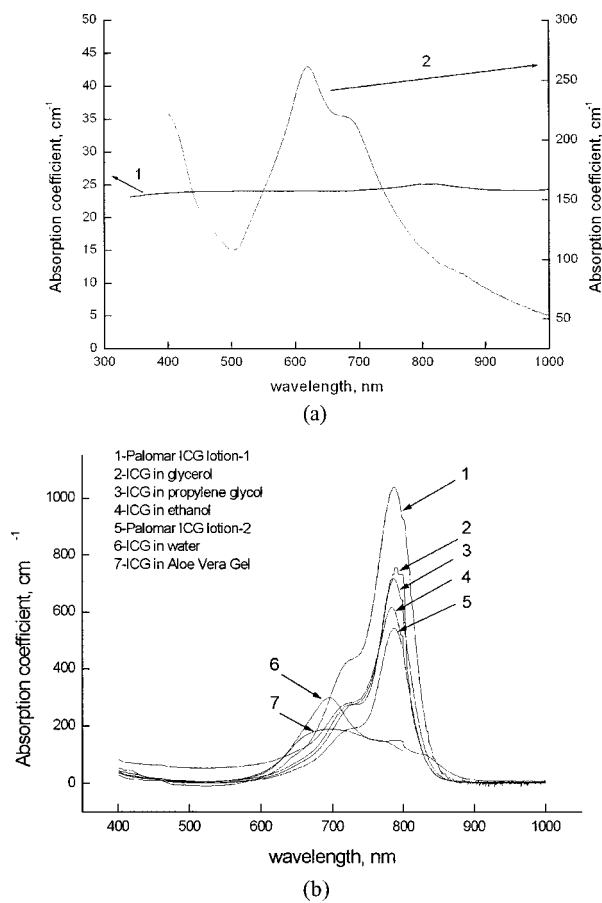
Optical color imaging microscopy was used for the study of the epilated and biopsy hair samples. Photos of all the samples were done by the video-microscopic system based on a VHS color camera Panasonic NV-RX70EN (Tokyo, Japan) interfaced with a personal computer.

The dyeing of the human skin surface was also examined. ICG and skin interaction were studied using a tape stripping method.<sup>25</sup> The skin surface was dyed with a Palomar ICG-lotion, and then the lotion was deleted. A few skin strips of 3–5  $\mu\text{m}$  of thickness were done by adhesive tape (Multi-Film, TESA, Beiersdorf, Hamburg) from the same place on the inner side of a forearm of volunteer and stuck on a subject glass. Spectra of total transmission were recorded by the spectrophotometer CARY-2415.

### 4 Results

Figure 2(a) presents the absorption spectra of Phospholipid Nanoemulsion with carbon particles (PN) dissolved in vegetable oil (1) and IC dissolved in glycerol (2). The spectrum of PN has a broad absorption band and no peaks in the visible and NIR areas. IC has a broad absorption band. The peak of absorption is about  $260\text{ cm}^{-1}$  at a wavelength of 620 nm. In Figure 2(b) the absorption spectra of ICG in different solvents are shown. They have two peaks in red (696 nm) and infrared (IR) areas (789 nm). These dyes can be applicable for a thermal treatment provided by He–Ne (633 nm), ruby (694 nm), or diode (800 nm) lasers. The analyzed dyes do not have absorption peaks near 1064 nm.

Thermal degradation of ICG dissolved in a glycerol–ethanol–water mixture was studied. We did not find any noticeable degradation of the solution and changes of its spec-



**Fig. 2** Absorption spectra of dyes recorded by a spectrophotometer: (a) Phospholipid nanoemulsion solution in vegetable oil at concentration 0.1 (vol/vol) (1—left axis), and Indigo Carmine solution in glycerol at concentration 10 mg/ml (2—right axis); (b) Indocyanine Green in various solvents at concentration of 1 mg/ml and in two Palomar ICG lotions (1—Palomar ICG lotion-1, 2—ICG in glycerol, 3—ICG in propylene glycol, 4—ICG in ethanol, 5—Palomar ICG lotion-2, 6—ICG in water, 7—ICG in aloe vera gel).

trum when increasing the lotion temperature up to 100 °C during a half hour.

In Figure 2(b) it is easily seen that in water and aloe vera gel (based on water) the IR ICG peak is suppressed for the chosen ICG concentration. A more pronounced IR peak can be seen in water solutions at the less concentrations of ICG.<sup>24</sup> Extremely high absorption above 1000 cm<sup>-1</sup> has Palomar ICG lotion-1 based on a glycerol–ethanol–water mixture and DMSO. It is very remarkable that this lotion has a 1.8-fold higher absorption coefficient than ICG dissolved in any of solvents at the same concentration of ICG. Absorption coefficients of Palomar ICG lotion-1 and lotion-2 are 1037 and 540 cm<sup>-1</sup> at 789 nm, respectively.

Two Palomar ICG lotions, IC solution in glycerol, and PN with carbon particles were tested. Figures 3–6 illustrate dye penetration into the human skin for *in vivo* experiments. In Figure 3 the image of a follicle in the telogen stage is shown. The skin was dyed by Palomar ICG lotion-1 with the usage of massage and heat. For calibration of linear sizes of the image a precise testing object (scale, shown in the image) was used. The skin surface is well seen in the image. It corresponds to

the top of the upper dyed area of the epilated hair (the length of this area is about 0.3 mm), which presents the dyed sebaceous discharge attached to the hair shaft. Sebaceous discharge filling the hair canal is well dyed due to the interaction between the dye and protein components of the sebum. Then an area of the hair without dye follows (about 0.5 mm in length) because the dye does not diffuse into dense keratinized tissue of a hair shaft. The dyeing lotion penetrates along a hair shaft to the soft nonkeratinized tissues. This dyed area is in the top of the hair club and is about of 0.2 mm in length. The penetration depth of dye can be estimated from the image as 0.95 mm. Dye distribution is well seen as green areas in the color image within the hair canal, the sebaceous gland, and the upper part of the club. It should be noted that we see only areas with a rather high concentration of dye, evidently the penetration depth of the dye is much higher. Due to the high absorption of ICG we can expect effective thermolysis even at depths bigger than 1 mm, where concentration of the dye is not so high.

Figure 4 shows IC dyed hair in the telogen stage. The dye was dissolved in glycerol. In the figure the sebaceous gland area (blue area) is seen. The dyed area of this follicle is approximately 0.3 mm and the depth of dyeing is correspondingly about 0.7 mm.

The image of hair in the anagen stage treated by PN is shown in Figure 5. Carbon nanoparticle distribution is well seen as the black area. The penetration depth of PN is about 0.7 mm.

Figure 6 shows a hair biopsy sample with hair shafts in the anagen and catagen stages done after ICG lotion application. Dye distribution is seen as the green areas within the hair canals and sebaceous glands.

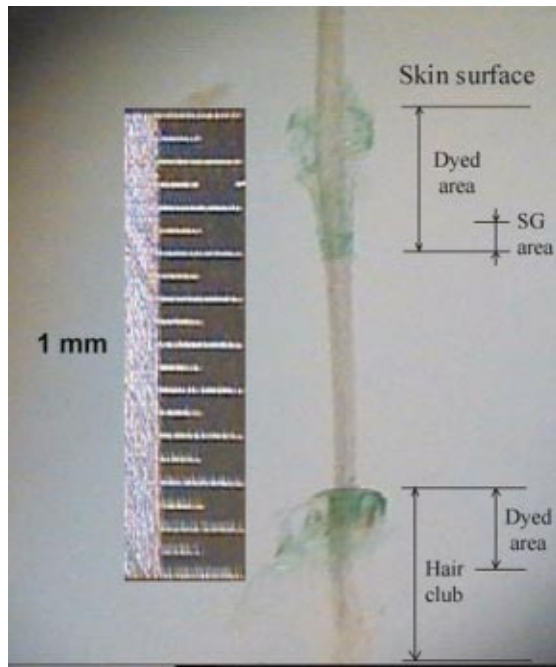
It was found that the penetration depth of the dyes is different for hairs in the anagen, catagen, and telogen stages. The depth is about  $0.5 \pm 0.12$  (sd) mm (averaged for 36 samples) for anagen hairs and  $0.9 \pm 0.18$  (sd) mm (averaged for 35 samples) for catagen and telogen hairs.

71 hair samples (7 biopsy and 64 epilated) were studied. 36 samples were in the anagen stage (26 were dyed by ICG, 3—IC, and 7—PN) and 35—in the telogen or catagen stages (16—ICG, 9—IC, and 10—PN). The mean depths of dye penetration for the follicles are presented in the Table 1.

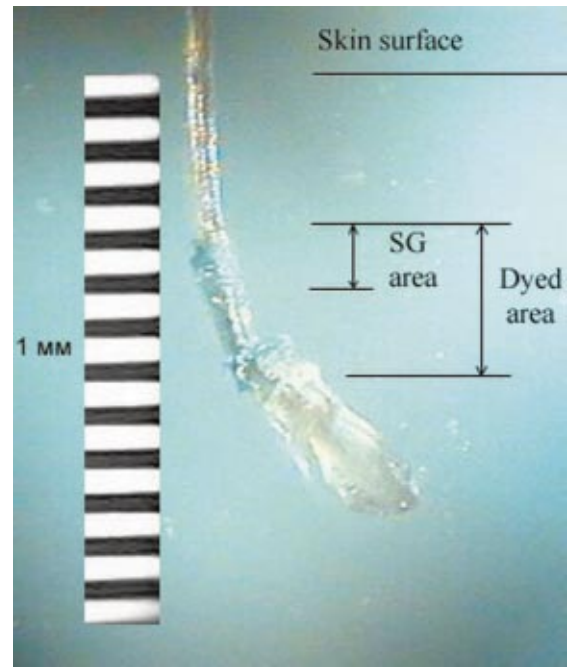
Figure 7 shows the optical spectra of the first and the second strips of skin dyed by Palomar ICG lotion-2 and the spectrum of this lotion. The shift to the longer wavelengths of about 16 nm (for the first strip of the skin), and about 21 nm (for the second strip of the skin) for the main absorption peak of ICG in the stratum corneum was defined.

In comparison with a living tissue the ICG distributions in the *post mortem* dyed skin samples were more diffusive and not so deep [the depth is not more than  $0.6 \pm 0.1$  (sd) mm, averaged for six samples]. The transverse diffusion of dye around the hair (determined from the *post mortem* skin biopsy) is much bigger [area around was up to  $0.4 \pm 0.1$  (sd) mm, averaged for six samples] than for living tissue [ $0.1 \pm 0.05$  (sd) mm, averaged for seven samples].

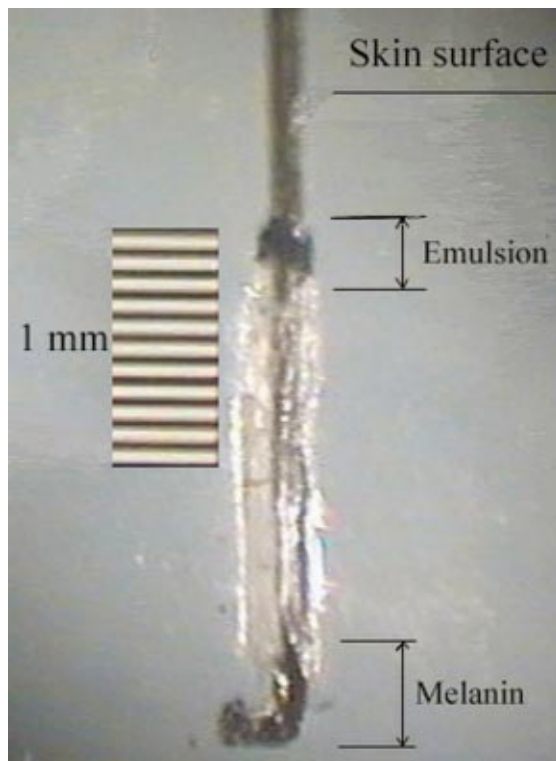
The study of time-dependent penetration of the dye (hair epilation on 15, 30 min, 1, 2, and 5 h after dyeing) at usual procedure of dyeing (15–20 min at massage and heating) has shown that the most intense dyeing of hair follicles was just after the procedure. The high intensity of dyeing remains for



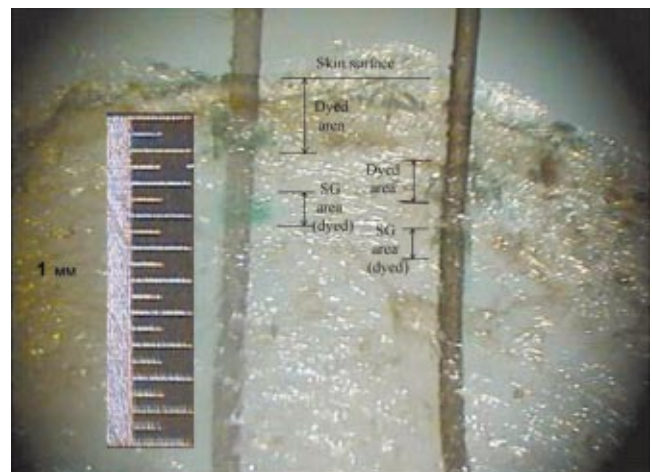
**Fig. 3** Image of the hair in the telogen stage epilated from the dyed by Palomar ICG lotion-1 forearm of the volunteer just after dyeing. Dye distribution is shown as green areas within the hair canal, the sebaceous gland (SG), and the upper part of the club.



**Fig. 4** Image of the hair in the telogen stage epilated from the dyed skin of volunteer's forearm just after dyeing (Indigo Carmine in glycerol). Dye distribution is shown as the blue areas within the hair canal, the SG, and the upper part of the club.



**Fig. 5** Image of the hair in the anagen stage epilated from the skin of volunteer's forearm just after treatment by Phospholipid Nanoemulsion of carbon particles. Emulsion distribution is well seen as slightly dark areas within the hair canal, the SG, and the upper part of the club.



**Fig. 6** Image of biopsy of the skin from dyed by Palomar ICG Lotion-1 forearm of the volunteer just after dyeing. The left hair shaft is in the anagen stage and right one is in the catagen stage. Dye distribution is shown as green areas within the hair canal and the SG.

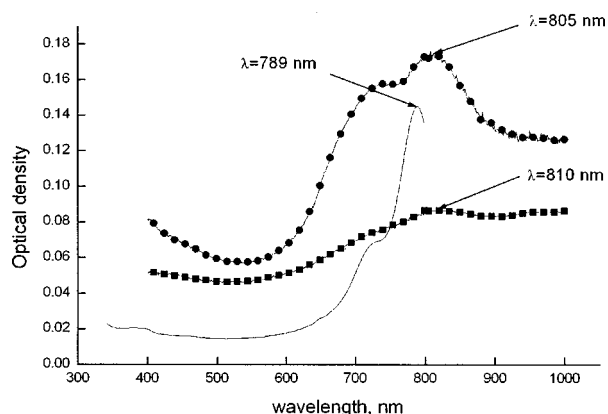
**Table 1** Mean depth of the dyed area of the follicles after action of some dyeing solution.

Stage of hair follicle	Mean depth of dyed area of follicle (mm)		
	ICG-lotion	IC in glycerol	PN
Anagen	0.6±0.1 (sd)	0.5±0.1 (sd)	0.55±0.15 (sd)
Telogen and catagen	1.1±0.1 (sd)	0.9±0.1 (sd)	0.85±0.25 (sd)

an hour, but then the intensity decreases. It is possible that the dye diffuses in ambient tissue. 5 h later after the procedure the dye on the extracted follicles was imperceptible.

## 5 Discussion

Some understanding of the presented results follows from the hair follicle and surrounding tissue structure for various stages of a life cycle. The follicle is bound by a basement membrane (“glassy membrane”). The ORS is the most peripheral of the cellular compartments. The permanent portion of the follicle begins with the hair canal region, extending from the skin surface to the level of the epidermal-dermal junction. Its lower part later becomes the “infundibular unit.” That means, that the region between epidermal-dermal junction (opened to the skin surface) and intraepidermal infundibulum is the path for lotion penetration. Only a few cell layers of living cells of the epidermis and the basement membrane (very thin) separate hair canal filled with lotion from dermis, which should be, due to its fibrous structure (collagen fibers), enough permeable for the lotion. Thus, the dermis around the ORS, the ORS itself, as a living cell structure, can be dyed. In fact, in the experiments sometimes the dyed area around the hair shaft was about two times bigger than the diameter of the hair shaft. But usually the dyed area around the hair shaft was only slightly bigger than the diameter of the hair shaft, so ICG does not penetrate deeply within the dermis at least in high concentration, but the living cells of the inner and outer root sheaths were always dyed. The transient portion of the follicle begins at the area of the bulge and extends to the deepest



**Fig. 7** Optical spectra of Palomar ICG lotion (solid curve, optical density is divided by 5) and two first strips (circles—the first strip, squares—the second strip) obtained from the skin dyed by Palomar ICG lotion-2.

levels of the follicle. The epilated hair contains: the whole follicle, including the ORS as an outmost part of epilated follicle. The upper part of the follicle and the ORS cells are dyed. The basement membrane and dermis around the hair follicle for some hairs also should be dyed.

The IRS, which normally disintegrates in the anagen follicle at the level of the sebaceous duct, is totally absent in the telogen follicle. That means that the keratinized portion of a hair follicle increases with the forming of a hair club and a gap (channel) between the hair shaft and the keratinized ORS forms. Thus, a dyeing substance diffuses more deeply. Therefore, the penetration depth of the lotion for the catagen and telogen hairs should be higher (as a distance from the skin surface). It is very important to say that the upper part of the hair follicle, a so-called permanent portion, especially the area around the sebaceous gland and even the isthmus, is always dyed for all phases of hair growing (catagen, telogen, or anagen).

To clean the skin surface thoroughly is important to eliminate overheating of the skin upper layers when laser hair removal is processed. The shift of the absorption bands of ICG in strip optical spectra farther to the IR region (see Figure 7) is caused by ICG binding with organic molecules of a living tissue. It will provide a more precise correspondence of the acting laser radiation and dyed tissue target. In some papers it was reported that the IR peak of ICG is moved due to binding ICG with cell proteins: to 805 nm for the blood<sup>26</sup> and rabbit skin *in vivo*,<sup>27</sup> to 810 nm for epidermal cell cultures.<sup>28</sup> In the present study for the stratum corneum of the human skin (*in vitro* measurements of *in vivo* dyed skin) the peak was moved to 805 and 810 nm (see, Figure 4). Our study and the Weer-sink et al. paper<sup>27</sup> show the influence of a free ICG component taking place, because nothing was done to wash out free ICG, where the IR peak is observed at 789–790 nm. Fickweiler et al.<sup>28</sup> washed out carefully free ICG from the studied samples, that is why they exclude the influence of free ICG and received pure spectrum of the bounded ICG at 810 nm. Spectra for Palomar ICG lotion-2 [see, Figure 2(b)] demonstrate that at the skin surface (the first strip) we have some mixture of free and bounded ICG (peak is at 805 nm) and at the depth of 3–5  $\mu\text{m}$  (the second strip) all ICG is bounded (peak is at 810 nm, corresponds well to the cell culture measurements of Fickweiler et al.).<sup>28</sup>

In comparison with a living tissue the ICG distributions in the *post mortem* dyed skin samples were more diffusive and not so deep [the depth is not more than  $0.6\pm 0.1$  (sd) mm, averaged for six samples]. The transverse diffusion of dye around the hair (determined from *post mortem* skin biopsy) is much bigger [area around was up to  $0.4\pm 0.1$  (sd) mm, averaged for six samples] than for living tissue [ $0.1\pm 0.05$  (sd) mm, averaged for 7 samples]. This might be explained by some degradation of the cells and the basement membrane of hair follicle. Nevertheless, the main pass of a dye penetration is through the hair canal. The sebaceous gland serves as a reservoir.

## 6 Conclusion

In this study we have investigated some dyes and absorbing emulsion for potential use in laser selective thermolysis. The values of the absorption coefficient of the dyes and emulsion

were obtained. Indocyanine Green is safe for humans. It has the greatest absorbency at 800–810 nm. Indigo Carmine is suitable for use of the He–Ne laser (633 nm). Phospholipid Nanoemulsion with carbon particles can be used for visible and NIR irradiation.

We have developed ICG lotions and a method of dyeing and have tested them *in vivo*. By the studying of epilated and taken by biopsy hair follicles it was shown that the ICG penetration depth is in the range from 0.3 to 1.2 mm depending on the hair growing stage.

It was found that dyes penetrate into the hair follicle through the hair canal and that the sebaceous gland serves as a reservoir for more deep dye penetration. For *in vivo* skin dyes are mainly distributed within a small area around the hair shaft, which includes the inner and outer root sheaths cells that should provide a high concentration of a dye in the area around the hair shaft, i.e., more effective photothermolysis under laser irradiation.

The shift on 21 nm of the absorption peak of ICG to the longer wavelengths due to ICG binding with ceratinocytes in the human skin was found.

Designed lotions and dyeing technology should be applicable for laser photoimmunotherapy<sup>3–6</sup> and photodynamic therapy<sup>29–31</sup> of hair follicle lesions as ICG is a photodynamic dye.<sup>27,30,31</sup>

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