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Fluorescence Spectroscopy of Biological Tissues—A Review

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Abstract: Fluorescence of the skin, enamel, dentin, and bone are reviewed. Fluorescence spectroscopy is one of the noninvasive methods that can identify diseases and promote increasing the knowledge in medical diagnosis. The microstructure and composition of biological tissues are presented, followed by a description of chromophores, fluorophores as identified by use of applied fluorescence techniques.

Keywords: Fluorescence, skin, enamel, dentin, bone

INTRODUCTION

Biological tissues consist of heterogeneous structures that promote light scattering. They contain chromophores that absorb light, as well as fluorophores

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that absorb and reemit light. The consequent tissue optical properties such as scattering, absorption, reemission, and reflection can help us characterize the tissue and identify diseases by noninvasive methods. In this article, several tissues are studied (skin, enamel, dentin, and bone) and their excitation and fluorescence peaks will be compared with the peaks observed for biological molecules (fluorophores).

BIOLOGICAL TISSUE ARCHITECTURE AND COMPOSITION

Skin

The human skin is divided into layers. Each has a specific composition that provides it with a characteristic optical profile (1). The outermost layer of the skin is the stratum corneum. This layer is composed mainly of dead cells embedded in a lipid matrix (2). The second layer, beneath the stratum corneum, is the epidermis. In this layer, melanin absorbs a great part of the light. Following the epidermis comes the dermis, which is composed of connective tissue, nerves, and blood vessels. Under the epidermis, the hypodermis is composed of adipose tissue.

Hard Dental Tissues

The tooth consists of four tissues: enamel, dentin, cement, and pulp tissue. The pulp irrigates the tooth interior with nutrients originating from the blood. This tissue is composed of blood vessels, nerves, odontoblasts, and fibroblasts. The dentin surrounds this tissue and is recovered by the cement at the subgengival region of the tooth and by the enamel at the supragengival region. The dentin is structured with tubules (diameters between 1 and 5 μm) that arise at the Q1 pulp-dentine interface and belong up to the dentin-enamel interface. These tubules are filled with water and odontoblastic processes (cells responsible for dentin production) occur there. The collagen molecules are oriented orthogonal to the tubules, and hydroxyapatite crystals are inserted within the collagen matrix. In the enamel, the crystals are bound together, forming bundles called prisms (diameter of about 5 μm). These prisms start at the dentin-enamel interface and extend up to the tooth external surface. The enamel organic material and water are concentrated in the interprismatic regions, while the prisms bulk are composed mainly of hydroxyapatite crystals.

The biological hard tissues, enamel, dentin, and bone consist of a mineral matrix (hydroxyapatite), water, and an organic matrix (collagen and a small fraction of non-collagen proteins, lipids, citrates, and sugars) (3) (4). The chemical composition of the three tissues are compared in Table 1. The enamel has a small organic matrix (1 wt%) and a major inorganic matrix (97 wt%), while the dentin and the bone have a larger organic matrix

Table 1. Percentage values of the organic matrix, mineral matrix, and water present in the human enamel and the dentin tissue (4) and references Q2

	Dentin		Enamel		Bone	
	vol%	wt%	vol%	wt%	vol%	wt%
Inorganic matrix	47	70	87	97	36	65
Organic matrix	30	20	2	1.5	35	25
Water	21	10	11	1.5	28	10

(respectively, 20 wt% and 25 wt%) and an inorganic matrix of about 69 wt% for the dentin and 65 wt% for the bone.

Bone

The bone structure and composition vary between different parts of the skeleton and with age, but some general bone features can be described (5). Contrary to the enamel and the dentin, the cells in the bone are continually dissolving and forming the hydroxyapatite crystals, so that this tissue is remodeled during life. The microscopic base structures of the bone are the tubular elements, also called osteons, with a combination of collagen and hydroxyapatite crystals (6).

BIOLOGICAL CHROMOPHORES

The main biological molecules that absorb the light in the ultraviolet, visible, and near infrared spectral regions are listed in Table 2. Proteins, collagen,

Table 2. Main chromophores present in biological tissues and their absorption peaks in the ultraviolet, visible, and near infrared spectral region

Absorption peaks (nm)	Chromophore	Reference
412, 542, 577	Oxyhemoglobin	(13) (25) (26)
430, 555, 760	Deoxyhemoglobin	
Increase to short wavelengths	Melanin	
760, 900, 1250, 1400, etc.	Water	
460	Bilirubin	
260	DNA/RNA	
280	Urocanic acid	
~290, ~320	Collagen	(27)
~325	Elastin	

elastin, DNA/RNA and urocanic acid absorb in the ultraviolet region (wavelength shorter than 400 nm), while oxyhemoglobin, deoxyhemoglobin, melanin, and bilirubin absorb light in the visible region (400–700 nm). In the infrared region (wavelength longer than 700 nm), we can observe the deoxyhemoglobin band (760 nm), water bands (760 nm, 900 nm, 1250 nm, 1400 nm, etc.), and other vibrational absorption bands at higher wavelengths (not listed).

The two types of hemoglobin are found in blood cells and the high absorption band observed near 400 nm gives blood its reddish color. Melanin is observed in the epidermis and is responsible for the skin color (7). Bilirubin and β-carotene can be found in all skin layers: stratum corneum, epidermis, and dermis; and the structural proteins, collagen and elastin, are found in soft tissues as well as in hard tissues such as dentin and bone.

OPTICAL SPECTROSCOPY TECHNIQUES

Changes with age, diseases, or other cellular processes will also change tissue properties, so these changes can be used to distinguish between these tissues and the healthy ones. Different optical techniques have the potential to access the tissue optical characteristics by noninvasive procedures.

When light interacts with the tissue it can be absorbed, reflected, reemitted, or scattered. Absorbed light can be measured by ATR (attenuated total reflection) or photoacoustic techniques; reflected light can be measured by diffuse reflectance spectroscopy, visible-infrared images, OCT (optical coherence tomography), or confocal microscopy; re-emitted light can be measured by fluorescence-excitation spectroscopy, two-photon microscopy, or confocal microscopy; and scattered light can be measured by scattering spectroscopy or Raman spectroscopy.

When molecules are stimulated by light in the cells, they respond by becoming excited and can thus re-emit light of varying wavelengths, which can be measured. Just as a prism splits white light into a full color spectrum, laser light focused on the tissue can be reemit in colors determined by the properties of the molecules and its environment.

FLUORESCENCE SPECTROSCOPY TECHNIQUES

While it is beyond the scope of this review to fully describe fluorescence spectroscopy, the following will provide an overview for the readers. Detailed description is to be found elsewhere (8).

Usually simplified diagrams such as that presented in Figure 1 are used to represent the energy levels of a molecule. These energy levels include the ground electronic state (S_0) and higher energy electronic states (e.g., S_1, S_2) reached upon the absorption of light and are represented by thick lines. Each electronic state of a molecule also contains numerous vibrational and

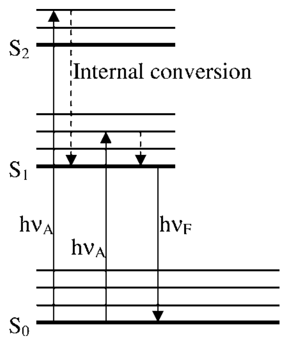

Figure 1. Schematic representation of a fluorophore energy levels.

rotation energy levels that fully describe the energetic of the system and is represented by thin lines in the figure.

The absorption of a photon with energy $h\nu_A$ excites the fluorophore from its electronic ground state (S_0) to upper electronic states ($S_1, S_2, \ldots$). The exact vibrational and electronic level reached will depend upon the energy content of the light absorbed. Regardless of the excited level reached, the molecule will rapidly lose energy to its environment through non-radiative modes (internal conversion) and will revert to the lowest vibrational level of the lowest electronic excited state. The transition from this state to the ground state may be accompanied by the emission of a photon with energy $h\nu_F$ in the process called fluorescence emission. The molecule may persist in this lowest level of the S_1 state for a period of time known as the fluorescence lifetime, which, for most fluorophores of interest in tissues, are in the range of several nanoseconds to a few tens of nanoseconds.

The main components for a fluorescence spectroscopy instrument are represented in Figure 2. The excitation source can be lamps (deuterium,

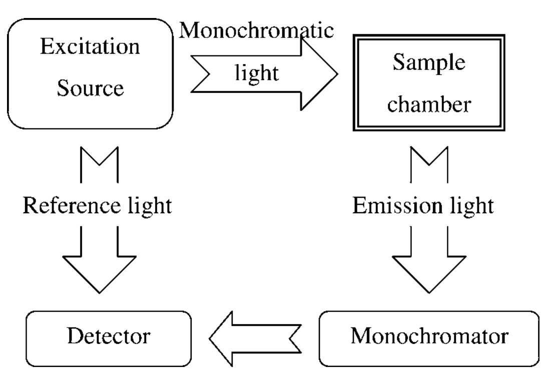

Figure 2. Schematic illustration of the instrumentation for fluorescence spectroscopy.

Table 3. Fluorescence peaks of soft tissue samples

Excitation (nm)	Florescence peaks (nm)						Sample description	Reference
270	300	315	325	350			Type I acid-soluble collagen (0.05% in 0.5 M acetic acid)	(28)
350				430				
337			390	460			Normal colonic tissue	(29)
			390	460	630	680	Hyperplastic polyps; adenomatous polyps	
			394				Collagen type I	
—			385	460			Aging human insoluble collagen-rich tissue	(9)
370				440			Collagen from rat and human tissue	(10)
337			380				Type I collagen (achilles tendon)	(19)
				420			Type I collagen (calf skin)	
				400			Type I collagen (rat tail)	
			380				Type II collagen (bovine tracheal cartilage)	
			380				Type II collagen (bovine nasal septum)	
			385				Type III collagen (human placenta)	
				410			Type IV collagen (human placenta)	
				405			Type V collagen (human placenta)	
280				410			Skin	(30)
325				405				
365				440			Rat tail tendon	(31)
330	~427							
480		~540						
510			~560					
570				~620			Native collagen	(32)
630					~670			
680						~690		

xenon, tungsten), light-emitting diodes (LEDs) or lasers systems. If the source is not monochromatic, like the lamps, additional components such as monochromators or filters must be used to select the desired excitation wavelength or range of wavelengths.

After selecting the excitation wavelength, the beam can be handled using lens, mirrors, or fibers to irradiate the sample. The emitted light from the sample will be selected by a monochromator and detected through photodiodes, charge-coupled devices (CCDs), InGaAs detectors, or photomultipliers. Usually the emission beam is examined in a direction that makes an angle of 90° from the excitation beam direction, in the so-called L geometry, to avoid detection of transmitted light. Emission (or excitation) spectra are obtained fixing the excitation (or emission) wavelength and measuring the intensity of the light coming from the sample as a function of the emission (or excitation) wavelength.

FLUORESCENCE OF SOFT TISSUES

Fluorescence peaks of soft tissues are summarized in Table 3 and the excitation peaks in Table 4. Fluorescence peaks of normal soft tissues occur in

Table 4. Excitation peaks of soft tissue samples

Fluorescence (nm)	Excitation peaks (nm)						Sample description	Reference
360	265	280					Type I acid-soluble collagen (0.05% in 0.5 M acetic acid)	(28)
435			350–360					
—			335	360			Aging human insoluble collagen-rich tissue	(8)
440				370			Collagen from rat and human tissue	(9)
—	295	335	350	370			Human skin (27-year-old volunteer)	(13)
	295	335	350	370	380	420	Human skin (70-year-old volunteer)	
350	264						Skin	(30)
410		322						

Table 5. Fluorescence peaks of natural and carious hard tissues

Excitation (nm)	Fluorescence (nm)						Sample description	Reference
280		407					Natural dentine	(30)
325		413						
365				440	590	640	Whole bone; collagen and apatite extracted from bone samples	(31)
375				460	560		EDTA dissolved human dental enamel	(33)
				460	560		EDTA dissolved synthetic hydroxyapatite	(33)
250–320		397–402					Sound human dentin	(34)
285	355						Fluorophores extracted from normal dentin with HCl	(35)
350		410–440						
280–370	350	405		450	520		Solid human/bovine enamel	(36)
	360	410		455			Organic component extracted from enamel	
295	360	400					Hydrolyzed enamel and dityrosine	(37)
365		430–450					Natural dentin	(11)
480			550				Carious and non-carious enamel	(38)
337		400					Carious and non-carious enamel	(33)
488			540					
407			590	625	635	700	Carious enamel and dentin	(16)
250–320		425					Carious human dentin	(34)
400		480		624	635	690	Carious enamel	(39)
400		480		624	650	687	Root carious	(40)
405		455	500	582	622		Sound and carious enamel	(41)
337		440	490	590	630		Carious enamel	(42)

(*continued*)

Table 5. Continued

Excitation (nm)	Fluorescence (nm)						Sample description	Reference
337	405	435	490	525			Sound and tooth	(43)
	405	435	490	555			Dentin level caries	
	405	435	490	530	635		Pulp level caries	
420		495	595		635	695	Dental calculus (supragingival)	(44)
420		495	595		650	695	Dental calculus (subgingival)	
635					700	783	Dental calculus (subgingival)	(45)
655					720	810	Carious dentine	(45)
405			500				Sound and carious enamel	(46)

the spectral region between 300 nm and 460 nm, and the excitation peaks occur in the region between 265 nm and 370 nm. During aging of human collagen in the skin tissue, an increase in the fluorescence and excitation peaks occurs (9, 10). The collagen excitation (350 nm) and fluorescence (430 nm) maxima increase significantly between young (age 19) and old (age 81) humans. An absorption band is also observed in the ultraviolet region (250–400 nm) (11, 12) and additional excitation peaks are observed at 380 nm and 420 nm in old human skin (13).

FLUORESCENCE OF HARD TISSUES

Wavelength of fluorescence peaks of natural hard tissues are summarized in Table 5 and of the excitation peaks in Table 6. The major fluorescence peaks of natural tissues occur at the spectral region between 350 nm and 560 nm, and the excitation peaks between 268 nm and 375 nm. The fluorescence and excitation peaks of carious tissues occur at longer wavelengths: fluorescence in the region between 540 nm and 700 nm, and excitation in the region between 398 nm and 632 nm. In general, it is possible to observe that after caries attack, the fluorescence and excitation peaks change to wavelengths of lower energy.

AMINO ACIDS

As the fluorescence persists after collagen degradation (14), the natural collagen fluorescence must arise from fundamental units of this molecule

Table 6. Excitation peaks of natural and carious hard tissues

Fluorescence (nm)	Excitation (nm)							Sample description	Reference
350	268							Natural dentine	(30)
410		324							
320	285							HCl dissolved normal dentin	(35)
350–460	285	335	375					Solid human/bovine enamel	(36)
	285	330						Organic component of enamel	
360	285	315						Hydrolysed enamel	(37)
700		400	466	505	540	582	632	Carious lesion (enamel/ dentin)	(16)
635		400	465					White spot lesions	(39)
624		405	465					Light brown; dark brown lesions	
633		398	405	507	538			Dental calculus (supragingival)	(44)
700			402	412	506	577	628	Dental calculus (subgingival)	(45)
700		394		527			629	Carious dentine	(45)

Table 7. Fluorescence peaks at two fixed excitation wavelengths of various amino acids and peptides (15); the peaks are compared with dentin and skin samples.

Excitation (nm)		
280	325	Amino acids/peptides
Fluorescence (nm)		
360	367	Tryptophan
—	410	Hydroxylysine
321	—	Phenylalanine
325	—	Histidine
312	414	Tyrosine
408	410	Tetraglycine
414	413	Triglycine
410	405	Glycyl-aspartic acid
415	410	Glycyl-serine
432	451	Histidyl-histidine
400	416	Glycyl-asparagine
—	408	Glycyl-proline
—	420	Glycyl-prolyl-glycyl-glycine
410	410	Dentin
410	410	Skin

such as amino acids or peptides. Dentin and skin fluorescence peaks are compared with different amino acids and peptides fluorescence peaks in Table 7 (15). For the amino acids, only hydroxylysine and tyrosine display emission near the collagen fluorescence peak (410 nm). Certain peptides like glycyl-aspartic acid and glycyl-serine also display a peak near 410 nm. Other amino acids exhibit fluorescence not exactly at 410 nm, but near this wavelength, so they can also contribute to the final collagen fluorescence observed in the case of the dentin and the skin. To summarize, the main fluorophores present in soft and hard tissues are listed in Table 8.

PORPHYRINS

The fluorescence peaks of various porphyrins are summarized in Table 9 (16) and also compared with microorganisms fluorescence peaks (17). Porphyrins are products of microorganisms and can be one of the endogenous fluorescence observed in dental caries. In the literature, the caries fluorescence peak at 635 nm is attributed to protoporphyrin IX, the 625 nm peak to coproporphyrin, and the 590 nm peak to Znproto- porphyrin (16). The main microorganisms responsible for the plaque flora are *Streptococci*,

Table 8. Assignment of the main fluorophores present in soft and hard tissues

Excitation peak (nm)	Fluorescence peak (nm)	Fluorophores	Reference
270	320	Tyrosine	(47)
295	345	Tryptophan	
335/370	390/460	Collagen cross-links	
420/460	500/540	Elastin/collagen cross-links	(13)
405	600	Porphyrins	
350	460	NAD/NADH	
370	460	Keratin, horn	
280	350	Tryptophan	(27) and references cited therein
275	300	Tyrosine	
260	280	Phenylalanine	
325	400, 405	Collagen	
290, 325	340, 400	Elastin	
450	535	FAD (flavin adenine dinucleotide), flavins	
290, 351	440, 460	NADH (reduced nicotinamide adenine dinucleotide)	
336	464	NADPH (reduced nicotinamide adenine dinucleotide phosphate)	
327	510	Vitamin A	
335	480	Vitamin K	
390	480	Vitamin D	
332, 340	400	Pyridoxine	
335	400	Pyridoxamine	
330	385	Pyridoxal	
315	425	Pyridoxic acid	
330	400	Pyridoxal 5-phosphate	
275	305	Vitamin B_{12}	
436	540, 560	Phospholipids	
340–395	540, 430–460	Lipofuscin	
340–395	430–460, 540	Ceroid	
400–450	630, 690	Porphyrins	
270–280	360	Excimer-like species	(32) (48)
325	400	Dityrosine	(49)
370	450	Age-related modification	(48)? **Q3**

Actinomyces, and *Bacteroides*. No typical fluorescence in the red spectral region was found in the case of bacterial strains *Streptococcus mutans* and *Lactobacterium*. Fluorescence was observed only in the case of *Actinomyces odontolyticus*, *Bacteroides intermedius*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Corynebacterium* (17).

Table 9. Fluorescence peaks of various porphyrins in solutions (16) and microorganisms that can be found in dental caries (17). The spectra were recorded at a 407 nm excitation wavelength

Fluorescence peaks (nm)		Sample description
Porphyrins		
633	700	Protoporphyrin IX
623	690	Coproporphyrin
593	646	Zn-protoporphyrin
Microorganisms		
636	708	Actinomyces odontolyticus
635	708	Bacteroides intermedius
618/635	703	Pseudomonas aeruginosa
620	~700	Candida albicans
600	~680	Corynebacterium

FLUORESCENCE LIFETIME

Fluorescence lifetime helps distinguish between two or more compounds that emit at similar wavelengths. The fluorescence decay time of natural hard

Table 10. Fluorescence lifetime of natural hard dental tissues, carious enamel, and different collagen types of soft tissues

Lifetime (ns)	Sample description	Reference
0.1–0.2; 5.7–6.3; 17.5–19.0	Natural dentine	(11)
0.5 (15%); 3.18 (46%); 9.76 (39%)	Natural enamel	(18)
0.31 (7%); 2.27 (11%); 17.25 (82%)	Carious enamel	
20 (100%)	Coproporphyrin	(18)
3 (11%); 17 (89%)	Protoporphyrin	
2 (92%); 13 (8%)	Zn-protoporphyrin	
5.2	Type I collagen (achilles tendon)	(19)
1.05	Type I collagen (calf skin)	
1.45	Type I collagen (rat tail)	
6.1	Type II collagen (bovine tracheal cartilage)	
6.2	Type II collagen (bovine nasal septum)	
2.95	Type III collagen (human placenta)	
1.25	Type IV collagen (human placenta)	
1.05	Type V collagen (human placenta)	

dental tissues, the carious enamel, and soft tissues are summarized in Table 10. Both the enamel and the dentin exhibit a fluorescence spectrum consisting of three different fluorescence lifetimes (11, 18). In the carious enamel it is possible to observe an increase in those lifetimes. As protorphyrin IX is considered one possible source of the carious fluorescence (18), in the same table we compare the fluorescence lifetime of different porphyrins. In soft tissues, the different collagen types (Table 10) have fluorescence with lifetimes roughly between 1 ns and 6 ns (19), similar values to that of the three lifetimes observed in dentin. Another group of molecules, the metal-free porphyrin monomers, have a long fluorescence lifetime, of about 10–20 ns (20–23). In contrast, most of the endogenous fluorophores like the fluorescent coenzymes NADH and flavin molecules or the amino acid tryptophan have lifetimes shorter than 6 ns (24).

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