Added-value of non-invasive fluorescence lifetime imaging in wound healing studies

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Small literature overview in context of the FWO interdisciplinary grant submission: Enabling Wound Monitoring Through Real-time Fluorescence Lifetime Imaging with Novel Time-Gated Image Sensors.

The various phases of wound healing include the **hemostasis** or coagulation phase, an **inflammatory** phase in which white blood cells clean the wound from dirt and bacteria, a **proliferative** phase in which the wound bed is filled with connective tissue, new blood vessels are formed, and epithelial cells migrate across the wound bed, and, a **maturation** or remodeling phase in which the newly formed tissue matures and increases in strength [1]. In each and every phase a single deviation can result in impaired wound healing, which can result in the development of a **chronic wound**. By definition, chronic wounds are wounds that fail to proceed through the normal wound healing phases in an orderly and timely manner [2]. Chronic wounds are often referred to as **hard-to-heal** or **difficult to heal wounds** [3]. Because these wounds are typically managed as a co-morbidity of other conditions, clinicians often lack specialized training in the diagnosis and treatment of wounds [4]. Subjective evaluation and high risks of over- and under-debridement of tissue during wound assessment can impair wound healing [5]. There is a **need for objective quantification of the wound healing process**.

In attempt to provide clinicians with a tool that can quantify wound bed characteristics to offer dynamic and individualized wound care, **non-invasive fluorescence intensity (FI) imaging studies** have been ongoing. **FI** imaging involves the excitation of **endogenous** or **exogenous** administered fluorophores, with light from which the wavelength is tailored to the match the fluorophore absorption spectrum. When absorbing photons, the fluorophore is excited into a higher state of energy, from which it almost immediately returns back to its ground state by re-emission of photons. This light consists of a longer wavelength than the excitation light and can be visualized with an advanced imaging system, designed to detect the fluorescence emission [6]. **Autofluorescence** or **endogenous fluorophores**, require excitation within ultra-violet or visible wavelengths. In contrast, **exogenous fluorophores** are preferably developed for excitation in the **near-infrared region (NIR)**, where endogenous fluorescence of tissue is very weak to absent [7]. Because of the natural ability of tissue and cells to emit light after excitation in the **ultra-violet** and **visible wavelength**, endogenous fluorescence is seen as a potential tool for research and diagnosis of the healing of the wounds [8].

In vitro, cell studies are often focused on a single biomarker and a chosen emission window which can provide the user on cell location and cell metabolism. For example, **NAD(P)H** and **FAD** are key determinants of cellular metabolism and emit peak fluorescence at violet wavelengths [9]. In contrast, **bacteria** emit fluorescence at visible wavelengths [10]. **Exogenous** contrast agents can emit fluorescence in the **NIR,** but also at lower wavelengths depending on the chemical structure (Figure 1).

It should, however, be noted that the emission spectra of various fluorophores overlap.

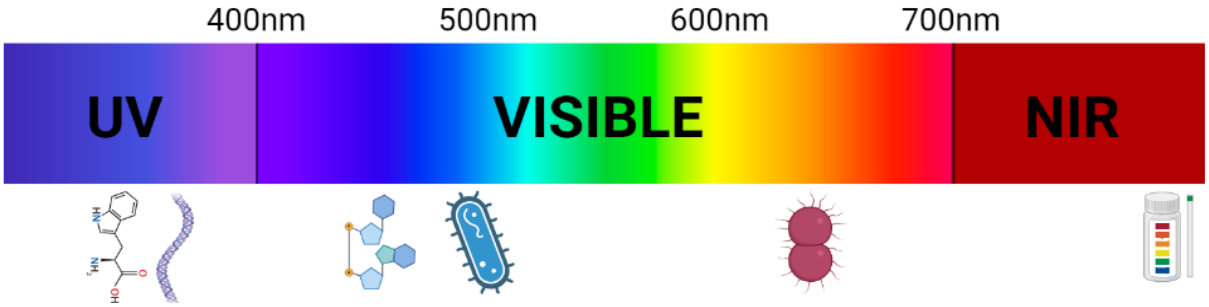

Figure 1: Possible fluorescence emission wavelengths from endogenous (UV-Visible) and exogenous fluorophores (UV-Visible-Near-Infrared(NIR))

An overview of various biomarkers involved in wound healing and corresponding emission spectra, based on intensity microscopy studies or fluorometer measurements, can be found below (Table 1).

Table 1 Overview of autofluorescence intensity signals arising from cells involved in wound healing

Cells	Biomarker correlations	AF emission	Wound healing phase	References
Red blood cells	Hemoglobin	420-600nm	Hemostasis	[11]
Platelets	NAD(P)H FAD	513-673nm 475-600nm	Hemostasis	[12]
Eosinophils (1), Neutrophils (2), Macrophages (3)	NAD(P)H (1,2,3) FAD (1,2,3) Tryptofaan (1,2,3)	440-600nm 475-600nm 300-400nm	Inflammatory	[13]
Apoptotic cells	N/A	N/A	Inflammatory	
Fibroblasts	NAD(P)H Flavin Lipopigments	400-750nm 475-600nm	Inflammatory Proliferative	[14, 15]
Collagen	Hydroxyproline Pepsine	380-410nm	Proliferative	[16, 17, 8]
Dermal adipocytes	N/A	N/A	Proliferative/ Inflammatory	[18]
Epithelial cells	Cytokeratin Tryptofaan	495-525nm 300-400nm	Proliferative	[8]

When using fluorescence intensity on a macroscopic level, the **intertwined fluorescence emission levels** from multiple biomarkers **limit the use of intensity-based imaging** to provide information on the wound healing phase, the direction in which the wound healing phase is going, and the vitality of the wound bed. **In contrast**, the potential use of **FI** imaging in the detection of **bacteria in the wound bed** has been assessed in multiple ***in vivo*** studies (table 2). The determination of the presence of bacteria is made by searching for locations with fluorescence emitting in the cyan (485-500nm) or **red (625-750nm) emission window** due to the bacterial production of porphyrins or pyoverdine. While in the red emission window, autofluorescence from tissues within the wound bed is weak, the signal arising from bacteria in the cyan window has additional overlap with AF signals arising from various biomarkers in the wound bed. Furthermore, **subtyping of bacteria in the wound bed remains difficult and the bacterial load of the wound bed cannot be assessed** [19]. Additionally, the bacterial load is only detected when higher than 104 CFU/g, which is seen as the tipping point between requiring careful alertness of the wound to requiring intervention [19]. Difficulties in the interpretation of results can introduce **variability and subjectivity** during wound diagnosis and prediction of prognosis.

Fluorescence lifetime imaging (FLT) imaging measures fluorescence in the time-domain. Because the **FLT** can reveal information on metabolism [20] of cells or tissues [21], as well as is influenced by the environment of cells and tissue [22], **FI** signals can be differentiated when measuring the signals in the time-domain. Because **wound healing is a highly metabolic** demanding process [23], in which **cells** and **clinical signs** (rubor, calor, tumor, dolor) **transform** over time, **FLT** imaging has potential to guide clinicians during wound assessment and care as an objective quantitative tool that can provide information on the vitality of the wound bed [24, 25], contamination of the wound bed with subtyping of bacteria [26], and predict in which direction the wound healing phase is turning as the metabolic pathways change.

Table 2 Overview of fluorescence studies focusing on wound healing in vitro and in vivo

Cell study	*In vitro* *in vivo?*	*Healing phase*	*Wound biomarker*	*Emission*	*FI or FLIM*	*References*
Evaluation of keratinocyte function	*In vitro – 2D*	Proliferative	NAD(P)H FAD	490-252 490-525	FLIM	[27]
Evaluation of cellular epithelization	*In vitro – 3D*	Proliferative	Pepsine Tryptophane	390nm 340nm	FI	[8]
Evaluation of bacteria in the wound bed	*In vivo*	Inflammatory/ Infection	N/A; bacteria	N/A Cyan/red	FI	[19, 28, 29]

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