Composite phantoms for quality control and standardization of fluorescence molecular imaging systems

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1. Introduction

Despite the poor ability of human vision to detect disease, it still remains the main diagnostic tool until now. Fluorescence molecular imaging (FMI) is an emerging technique that has shown a great potential to assist visual inspection of tissue and to improve early diagnostics and disease management. Near-infrared (NIR) FMI enables in vivo visualization of molecular processes in whole tissues by targeting upregulated proteins, overexpressed receptors, or disease-specific biomarkers. Therefore, it enables real-time highlighting of the targeted lesions, functioning as a “red-flag” identification method that can guide diagnostic or surgical interventions. Up to date, there have been various fluorescent agents introduced that can target different biochemical and molecular disease features. The performance of FMI is currently assessed through numerous FMI Phase I and II clinical studies, many of which have demonstrated greater detection sensitivity and subsurface imaging capability than the gold-standard human vision. Examples include the use of folate receptor targeting agents to improve detection of ovarian cancer [1], fluorescently labelled cetuximab to improve surgical accuracy in oral cancer [2], and protease-activated agents to detect soft tissue sarcomas [3]. In addition, FMI has shown promising results in the detection of colorectal dysplasia and Crohn's disease in humans, as well as for the early detection of esophageal cancer in Barrett’s patients [4, 5].

One of the challenges towards translation of FMI to clinics and becoming an established diagnostic tool is the need for system standardization[6]. Different FMI systems employ different illumination and excitation sources, detectors, experimental parameters and visualization/processing techniques. In addition, fluorescent agents cover different spectral ranges including visible, near infrared, and shortwave infrared, further complicating the characterization of FMI performance.

In 2018 Koch et al.[6] introduced the concept of high-fidelity fluorescence imaging (HiFFI) to provide accurate clinical FMI. This concept is proposed to ensure that the FMI measurements are independent of particular systems, experimental and tissue conditions. Therefore, it is expected that recorded fluorescence images do not change with modification of acquisition settings and represent consistently and accurately fluorochrome biodistribution within the tissue.

In the real world, many parameters related to the camera system and its operation (i.e. invariable) or to the imaging procedure and target (i.e. variable) affect the image quality and the signal-to-noise ratio of the acquired fluorescence and potentially could lead to

data misinterpretation. Therefore, HiFFI requires a technology that can consistently provide stable fluorescence readings regardless of the invariable and/or variable parameters.

While variable parameters vary from measurement to measurement or even during the same measurement and may require continued monitoring throughout the experiment, differences in the readouts due to unchanged parameters can be characterized once or repeatedly to assess the system and its performance. Along with HiFFI, two key principles were proposed to improve FMI fidelity: standardization and reversion. Standardization involves the system selection and/or control according to the parameters that influence the measurements, while reversion involves correcting the effect of these parameters on the fluorescence image and quantification of the acquired data [6].

One of the commonly used tools for device/method performance assessment in biomedical imaging is the use of imaging phantoms mimicking tissue features. For optical imaging, these phantoms should meet three criteria: 1) reproducibility of the target optical (absorption and scattering) and fluorescence properties; 2) maintain photostability in different environmental conditions over time; 3) maintain a constant shape without mechanical deformation. To meet these requirements, optical phantoms are typically based on such materials as epoxy, polyester resin, or polyurethane. The optical properties can be flexibly adjusted by adding absorbers, scatterers, and fluorophores before curing. Typically, absorbing properties are based on the addition of absorbing dyes (e.g., India ink, nigrosine) and scattering properties are based on the addition of TiO_2 particles to the material. To avoid rapid photobleaching and provide better photostability, instead of organic fluorophores, quantum dots are used to adjust fluorescence properties of the phantom. Sonication enables homogeneous distribution of the components. [6, 7]

Our group has been introduced several phantoms over the past years that can be effectively used for the characterization and correction of FMI systems performance as well as data referencing and comparison of markedly different systems. In this practical lab course, you are going to use the most recent model [8] of the composite phantom developed by our group (described in the next Section) and apply an experimental protocol to standardize a hybrid FMI system.

2. Experimental setup

2.1. Fluorescence Molecular Imaging (FMI) System

During the experiment for the data acquisition you are going to use a hybrid fluorescence molecular imaging (FMI) system developed by our group (Figure 1a). It allows simultaneous acquisition of fluorescence and reflectance (color) images. The FMI system consists of an electron-multiplying charge-coupled device (EMCCD, DV897DCS-BV, Andor Technology, Belfast, UK) and a charge-coupled device (CCD, pixelfly qe, PCO AG, Kelheim, Germany) for near-infrared (NIR) fluorescence detection and color imaging, respectively. For field illumination a 250 W halogen lamp (KL-2500 LCD, Schott AG, Mainz, Germany) is used, while fluorescence excitation is achieved by a 750 nm continuous wave (CW) laser diode (FLX-750-1500M-100-9MM Frankfurt Laser Company, Friedrichsdorf, Germany) with a maximum power of 1.500 mW. At the minimum working distance (i.e., the distance between the camera lens and the tissue) the radiance of the excitation source is equal to 15.5 mW/cm^2, well below the maximum permissive exposure (MPE) according to the American National Standards Institute (ANSI) Z136 guidelines for the MPE for skin. The system operation and data acquisition can be performed through the graphical user interface (GUI) developed for the system.

2.2. FMI Composite Phantom

Within this lab training you are going to get familiar with one of the composite phantoms developed by our group [8]. Figure 1b shows the scheme of the phantom design with compounds used for each feature and Figure 1c illustrates the phantom and its features with their corresponding dimensions.

The phantom consists of four basic compounds listed in Table 1, by varying which the optical and fluorescent properties of each element can be controlled:

Table 1. Phantom materials.

Property	Compound	Producer
Scattering	TiO2 nanoparticles	Sigma Aldrich, St. Louis, MO, USA
Absorption	alcohol-soluble nigrosin	
	bovine hemin (≥90% pure)	
Fluorescence	organic quantum dots (Qdot 800 ITK)	Thermofisher Scientific, Waltham, MA, USA

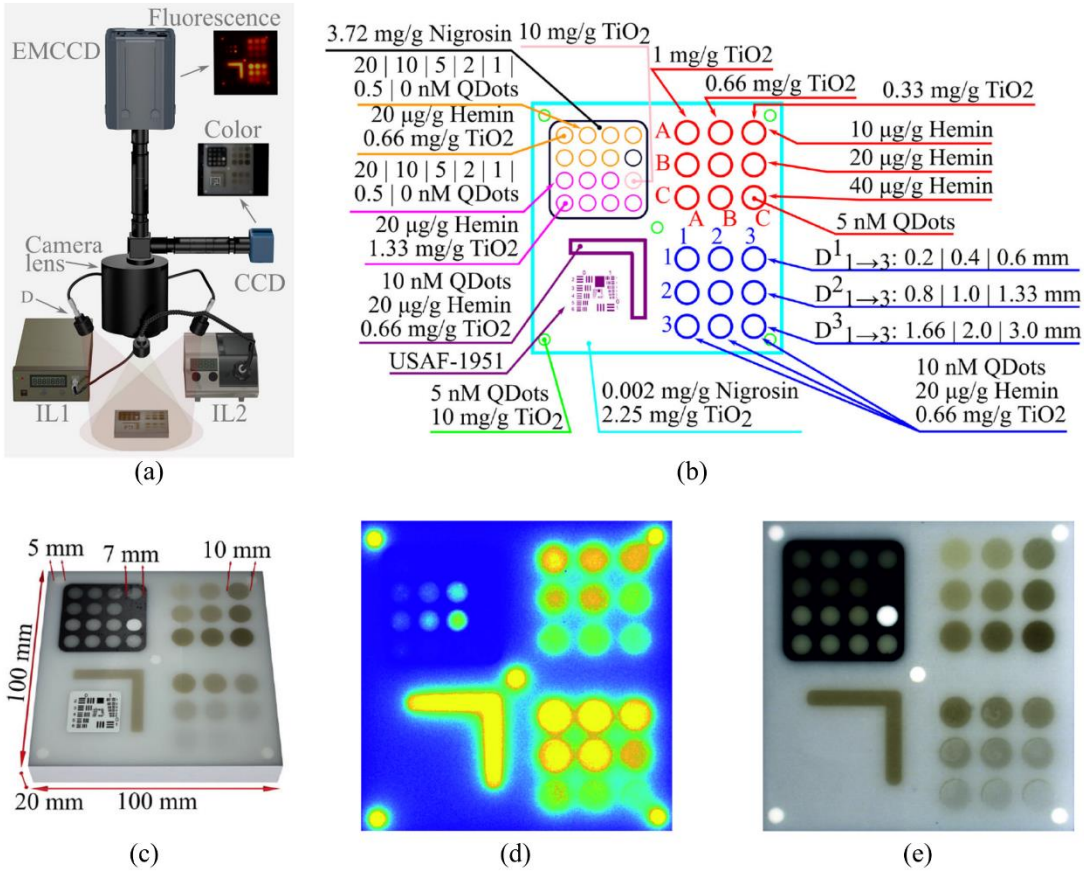

Figure 1 (a) The hybrid fluorescence/color fluorescence imaging system imaging the composite phantom; (b) Materials used to build the phantom and their concentration in each phantom element. Arrows indicate the presence in the group of elements (in a row, column or color) of compounds indicated by the tail of the line; (c) The composite phantom and its structures with corresponding dimensions. (d) Fluorescence image acquired with FMI system (presented using colormap); (e) Reflectance (color) image acquired with FMI system; (Panels a-c copied from Gorpas et al. (2020)[8]

Every phantom feature has a certain function and enables FMI system assessment based on a certain characteristic as it is described in Table 2.

Table 2. Phantom elements and quantifiable metrics.

Phantom element	Function / System characteristics	Color Code in Figure 1b
Phantom matrix with high scattering and low absorption	Simulates a realistic scenario for NIR imaging	Cyan
Nine wells with the same QDots concentration but different scattering (across columns) and absorption (across rows)	Sensitivity of the system under different optical properties	Red
Nine wells with the same optical properties at the various distances from the top surface of the phantom	Sensitivity of the system versus depth	Blue
A 1951 United States Air Force chart (USAF-1951) and L-shaped fluorescent structure	Resolution • Optical (USAF-1951) • Fluorescence (L-shaped element)	Deep purple
A grid of 14 wells with identical optical properties but gradually increasing QDot concentration	Dynamic range and light leakage • At low scattering • At high scattering	• Orange • Purple
Highly absorbing (black) block	Limits diffusion and cross-talk between neighboringwells	Black
5 wells at the phantom's corners and center with relatively high TiO2 concentration	Image uniformity	Green

By placing the phantom in the field of view (FOV) of the system and acquiring single or multiple images (Figure 1d and e), we can assess the system performance based on multiple parameters that were in detailed described in **Section 2**.

3. The experiments and data analysis

Before starting the experiment, you will get familiar with the experimental setup and the safety instructions. If you have any questions or doubts, first of all, ask your tutor.

3.1. Phantom measurements

During this practical lab course, you will gain hands-on experience on fluorescence molecular imaging by performing standardization measurements of the phantom using the system described in **Section 3.1**. The following procedure should be done to record the data:

- Measure the power of the excitation source using a power meter;
- Place the phantom in the FOV of FMI system and record the working distance;
- Start the acquisition process, make sure that both fluorescence and reflection images are obtained, and adjust the acquisition parameters of the system (gain, exposure time) so that a clear signal is obtained but saturation is avoided. Identify 2-3 sets of parameters that produce good images. For every set of acquisition parameters save 10-20 still images and a video recording.
- For every acquired image record a corresponding dark image with the excitation source disabled keeping the same acquisition parameters.

To perform processing and analysis of the acquired images, you will get a MATLAB live script (Mathworks, Natick, MA, USA) with parts of the code you need to complete for tasks **4.2-4.4** listed below.

3.2. Phantom features segmentation

As the first step, following by the data acquisition, you need to register the fluorescence and color images. For this purpose, you will use a script that allow you to manually select five fiduciary marker in each of the two images. Using those markers, you will estimate the translation matrix that will allow you to project one image to the coordinates system of the other. Then by using the fluorescence and color images you will need to manually segment the different phantom features in order of the different invariable parameters listed in Section 3.3.

3.3. Quantification of quality control metrics

After extracting all phantom components, you can start consequently quantify all camera performance metrics, listed in **Section 3.2 (Table 2)**:

- Magnification: approximate for both fluorescence and reflectance images using the following formula

$$M = \frac{D_{phantom}}{\sqrt{D_{pw}^2 \cdot (u_1 - u_2)^2 + D_{ph}^2 \cdot (v_1 - v_2)^2}}, (Eq. 1)$$

  where D_{pw} and D_{ph} are pixel width and height, respectively, and (u_1, v_1) and (u_2, v_2) are pixel coordinates of the two phantom corners.

- Optical Resolution: calculate using the USAF-1951 target at reflectance image that consists of series of elements, organized in groups. As a first step, for each one of the bounding boxes of the USAF-1951 target, calculate the contrast transfer function (CTF) by the Michelson's formula:

$$CTF_i = \frac{\max(I_i) - \min(I_i)}{\max(I_i) + \min(I_i)}, (Eq. 2)$$

  where I_i refers to the intensities of the pixels inside the i-th bounding box. By comparison of the resulting CTF to the limit value ~26.4%, we can define the target's elements resolved by the system. As a following step, you can calculate the frequency of these elements using the formula $F = 2^{group + (element-1)/6}$ and corresponding resolution value as $L_o = (2 \cdot F)^{-1}$ in mm.

- Excitation light leakage: can be calculated as $R_{exc} = {}^{S}/_{N}$, where S is average signal intensity in the high scattering element (pink well in Figure 1b) and N is average signal intensity in the high absorbing element (black well in Figure 1b).

- Parasitic illumination: assessed the same way as the excitation light leakage, using the dark images.

- Sensitivity: estimate sensitivity of the system as a function of optical properties and as a function of depth based on two metrics: signal to noise ratio (SNR) and contrast. As the first step, for every element in every group of wells calculate the average and standard deviation (std) of the signal. Using this values, calculate SNR and contrast via following formulas:

$$SNR_{dB} = 20 \cdot \log\left(\frac{S}{RMSN}\right), (Eq. 4)$$

$$C = \frac{S - N}{N}, (Eq. 5)$$

  where S is the average intensity within the well, RMSN corresponds to the root mean square noise approximated from the background and N is average signal intensityof the background. Background can be defined as the area of the phantom matrix next to the wells. Estimating sensitivity as a function means that the result should be illustrated as a plot showing the dependency of the metric on certain parameter.

3.4. Flat-fielding for spatial illumination correction

As the last step, you need to evaluate field illumination homogeneity. Use the average intensity from each of the five highly scattering elements at the four corners and the center of the phantom and apply bicubic splines interpolation to generate illumination profile. Then flat-fielding of the reflectance image can be done by dividing the image with the resulting profile.

4. Lab report

The report of the experiment should be the format of a short paper and consists of

- Short **introduction** and theoretical background;
- **Methods part:** very short description of the experimental setup and the experiments you have performed as well as information on the methods have been used for data processing and analysis;
- **Results part:** presentation of your main findings including images and plots you got;
- **Discussion part:** based on the results evaluation and answers to the following questions:
    - Why we need a dark environment for optimal FMI?
    - What can we achieve with the flatfielding?
    - What is the purpose of standardization?
    - What could be the meaning of quality control for FMI?

5. Laser safety instructions

Certain safety precautions should be maintained with the laboratory equipment used in this experiment. The primary associated risk is exposure to **laser radiation**. Therefore, every participant should get a laser safety training before the practical part of the lab training.

I understand and agree to follow the Laser Safety Guidelines explained to me during the experiment.

Student Signature: Date, place:

Student Signature: Date, place:

Student Signature: Date, place:

Instructor Signature: Date, place:

6. Literature

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