A matter of collection and detection for intraoperative and noninvasive near-infrared fluorescence molecular imaging: To see or not to see?

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Purpose: Although fluorescence molecular imaging is rapidly evolving as a new combinational drug/device technology platform for molecularly guided surgery and noninvasive imaging, there remains no performance standards for efficient translation of “first-in-humans” fluorescent imaging agents using these devices.

Methods: The authors employed a stable, solid phantom designed to exaggerate the confounding effects of tissue light scattering and to mimic low concentrations (nM–pM) of near-infrared fluorescent dyes expected clinically for molecular imaging in order to evaluate and compare the commonly used charge coupled device (CCD) camera systems employed in preclinical studies and in human investigational studies.

Results: The results show that intensified CCD systems offer greater contrast with larger signal-to-noise ratios in comparison to their unintensified CCD systems operated at clinically reasonable, subsecond acquisition times.

Conclusions: Camera imaging performance could impact the success of future “first-in-humans” near-infrared fluorescence imaging agent studies. © 2014 American Association of Physicists in Medicine. [<http://dx.doi.org/10.1118/1.4862514>]

Key words: optical imaging, molecular imaging, phantom, performance standard, charge-coupled camera (CCD)

1. INTRODUCTION

Near-infrared fluorescence (NIRF) imaging is evolving from small animal applications to clinical indications that seek to detect sentinel lymph nodes, visualize lymphatic and blood vasculatures^{1,2} as well as to detect tumor margins intraoperatively with nonspecific³ and molecularly targeting fluorescent imaging agents.^{4,5} NIRF excitation at >780 nm enables increased depth of tissue penetration due to minimal tissue absorbance and decreased background arising from autofluorescence. Prior to successfully translating NIRF molecular imaging agents into the clinic, the NIRF imaging device should be warranted as sensitive enough to detect emitted fluorescence signals from tissues.⁶ Measurements are typically performed using red-sensitive scanning or multianode photomultiplier tubes (PMTs) operating in analog mode or with charge coupled devices (CCDs) with quantum efficiencies of 40%–80% up to 800 nm. While PMTs offer the opportunity of highest dynamic range, the bit-depth of the CCD A/D converter limits dynamic range to at most four orders of magnitude. Dynamic range however can be expanded by varying integration CCD times, gain, and through pixel binning. Fast response times of CCDs are limited by virtue that they operate by integrating charge (with response times as fast as 100 Hz), while PMTs have pico- and nanosecond rise times, enabling modulated MHz operation for homodyne and heterodyne detection schemes. Nonetheless, under the conditions of low photon count rates expected in fluorescence molecular imaging applications for detecting trace amounts of targeted imag-

ing agents, integrating CCDs can offer significantly higher signal-to-noise ratios (SNRs) than that from amplified PMT anode currents. As a result of these considerations and their versatile area detection, CCDs have been the choice of detectors for the majority of fluorescence molecular imaging applications. Yet for NIRF applications that employ 780–790 nm excitation and collection of fluorescence at wavelengths >820 nm, the quantum efficiency of CCD chips fall precipitously, potentially impacting sensitivity for molecular imaging applications.

Recognizing the limitation in quantum efficiency at NIRF wavelengths and long response times of CCDs that prevent time-dependent fluorescence measurements, we adapted Gen III image intensifiers to CCDs⁷ to electronically amplify far-red (~680–780 nm) and NIRF fluorescence (~780–830 nm) signals (with gains as high as 10^6) and to convert them into green (550 nm) phosphor signals that could be collected more sensitively by a conventional integrating CCD. Due to the fast response time of the photocathode, the intensifier can be modulated as fast as 100's MHz for NIRF small animal tomography.^{7–9} In our prior work to conduct noninvasive NIRF imaging in humans, we focused upon using the intensifier as an amplifier, rather than a means to measure time-dependent processes.

Several choices of CCD detection schemes are used in far-red fluorescence (at wavelengths >650 nm) and NIRF molecular imaging devices: (i) electron multiplying CCDs; (ii) front-illuminated CCDs; (iii) back-illuminated CCDs; and (iv) intensified combinations (ICCDs) as described in Fig. 1.

FIG. 1. Schematics of various CCDs and intensifier and spectral response. (a)-(d) illustrate the schematics of front-illuminated CCD, back-illuminated CCD, electron multiplying CCD (EMCCD), and Gen III thin film intensifier, respectively. (e) Spectral response of the front- and back-illuminated CCD and Gen III thin film intensifier.

Yet, even though there has been a plethora of devices advanced for clinical translation, there has been no comparison of performance to determine the most suitable CCD configuration for fluorescence molecular imaging. In this work, we compare these CCD configurations using a solid phantom developed to mimic nM–pM concentrations of far-red and near-infrared fluorescent dyes in scattering media to differentiate performance for detecting targeted, NIRF imaging agents in tissues. We include far-red excited (>650 nm) fluorophores in our analysis since these fluorophores are frequently referred in the literature as near-infrared fluorophores.

In our past experience, the Food and Drug Administration's Center for Drug Evaluation and Research would not allow the "risk" associated with administering a "first-in-humans" molecularly targeted NIRF imaging agent until the potential "benefit" of it being detected in humans could be demonstrated with a specified device. As a result, the regulatory strategy and lack of standards for clinical studies of fluorescence molecular imaging requires the pairing of a specific device with a "first-in-humans" fluorescent imaging agent. Measurements to delineate device performance are therefore critical to emergence of clinical, fluorescence molecular imaging agents since there is no available platform of devices with known standardized performance as there are (for exam-

ple) in nuclear medicine. To demonstrate the potential performance variation of emerging devices, we conducted a systematic, controlled comparison between CCDs and an ICCD with the only viable being the CCD or ICCD camera type. To show the relevance of variable performance, we compared performance of an FDA market approved investigational device with an FDA market approved device, the NOVADAQ SPY, which is approved for intraoperative imaging and noninvasive surface vessel imaging following intravenous (i.v.) administration of the nontargeted fluorescent dye, indocyanine green (ICG). Recently the NOVADAQ has been suggested as a marketed device suitable for advancing "first-in-humans" imaging agents.¹⁰ Our results point to the importance of maximizing performance of imaging devices for successful translation of "first-in-humans" imaging agents through the use of performance measurements using standardized phantoms.

2. APPROACHES AND METHODS

2.A. Phantoms and their characterization

To enable quantification of device measurement performance associated with various CCD cameras, a fluorescent solid phantom was constructed of 6-wells machined from a

Microtest™ 96-well assay plate with 6.5 mm-diameter cylindrical wells filled with TiO₂ scattering particles to mimic exaggerated tissue scattering that can raise device noise floor¹¹ and with varying quantities of NIR fluorescent QDots 800 nanoparticles in a polyurethane solvent. During phantom construction, the QDots 800, TiO₂, and polyurethane hardener were first sonicated (Vortex Genie-2, Scientific Industries, NY) for 45 min to obtain maximum homogeneity, and then polyurethane base was added and mixed. To ensure a homogeneous mixture, the bubbles were removed through a degassing process. The mixture was then poured into the wells and set aside for approximately 48 h in order to allow complete curing.

The concentrations of QDots 800 in the phantom differ in order to mimic typical tissue concentrations of NIR fluorophore (such as ICG) used in the preclinical and clinical studies using the following relationship:

$$C_{\text{ICG}} = \frac{\varepsilon_{\text{QDots800}}(\lambda_x) \Phi_{\text{QDots800}}(\lambda_m)}{\varepsilon_{\text{ICG}}(\lambda_x) \Phi_{\text{ICG}}(\lambda_m)} C_{\text{QDots800}}, \quad (1)$$

where C is the concentration of the fluorescent dye, λ is the excitation (suffix “x”) and emission wavelengths (suffix ‘m’), ε and Φ are the extinction coefficient and quantum yield of the fluorescent dye, respectively ($\Phi_{\text{QDots800}} = 0.36$, $\Phi_{\text{ICG}} = 0.132$, $\varepsilon_{\text{ICG}} = 130\,000 \text{ M}^{-1} \text{ cm}^{-1}$, and $\varepsilon_{\text{QDots800}} = 129\,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 785/830 nm excitation/emission). From Eq. (1), one can compute that the concentration of QDots 800 corresponds to three times of that of ICG at 785/830 nm excitation/emission. It should be noted that the corresponding phantom concentration of fluorophore is not intended to reflect upon the amount of fluorophore administered to the subject needed to create contrast, but rather the amount of fluorophore within the tissues that is associated with the targeted disease marker and after its clearance from normal tissues. Due to the broad excitation and some far-red emission spectra of QDots 800, the constructed phantom can be also used for quantification of far-red fluorescence imaging systems.

2.B. CCD detector configurations

Conventional front-illuminated CCDs have relatively low quantum efficiency (QE) because their configuration requires light to pass through the polysilicon gates before reaching the photodiode, as shown in Fig. 1(a). Recently, back-illuminated CCDs were innovated by rearranging the gate structure to the back of the photosensitive area of the CCDs and by reducing the thickness of silicon layer via proprietary etching techniques, as shown in Fig. 1(b). Therefore, there is less light lost through absorption and reflection by the polysilicon gate structure in the back-illuminated CCDs, resulting in more than two fold improvement in QE compared to that of their front-illuminated counterparts. Herein we employed the existing front- and back-illuminated CCD cameras (customized fiber optic coupled CCD cameras with e2v CCD47-20 back-illuminated and CCD47-20 front-illuminated chips, Princeton Instruments, Trenton, NJ) that were cooled to -25°C . These customized fiber optic coupled CCD cam-

eras were designed to couple the intensifier as discussed below. The fiber optical taper offers better light throughput between the intensifier and CCD camera than lens-coupled configuration.

Unlike a conventional CCD, an electron-multiplying CCD (EMCCD) is installed with an additional electron multiplying (EM) register between the end of the normal serial register and the output amplifier to multiply weak signals, as shown in Fig. 1(c). The EM register consists of several hundred stages that use higher-than-typical CCD clock voltages. An impact-ionization process is initiated to produce secondary electrons as charge is transferred through each stage. The degree of multiplication gain can be controlled by varying the clock voltages applied to the EM register. Herein we used the existing back-illuminated EMCCD camera (Photon Max 512, Princeton Instruments, Trenton, NJ) that was cooled to -70°C .

Compared to EMCCDs, ICCDs have a different multiplication mechanism, in which an image intensifier amplifies the collected image before it is registered by a CCD. The intensifier is comprised of a photocathode input, an electron-multiplying microchannel plate (MCP), and a phosphor screen, as shown in Fig. 1(d). At the photocathode, the light signal is converted into electrons, which are then amplified at the MCP, and finally the amplified signal is converted back to a light signal at the phosphor screen ready for the CCD acquisition. For a standard Gen III intensifier, a thin film is positioned between the photocathode and MCP to protect spurious backscattered ions that damage the photocathode. Herein we employed the Gen III thin film image intensifier (model: 273686, ITT Night Vision, Roanoke, VA). The spectral responses of front- and back-illuminated CCD cameras and image intensifier are shown in Fig. 1(e).

To date, it has not been clear whether the conversion of near-infrared fluorescence photons to electrons at the intensifier photocathode, of electrons to photons at the intensifier phosphor, and finally photons to electrons at the CCD has signal to noise advantages of nonintensified CCD systems that directly collect near-infrared fluorescent photons for CCD output. This study is designed to compare the performance of these systems for fluorescence molecular imaging.

2.C. NIRF and far-red fluorescence imaging

For NIRF imaging of ICG, a laser diode operating at the wavelength of 785 nm (HPD 1005-9mm-78503 model, Intense, North Brunswick, NJ) was used and the laser output was filtered with a “clean up” filter (LD01-785/10, optical density >5 at 705-765 nm and 803-885 nm, Semrock, Rochester, NY) to remove undesirable sideband components. The laser output power was stabilized by a temperature controller and an optical diffuser was placed in front of the laser diode output to provide uniform illumination. The collection of fluorescence signals was implemented by using two 830 nm band pass filters (830FS10, optical density >4 , FIR-Xray, Andover, Salem, NH) to reduce the excitation light leakage and improve measurement contrast and SNR. For far-red fluorescence imaging corresponding for

FIG. 2. Evaluation of fluorescence imaging systems using phantoms. To evaluate the performance of various CCD based NIRF or far-red fluorescence imaging systems, a series of QDots 800 based fluorescence phantoms were placed under the field of view.

example to a Cy5.5 or Alexa680 dye, the corresponding laser diode and filters in NIRF imaging system were replaced with a 690 nm laser diode (HPD 1305-9mm-69005 model, Intense, North Brunswick, NJ), a 690 nm “clean up” filter (690FS10-25, optical density >4, FIR-Xray, Andover, Salem, NH) and 720 nm emission filter (720FS10, optical density >4, FIR-Xray, Andover, Salem, NH), respectively. As illustrated in the schematic of Fig. 2, the various CCD and ICCD detectors were used to record the collected fluorescence signals from the solid phantoms. The laser power incident on the illumination surface for both NIRF and far-red fluorescence imaging systems was set to 2.75 mW/cm^2 , and the field of view (FOV) is adjusted to $10 \text{ cm} \times 10 \text{ cm}$. The control of devices and data acquisition was implemented under a custom LabVIEW interface (National Instruments, Austin, TX).

To evaluate integrating detectors for both NIRF and far-red fluorescence molecular imaging, the constructed phantoms were placed in the FOV and images were acquired with different integration times (200 ms, 800 ms, and 8000 ms). These times were chosen as the typical integration times used in previous studies for EMCCD-based small animal studies (800 ms) and for ICCD-based clinical studies (200 ms). We chose the longest 8000 ms integration time as ten times that employed by the EMCCD in order to attempt to improve its performance, since in hindsight, we found it to be the least sensitive device. The gain of EMCCD camera was set to a value of 3500 as typically used in our small animal imaging studies, and the gain of ICCD camera was adjusted to obtain the counts of >30 000. While filtration and optical path designs

also impact performance, we chose to assess the impact of the detector only with all other design features held constant.

2.D. Image analysis by figures of merit

The acquired images were unsigned data type with integer values ranging from 0 to 2^{16} and processed using ImageJ software (National Institutes of Health, Washington, DC). Intensity values along a line transecting the centers of different concentrations of QDots 800 in the phantom were plotted. To quantify the measurement sensitivity of a device, the SNR was calculated two ways: (i) first employing the average intensity and standard deviation of intensity values at pixel (i, j) , $S^b(i, j)$ across the well area denoted by pixels $i = 1, 2, \dots, m$, and $j = 1, 2, \dots, n$ as is done in nuclear imaging:^{12–14}

$$\text{SNR} = \frac{\bar{S}^t(C) - \bar{S}^b(0)}{\sqrt{\frac{\sum_{i=1}^m \sum_{j=1}^n [S^b(i, j)(0) - \bar{S}^b(0)]^2}{mn}}}, \quad (2)$$

where $\bar{S}^t(C)$ and $\bar{S}^b(0)$ are the average intensity for the area corresponding to the entire well containing C concentration of QDots 800 and to the well without QDots 800, respectively; and (ii) using the following equation:

$$\text{SNR} = 20 \log_{10} \frac{\bar{S}^t(C)}{\bar{S}^b(0)}. \quad (3)$$

The achievable contrast of a device was calculated by the following well-known relationship:

$$\text{Contrast} = \frac{\bar{S}^t(C)}{\bar{S}^b(0)} - 1. \quad (4)$$

By using the same illumination configuration and filtration scheme, the performance of an imaging system as determined by the type of CCD detector employed was evaluated.

2.E. Assessment of NOVADAQ SPY system

The NOVADAQ SPY (NOVADAQ, Ontario, Canada) is a commercial, market approved NIRF fluorescence imaging device that has been used in a variety of applications including coronary artery bypass, cardiovascular, plastic, reconstructive, micro, organ transplant, and gastrointestinal surgery following i.v. administration of 5–25 mg ICG.¹⁵ The NOVADAQ SPYelite imaging system employs an 806 nm laser diode to illuminate a maximum field of $18.5 \times 13.5 \text{ cm}^2$ and a 1024×768 NIR camera to record the collected fluorescence signals with data-files indicating 8-bit dynamic range. The embedded laser source has the maximum power output of 15000 mW, a beam divergence of 25° (Ref. 16) and an incident laser power at the operational tissue surface of approximately 12 mW/cm^2 . The NIR camera has a fixed FOV of $19 \text{ cm} \times 12.7 \text{ cm}$ at a fixed 30 cm working distance. The NOVADAQ SPY system collects fluorescence imaging at 30 frames per second, or with an integrating time of approximately 33 ms. Other specifications such as the CCD type, A/D conversion, f number, optical spectral response, and the detection scheme are not specified in the NOVADAQ SPYelite

FIG. 3. Phantom fluorescence images. (a)–(c) illustrate phantom fluorescence images acquired using the various integrating detectors at 200, 800, and 8000 ms integrating times, respectively.

manual and were not made available to us upon request (personal communication with M. Koyfman, 12/2/2013). The performance of the NOVADAQ SPY Imaging System was evaluated at its factory settings using the constructed fluorescent solid phantom for clinical imaging and compared with our investigational device based on back-illuminated ICCD camera operated with 33 and 200 ms integration times. To secure comparable intensity, we decrease the gain of intensifier of the investigational ICCD when the integration time is increased in order to avoid oversaturation of the CCD. Because the number of pixels in the binned images obtained from the NOVADAQ SPY system differed from our CCD based systems, the fairest comparison of SNR was performed using Eq. (3).

2.F. Statistical analysis

Two-way analysis of variance (Microsoft Office Excel 2010 software) was performed to compare the SNR and contrast of two different CCD-based imaging systems across the concentration ranges of Qdots 800. Differences were considered statistically significant when $p < 0.05$.

3. RESULTS

3.A. Stability of QDots 800 based fluorescence solid phantoms

While we are currently attempting to certify the solid phantom with collaborators at National Institute of Standards and Technology (NIST), we found that it exhibited less than 1.0% variation in fluorescent intensity over 50 days. Therefore, it

has greater stability than solutions of ICG in lipid or in TiO₂ suspensions that have been used to assess device performance in the presence of tissue mimicking scatter¹⁷ and allows a robust comparison of devices.

3.B. Performance of various CCD detectors based NIRF and far-red fluorescence imaging system

The phantom images acquired using the various CCD detectors under different integrating times are shown in Fig. 3, demonstrating the differences in dynamic range. From the intensity profiles shown in Fig. 4(a), the higher concentration of QDots 800 in the phantom gives stronger fluorescence signals, as expected. At short integration times (200 ms), the measured fluorescence intensity from ICCD detectors is stronger than that from unintensified, front- or back-illuminated CCD or EMCCD detectors, indicating the added value of light amplification by the intensifier prior to collection by the CCD. However, as the integration time is increased to 800 and 8000 ms, the measured fluorescence signals from the front- and back-illuminated CCD and EMCCD detectors approach that of the intensified CCDs because the gain of the intensifiers had to be reduced to prevent saturation of the coupled CCD.

Figure 4(b) illustrates the plots of the SNR versus contrast obtained from phantoms conducted at varying integration times. For example, at the short 200 ms integration times, the SNR and contrast of ICCD camera based NIRF imaging systems are more than 6 and 13 times higher than that of their corresponding CCD cameras, respectively, and the

FIG. 4. Quantitative analysis of various CCD based NIRF imaging devices. (a) illustrates the measurement fluorescence intensity profiles using various CCD cameras under different integration times. (b) illustrates the plots of SNR vs contrast using various CCD cameras under different integration times.

EMCCD camera gives the lowest SNR and contrast. The performance of unintensified CCDs improves with longer integration times of 800 ms or longer. These observations imply the critical nature in the choice of integrating CCD detectors for specific low-light applications that require rapid image acquisition. For example, an ICCD camera is the best choice for real-time, video-rate imaging while EMCCD and unintensified cameras may be more suitable when fluorescent dye is available at higher concentrations than is typical of molecular imaging applications or when long image acquisition times can be tolerated.

Compared to quantum efficiencies (QE) at NIR wavelengths, the CCD chip is more sensitive at far-red wavelengths while the intensifier has a comparatively constant QE across the far-red and near-infrared wavelengths [Fig. 1(e)]. Nonetheless at far-red wavelengths, the ICCDs still retain higher SNR and contrast at shorter integration times (200 ms) compared to their unintensified counterparts, as shown in Fig. 5. Therefore, the primary advantage of the intensifier may not be due to the blue shifted wavelengths produced by the phosphor screen and acquired by CCD chip, but more appropriately due to the light amplification by the intensifier and the reduction of random noise through integration that occurs at the CCD.

Figure 6 shows the detection limits of the back-illuminated ICCD based NIRF imaging system. Phantoms with the highest concentrations of QDots 800 resulted in the strongest fluorescence intensity with little discernible signal from the control well without QDots 800. Phantoms with QDots 800 concentrations ranging from 0.02 nM to 0.1 nM also produced fluorescent signals distinctly brighter than the control well. However, the lower concentrations of QDots 800 (from 2 pM to 10 pM) are associated with fluorescence intensities that were progressively weaker and become comparable with the background signal of the control well. The calculated SNR and contrast from 10 pM concentration of QDots 800 is less than 5 and 1, respectively, and may represent the detection limits of the configuration/operation of the NIRF imager, which corresponds to 30 pM of ICG specifically for this phantom.

3.C. NOVADAQ SPY versus and investigational ICCD device

Figure 7 illustrates the comparison of our investigational ICCD device and a marketed CCD based NOVADAQ SPY clinical imager and demonstrates dramatic differences in SNR and contrast between the two devices. The ICCD device is

FIG. 5. Performance of far-red fluorescence imaging systems. (a)-(b) show phantom fluorescence images acquired using back-illuminated ICCD (BICCD) camera and back-illuminated CCD (BCCD) camera, respectively. (c)-(d) illustrate the plots of the fluorescence intensity profiles and the SNR vs contrast, respectively.

FIG. 6. The measurement limitation of the back-illuminated ICCD based NIRF imaging system. (a) Fluorescence images acquired from different ranges of QDots 800 based phantoms. (b)-(c) illustrate plots of the fluorescence intensity profiles and the SNR vs contrast, respectively.

FIG. 7. Performance comparisons between the investigation device and NOVADAQ SPY system. (a) illustrates fluorescence images acquired on the phantom 1 and 2. (b)-(c) illustrate plots of the fluorescence intensity profiles and the SNR vs contrast, respectively.

typically operated at 200 ms integration times, but can be operated at the similar 33 ms integration times as the NOVADAQ SPY device. SNR and contrast are related by a logarithmic function, and the range of values for each of the devices varies at different integration times. The SNR and contrast of the investigational device was found to be more than 3 to 50 times greater than that of the commercial, market-approved NOVADAQ SPY device as assessed from the phantom with 0-10 nM QDots 800. While two systems can detect the phantom at all concentrations, NOVADAQ SPY system failed to discriminate differences in QDots 800 concentrations lower than 4 nM. The investigational device shows similar SNR and contrast when operated at 33 ms (by increasing the gain of intensifier) as compared to 200 ms integration times.

Given the differences in SNR and contrast between the NOVADAQ marketed and the investigational imagers, a “first-in-humans” imaging agent which is detected in our investigational device may likely not be detected by the NOVADAQ SPY system at its factory settings. These compar-

isons are not made to claim superiority of one device over another, but rather to point out the variability of detection limits and contrast sensitivity that could reflect differences in device performance in the clinical setting or indication, such as “first-in-humans” molecular imaging for which it may not be approved.

3.D. Comparison of various CCD camera based imaging systems

Significant differences in both SNR and contrast were observed among all CCD camera based imaging systems except for the SNR of the EMCCD vs the FCCD at both 200 ms and 800 ms integration time and the FICCD vs the BICCD at 800 ms integration time (see Tables I–III). Because the CCDs integrate signals, random noise is reduced while signal is amplified by the intensifier in ICCD systems. As the integration time of an ICCD is reduced, the averaging of random noise is less effective, although the SNR remains significantly greater than nonintensified CCD systems.

TABLE I. A two-way analysis of variance of SNR among two different CCD camera based imaging systems at different integration time t ($\alpha = 0.05$ for a 95% confidence).

t (ms)	EMCCD vs FCCD	EMCCD vs BCCD	EMCCD vs FICCD	EMCCD vs BICCD	FCCD vs BCCD	FCCD vs FICCD	FCCD vs BICCD	BCCD vs FICCD	BCCD vs BICCD	FICCD vs BICCD
200	0.219	0.015 ^a	0.006 ^a	0.011 ^a	0.012 ^a	0.006 ^a	0.011 ^a	0.005 ^a	0.011 ^a	0.019 ^a
800	0.133	0.015 ^a	0.005 ^a	0.013 ^a	0.010 ^a	0.004 ^a	0.013 ^a	0.004 ^a	0.013 ^a	0.106
8000	0.013 ^a	0.016 ^a	0.024 ^a	0.023 ^a	0.010 ^a	0.000 ^a	0.015 ^a	0.013 ^a	0.247	0.019 ^a

^aSignificant, $p < 0.05$.

TABLE II. A two-way analysis of variance of contrast among two different CCD camera based imaging systems at different integration time t ($\alpha = 0.05$ for a 95% confidence).

t (ms)	EMCCD vs FCCD	EMCCD vs BCCD	EMCCD vs FICCD	EMCCD vs BICCD	FCCD vs BCCD	FCCD vs FICCD	FCCD vs BICCD	BCCD vs FICCD	BCCD vs BICCD	FICCD vs BICCD
200	0.010 ^a	0.010 ^a	0.006 ^a	0.011 ^a	0.013 ^a	0.006 ^a	0.011 ^a	0.006 ^a	0.011 ^a	0.015 ^a
800	0.010 ^a	0.010 ^a	0.006 ^a	0.012 ^a	0.011 ^a	0.005 ^a	0.012 ^a	0.005 ^a	0.012 ^a	0.032 ^a
8000	0.010 ^a	0.010 ^a	0.006 ^a	0.013 ^a	0.010 ^a	0.000 ^a	0.015 ^a	0.036 ^a	0.018 ^a	0.021 ^a

^aSignificant, $p < 0.05$.

4. DISCUSSION

Currently, there are no standards for fluorescence imaging systems, and there is no objective specification as to how sensitive a fluorescent imaging system should be, whether for small animal imaging or for investigational or marketed systems for human use. Yet for successful fluorescence molecular imaging, the rationale for a minimum requirement for device sensitivity should be based upon its ability to detect contrast due to a targeted fluorescent imaging agent at nM–pM tissue concentrations that are relevant for the specific disease marker being targeted. Previously, we identified the confounding effects of excitation light leakage that arises from broad-band illumination and improper filtering schemes as the major contributory factor to the noise floors of optical imaging systems for whole body imaging^{11,18} and herein, extend the analysis to detector schemes using solid phantoms to assess task-specific performance relevant for detecting fluorescence in tissues. The current lack of task-specific, device specifications, and standards that are relevant for all other molecular medical imaging modalities jeopardizes the emerging translation and clinical adoption of fluorescence molecular imaging. For example, the failure to image diseased tissues on the basis of fluorescent contrast could be interpreted as a failure of the imaging agent, when in fact the unqualified imaging device was simply not sensitive enough for its detection. In the absence of standards that can qualify and predict device performance in the clinical setting, we previously used our front-illuminated ICCD system and ICG, a dye that has an established safety record in humans for over 50 years, to directly visualize tissues in an FDA approved dose escalation clinical study that employed microgram doses.¹⁹ Although this prior clinical study may have qualified our device for future “first-in-humans” studies, in the absence of

standards, it remains impractical to qualify individual devices through human ICG dose escalation studies as a criterion for future “first-in-humans” studies. Past investigations to assess performance have employed ICG in phantom studies that involve lipid or titanium dioxide scattering solutions, but have not presented figures of merit for comparison of devices or device design modification. Unfortunately, ICG possesses poor NIR fluorescent properties and is notoriously unstable, obviating its use for robust device qualification and assessment. The solid phantom presented herein has been shown to be stable and conceivably can be NIST certified for device comparison and assessment of engineering design in order to optimize imaging performance. These and potentially other phantoms are needed to accelerate clinical translation of “first-in-humans” molecularly targeting near-infrared and far-red fluorescent imaging agents by enabling assessment of device performance.

In the absence of community adopted standards, we evaluated the imaging performance of unintensified CCD systems (that constitute the majority of small animal and investigational clinical systems) with intensified CCD systems and showed that rather substantial differences in imaging performance can be attributed to the choice of detectors. Our results show that the amplification of light by an image intensifier coupled with the integration of the CCD dramatically improves SNR and fluorescent contrast. One drawback to the use of an intensified CCD system may be the reduction of resolution inherent from the current design of the MCPs²⁰ that can be mitigated in part through variation of FOV. Because the majority of cost associated with an ICCD system is the CCD, amplification through an intensifier maximizes performance without significantly impacting device cost.

Although autofluorescence is minimized through the use of NIR fluorophores excited at wavelengths >780 nm, several investigators also employ far-red fluorophores that are excited at wavelengths <780 nm. Our results show that intensification also significantly improves device performance in the far-red fluorescence range; however, it must be noted that the contribution of tissue autofluorescence is not considered herein but would deteriorate performance of all imaging devices regardless of whether amplification through a coupled intensifier is employed.

Finally, given the recent published suggestions for using the marketed NOVADAQ approved for i.v. administration of ICG as a device for intraoperative detection of diseased tissues using “first-in-humans” fluorescent contrast agents,¹⁰ we conducted a comparison between the CCD-based NOVADAQ

TABLE III. A two-way analysis of variance of both SNR and contrast among two different CCD camera based imaging systems ($\alpha = 0.05$ for a 95% confidence).

Performance parameters	Investigational device (UTHSCH) at 33 ms integration time vs NOVADAQ SPY at 33 ms integration times	
	BCCD vs BICCD camera based far-red fluorescence imaging system at 200 ms integration time	
SNR	0.019 ^a	0.002 ^a
Contrast	0.018 ^a	0.034 ^a

^aSignificant, $p < 0.05$.

device and our qualified, investigational ICCD device used clinically for noninvasive imaging. For the imaging task of detecting QDots 800 in multiply scattering media, we show that the NOVADAQ cannot differentiate differences in signal when the concentration of QDots 800 is less than 4 nM, although it can detect all the concentrations. Although the NOVADAQ device employs different excitation and fluorescence collection filtering schemes as employed in our comparison studies, the results nonetheless indicate reduced contrast sensitivity that may be due, not only to the lack of an intensified CCD, but due to other design factors as well. Our results do not suggest that the NOVADAQ SPY device could not be modified in design or in factory settings to improve contrast sensitivity, but rather to point out that standards are missing to guide such design work. It is important to note that the NOVADAQ SPY system is designed for and successfully operates to detect nontargeting ICG in the vascular space following intravenous administration of 5–25 mg.

Because we evaluated all the devices under identical conditions and system components, only the detector (CCD, EM-CCD, and ICCD) performance was considered for instrument qualification, i.e., readiness to perform molecular imaging. Of course, other design system features such as optical filtering and split optical paths that were not considered herein, could dramatically impact sensitivity performance. As an example of the potential heterogeneity of performance, we presented the contrast sensitivity and SNR performance measurements of the NOVADAQ as well as an investigational ICCD system. Operational qualification of an approved fluorescence imaging device using a standard phantom could ensure that there is no defect in uniform excitation light delivery, and that the detection system is capable of measuring fluorescent contrast in the presence of scattering and ambient room light (which could also contain wavelengths common to the emission and defeat imaging agent detection).

The use of adopted standards for assessing device performance could provide an economical and judicious approach for matching device requirements with translated fluorescent imaging agents in specific clinical indications. To date, with the exception of study published using a folate targeted protein labeled with fluorescein and collection of fluorescence in the visible wavelength region,²¹ there have been no fluorescence molecular imaging studies conducted in humans.

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