Digital Dynamic Discrimination of Primary Colorectal Cancer Using Systemic Indocyanine Green with Near-Infrared Endoscopy.

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Research Article

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Abstract

Introduction: As indocyanine green (ICG) with near-infrared (NIR) endoscopy enhances real-time intraoperative tissue microperfusion appreciation, it may also dynamically reveal neoplasia distinctively from normal tissue especially with video software fluorescence analysis.

Methods: Colorectal tumours of patients were imaged mucosally following ICG administration (0.25 mg/kg i.v.) using an endo-laparoscopic NIR system (PINPOINT Endoscopic Fluorescence System, Stryker) including immediate, continuous in situ visualization of rectal lesions transanally for up to 20 minutes. Spot and dynamic temporal fluorescence intensities (FI) were quantified using ImageJ (including videos at one frame per second, fps) and by a bespoke MATLAB® application that provided digitalized video tracking and signal logging at 30fps (*Fluorescence Tracker App*, FTA, downloadable via MATLAB® file exchange). Statistical analysis of FI-time plots compared tumours (benign and malignant) against control during FI curve rise, peak and decline from apex.

Results: Early kinetic FI signal measurement (n=20, 16 males, 9 cancers) delineated discriminative temporal signatures from tumours offering rich data for analysis versus delayed spot measurement (n=10 patients with cancer). Malignant lesion curves peaked significantly later with a shallower gradient than normal tissue while benign lesions showed significantly greater and faster intensity drop from apex versus cancer. Automated tracker quantification efficiently expanded manual results and provided algorithmic KNN clustering.

Conclusions Analysis of a continuous stream of intraoperatively acquired early ICG fluorescence data can act as an in situ tumour-identifier with greater detail than later snapshot observation alone. Software quantification of such kinetic signatures may distinguish invasive from non-invasive neoplasia with potential for real-time in silico diagnosis.

Article Summary

This work investigates the use of indocyanine green fluorescence interrogation as a pathological discriminator in colorectal cancer. The importance of the developed methods of kinetic quantification, identification of flow milestones and experimental results are reflected in the potential avenues for further advances in digital diagnosis.

Introduction

Direct visualization by endoscopy is an early component of clinical pathways for many patients with visceral cancer that directs subsequent care. At operation too, direct inspection assists quantification of overall cancer burden especially in areas poorly seen by non-invasive workup and impacts surgical strategy. However, determination of tissue nature by operator judgement alone is imperfect.¹ Improved intraprocedural tissue characterisation could allow better in situ primary tumour profiling and confirmation/exclusion of metastatic deposits acuminating cancer outcomes and healthcare efficiency.²

Surgical colorectal cancer care is ideally suited for minimally invasive technical advance with both endoscopy and laparoscopy embedded in its diagnosis and therapy. Its endolaparoscopic systems are capable of providing multispectral illumination including tissue-penetrating near-infrared (NIR) spectral vision. Such capacity can help characterise tissue intraoperatively with most clinical application thus far for tissue perfusion assessment using the non-selective fluorophore indocyanine green (ICG).^{3,4,5} Importantly here its deployment is at surgery with real-time impact on operator cognition and consequent procedural step-sequencing.⁶ The NIR-ICG visualization is dynamic occurring within seconds of agent administration fitting current surgical practice very well. In contrast, NIR cancer identification strategies have concentrated on advanced preprocedural fluorophore administration with visual interrogation planned for many hours or days later at a time when tissue agent concentrations are relatively static.⁷

Malignant tumours distinguish from normal tissue and benign neoplasia by hallmark metabolic behaviours including their neovascularization (angiogenesis) as well as cell-cell and host-tumour interstitial interactions.^{8,9} Their irregular vascular architecture and lack of a lymphatic recovery system leads to the tumoritropic accumulation of macromolecules such as albumin in solid tumours (in contrast to their rapid clearance in normal tissue).¹⁰ Such characteristics mean even a chemically non-selective perfusate, such as ICG, could functionally discriminate cancer at any site allowing in situ optical delineation. However, when applied simply by preoperative administration and later snapshot observation, high false positive rates pertain (a problem that also affects cell-targeting agents deployed similarly).¹¹

We hypothesized that kinetic characterisation of the evolving dynamic fluorescence intensity (FI) signature at and within cancer deposits could better provide discrimination of cancer versus non-cancer even with a passive, non-targeted fluorophore such as ICG.¹² As the processes being visualized are more complex than perfusion alone and non-tumour background noise may manifest, video-based analytics could de-risk image interpretation permitting greater interpretative clarity and even potentially a 'digital tissue biopsy' through software. Herein we pilot the use of systemic NIR-ICG as a licensed available-now technology for endoscopic tissue characterisation in human patients with colorectal neoplasia.

Methods

In accordance with relevant guidelines, regulations and with institutional (Mater Misericordiae University Hospital, Dublin, Ireland) ethics approval (1/378/2092, ClinicalTrials.gov Identifier: NCT04220242) was sought. This clinical experience involved adult patients already undergoing endolaparoscopic diagnostic or therapeutic procedures for colorectal neoplasia under general anaesthesia. Informed consent was obtained from all participants.

Patients were administered ICG intravenously at a dose of 0.25 mg/kg intraprocedurally. An approved endoscopic NIR system (PINPOINT Endoscopic Fluorescence System, Stryker Corp, Kalamazoo, MI, USA.) was used to directly visualise rectal tumours continuously transanally for up to 20 minutes after ICG administration.

Ex vivo resection specimens from a separate cohort were opened and their mucosal surfaces were examined post-operatively with the same camera for still image analysis.

All imagery contained both the neoplastic lesion and some adjacent normal tissue. ICG is licensed internationally for tissue perfusion characterisation and has an excellent safety profile. The PINPOINT Endoscopic System is a commercially available system that simultaneously displays white light and NIR appearances. Therefore, no experimental agents or devices were used and no clinical care decisions changed based on NIR-ICG fluorescence appearances.

Data Collection:

Simultaneous endoscopic white light and NIR videos along with similar high-resolution photographs of the opened mucosal surface of any resected specimens ex vivo were recorded for post-hoc FI analysis. Patient demographics and reactions possibly related to ICG usage were recorded prospectively along with the routine pre and postoperative pathological and radiological reports to capture cancer-specific details.

ICG still image analysis:

Fluorescence photographs of resected neoplastic specimens were processed post-hoc using ImageJ (NIH, USA)¹³. Spot mean FI in 25x25 pixel areas in the tumour were contrasted with normal mucosa (see Figure One).

ICG flow Data Analysis:

For recorded videos, a surgeon directly annotated regions of interest (ROI) on the endomucosal surface regions representing the tumour and adjacent normal tissue (control) avoiding obvious areas of surface blood and those only variably seen over time in movies. Post-hoc kinetic plotting of FI dynamic patterns of in situ lesions over time, was initially performed using Image-J on grayscale still image stacks serially sampled at one frame per second (fps) from the time of fluorescence signal onset to video end. Thereafter fluorescence measurement was digitalised and automated through the creation of a bespoke, standalone Fluorescence Tracker App (FTA), developed in collaboration with MathWorks®, Galway, Ireland (software source code and executable now publicly available via the MATLAB® File Exchange, www.mathworks.com). This allowed in-app surgeon-ROI-annotation for tumour and control areas from the white-light (RGB) images prior to the onset of non-zero fluorescence intensities in the NIR images with automatic detection and frame-by-frame tracking of surface feature points in the white light images (using minimum eigenvalue¹⁴ and Kanade–Lucas–Tomasi algorithms respectively¹⁵) to account for the changing pixel locations of these areas due to camera movement. By continuously tracking in the white-light image, the app mapped FI in the corresponding pixels of the complimentary NIR image for the ROIs throughout the video duration. For such automated analysis, five minutes of continuous signal quantification was set as the minimum goal. Also, following surgeon annotation of tumour versus control ROI, the software can group the dynamic intensity results using K-Nearest Neighbour (KNN) algorithm. (see Figure Two) In the event that tracking was compromised (e.g. due to instruments obstructing the

tracking points in the field of view or large camera movements forcing the points out of frame), ROIs were re-selected, automatically featured, and tracking resumed. Interruptions in individual patient readings were collated and gaps documented.

Statistical Methods:

Data collection and statistical analysis was carried out using Microsoft Excel 2016 and SPSS Software (IBM, Armonk, NY, USA) version 26 with statistical significance ascribed to p-values less than 0.05. Spot FI measurements and dynamic mean values were compared from tumours versus control tissue using Mann-Whitney U. FI signals from videos were plotted against time and examined for distinctions regarding specific curve milestones and variables adapted from previously described methodologies.^{16,17} An intensity rise of 5 grey units was marked as the end of the latency period and the starting block for the incline. Following the rapid inflow period, the point of maximum fluorescence intensity ($F_{\max}$), time to achieve this summit ($T_{\max}$) and respective gradient ($F_{\max}/T_{\max}$) were calculated along with the time taken ($T_{\frac{1}{2}\max}$) to achieve half the maximum ($F_{\frac{1}{2}\max}$) intensity. Following peak intensity, fluorescence clearance from the target tissue was flagged at 100 seconds (T_{100}) from the start of the descent (see Figure Three). Final intensity, gradients, duration of tracking, skew and kurtosis were transcribed. Mean intensities for the tumours and controls before and after KNN grouping were compared using the Wilcoxon signed rank test.

Results

In total, thirty patients were recruited into the study (see Table One and Figure Four). No patient suffered ICG reaction or toxicity. All procedures progressed as planned, including diagnostic biopsies or therapeutic endoscopic, laparoscopic, open resection, highlighting that the NIR-ICG assessment was coincidental to patient care. Twenty patients with rectal neoplasia underwent endoscopic primary tumour site assessment under simultaneous white-light and NIR examination immediately after ICG administration continually for up to 20 mins. Eight of these recordings (three cancers), were analysed manually by Image J (1 fps) and both these and the next twelve were analysed using the automated FTA. A separate cohort of colorectal cancer cases (n=10) had their resected specimens examined postoperatively (therefore 4-6 hours post ICG administration).

With respect to spot intensity measurement late after ICG administration, mean ($\pm$ standard deviation) cancer intensity was non-significantly raised (57.0 ± 61.3) compared with control (36.0 ± 28.3) (Mann-Whitney U) with considerable overlap of scores suggesting poor discrimination of cancer by single point in time analysis even some hours after ICG administration (see Figure Five). In contrast, both manual Image J analysis (n=8) and automated FTA analysis (n=20) of lesions in situ being dynamically profiled displayed potentially discriminative statistical differences between cancers and benign tumours from their FI temporal signature kinematics. Figure Six shows graphs of mean FI over time for such lesions grouped by their nature using both the manual Image J and FTA methodology while Figure Seven shows for illustration the FI time-plot for a representative benign and malignant lesion. Alongside providing

cleaner, richer data due to its higher sampling frequency (30 fps), the FTA method was faster, required less user input and sampled more intensely allowing discriminative curves with shorter video analysis (average continuous FTA tracking time 601 seconds vs 1903 seconds with manual ImageJ). Furthermore, only 142 of 24,051 seconds (0.006%) of videos had to be censored due to inability to track serially using the FTA.

Tables Two and Three show the measures of graph parameters from the lesions viewed endoscopically by both manual ImageJ and automated FTA FI curves. Statistical analysis of temporal kinetic patterns generated by the manual quantificative method with respect to flow milestones of lesions versus global control readings revealed significant peak intensity differences ($p=0.047$, Kruskal Wallis). Direct $F_{\max}$ comparisons showed that the cancer intensity curve zenith ($F_{\max} 202\pm35.68$) was significantly higher than for both the benign tumours (165.8 ± 11.69 , $p=0.02$) and control tissue (169.63 ± 12.84 , $p=0.04$) (Mann-Whitney U). With respect to the FTA graphs, three-way analysis using Kruskal-Wallis comparison of cancer, benign and control regions showed significant differences in upslope (gradient from baseline to $F_{\max}$ $p=0.039$) and for both the downslope and fall between $F_{\max}$ and T_{100} ($p=0.046$ for each). Direct comparison with Mann-Whitney U between cancers and control regions showed that cancers peaked significantly later, took significantly longer to rise from baseline to maximum and exhibited a shallower gradient to the peak. This resulted in a significantly later T_{100} . Skew for cancers was further to the right (-1.69 ± 1.17 vs -0.41 ± 1.37 $p=0.04$). In comparing cancers with benign lesions, there were also significant differences by T_{100} in terms of both fall and downward gradient. There was no statistically significant difference in these measures between all lesions together and benign lesions alone compared with the controls.

Within the group analysed by FTA, six cancer cases underwent application of the KNN classification algorithm. With surgeon ROI (lesion and control) selection, the algorithm was able to cluster the data into two groups segregating cancer from normal tissue automatically, in a fashion not significantly different to the manually segregated data (Wilcoxon signed rank resting). However, the automated classification was unable to reproduce the significantly raised '*time to rise*' demonstrated on neoplastic tissue ($p=0.041$) versus control when these were manually grouped.

Discussion

Fluorescence-guided surgery is proposed as the next progressive iteration in cancer surgery, especially endo-laparoscopic operations.^{2,3} Notably, it is already impacting decision-making and step-sequencing in reconstructive surgery both of the gastrointestinal tract¹⁸ and other tissues.¹⁹ As well as a means to demarcate watersheds between perfused and non-perfused intestine, other uses include critical boundary identifications such as ureters^{20,21} and fascial planes²² by direct instillation. For cancer and draining lymph node identification, ICG has been placed peritumorally submucosally by endoscopic tattoo.^{23,24,25} In situ revelation of cancer including tumour margination however requires labelling pathology rather than physiology. While cancerous tissue can retain fluorescent agents, there remains a significant issue

with false positive findings.^{26,27} Because of this lack of sensitivity, most research groups are now focusing on development of new cancer-selective agents although accuracy remains problematic.^{11,28, 29}³⁰ Given that cancers likely act to accumulate agents in their vicinity differently to other areas that trap agents more passively, we thought to observe dynamically the processing of ICG by tumours under direct observation different to others who have focused on single point in time observations.

In this experience, visually obvious differential clearance patterns distinguish cancers from non-malignant tissues soon after agent administration and persist thereafter. While additional distinguishing parameters likely also exist and could be identifiable via longer videos enabling analysis of dye processing and efflux patterns and/or by using more sophisticated analytical methods, this initial work is revealing. Overall the fluorescence signal in tumours takes longer to peak and retains this intensity longer (consistent with impaired local pharmacokinetics compared with healthy tissue). Such distinct FI signatures could be visually noticed in both grey-scale, composite and threshold modes and could be quantified with post-hoc image data analysis. Manual intensity quantification allowed mathematical comparisons of the FI time patterns although such data generation was labour-intensive. Furthermore, episodic sampling (even at 1fps) made smoothing of data difficult while the background noise impaired statistical analysis. Automating feature detection, tracking and quantification enabled higher sampling rates (30fps) and so provided richer data for analysis with processing time being a function of computational ability rather than human labour. Automatic classification showed encouraging discriminate abilities even without prior annotation. Together these findings have great application for artificial intelligence methods.³¹

ICG accumulates quickly and can be effectively imaged with a variety of commercial cameras. Its attractiveness versus fluorescein lies in its responsiveness to NIR wavelengths (750-800 nm) which enables tissue penetration to approximately 1cm depth without cell damage (methylene blue may work similarly but is restricted in its intravenous licensing and has no CE or FDA-approved imaging device). Non-specific tumour uptake mechanisms include enhanced permeability and retention (EPR), aberrant gap junctions and dysregulated pinocytosis.^{32,33,34} Recent reports of similar behaviour of ICG at sites of secondary disease support this work²¹ and together the data are encouraging that malignant lymph nodes could behave similarly regarding uptake of circulating ICG. Although not the primary focus of this pilot experience, several positive nodes were visualized in situ during this study, though a larger cohort of patients would be needed to establish the generality of these observations. There are though many more patients who could benefit from an effective digital malignant discriminator for primary colorectal lesions (including those with significant polyps) however than there are presenting for surgery for recurrent disease. It is also likely that the ICG uptake mechanisms through cancer metabolics seen here are shared across other common cancers.

Cell-specific targeting remains of great interest for future advances in this area but a deep understanding of tumoural functional characteristics is necessary to inform the purpose and level of probe engineering needed. While cancer cell selectivity can be engineered in cell culture, tumour imaging in vivo can also

encompass a variety of host:cancer interactions as well as additional cell signalling and response pathways. As ICG can be seen in this experience to passively concentrate in tumours it seems likely that so too will, at least in part, a “cancer-specific” agent. Furthermore, additional unrelated sites of inflammation³⁵ or injury will equally be prone to incidental, unintended probe accumulation. This indicates that a relatively high rate of false positive findings may continue despite any additional tumour targeting features. Rigorous preclinical and clinical study controls are required to minimize heuristic mechanistic targeting assumptions. While long-in-advance (24–48 hours) administration of the fluorophore has been proposed to offset non-cancer signalling (and allow signal strengthening by agent concentration), a significant downside exists with respect to the unpredictability of operative timing and patient-to-patient pharmacokinetic variances.

An inbuilt synchronous and continuous dynamic quantification algorithm and screen display can be of use to all fluorescence imaging agents and could even enable synchronous use of multiple probes of different wavelength colours. Furthermore, while cell-surface or ligand-specific agents could be limited in applicability due to variance in intra and inter-tumour target expression, internalisation of ICG is governed by the tumour microenvironment, so it could perform similarly across a broad spectrum of cancers irrespective of tissue site or cell origin once the optimal real-time method for interpretative analysis is in place. Addition of cancer-specific targeting could still accelerate agent accumulation at the ROI following systemic distribution, enabling short-window observations with the greatest sensitivity and specificity possible within minutes. This would be a use for the operator more akin to episodic perfusion assessment with the added biochemical, biological and imaging complexity hidden. “Off-on” fluorogenic compounds are especially attractive for this purpose as their signalling only switches on when they arrive at the appropriate microenvironment.³⁶

This study is of course limited by the small cohort studies although patients were recruited into the study without preselection. Fluorescence data is also dependent on the recording of the detected fluorescence signal. Video stability, suctioning, instrumentation and tumour desloughing impaired continuous tracking and required further user interaction. The distance and angulation of the camera as well as the depth of ICG have been found to influence the signal and need consideration in further protocols.³⁷ Furthermore, while other surgical camera manufacturers offer similar systems for ICG visualization by NIR, camera design and performance investigation has shown previously significant inter-manufacturer variation between signal and ICG concentration (including the tendency of some systems to boost the images for human interpretation) which limits the generalizability of these findings until further (ongoing) validation is performed.³⁸ While selection of the specific timepoints for analysis (e.g. 100 second post-peak timepoint) was somewhat arbitrary, they do provide milestones for statistical analysis enabling comparisons between lesions and adjacent healthy tissue as control. Statistical curve analysis is currently not calculated in real time and is limited to one curve per ROI. Simultaneous synthesis and dissection of multiple curves may further discern tumour heterogeneity. Although the automatic classifier diminished the differentiation ability of our metrics when compared to surgeon-based classification,

better classification algorithmic methods may to some extent replace operator input. Other tracking algorithms exist and further software development may address field of view interruptions.

This endeavour has nonetheless shown that ICG with NIR-illumination is capable of delineating cancer in human patients in this specific test-case in a straightforward manner deployable within real-world surgical practice. The previous focus on spot readings may alleviate the need for concurrent injection and recording but has overlooked the potential for incorporating dynamic ICG flow patterns to improve confidence of interpretation. Equally incorporation of computer-assistance can help and even potentially replace the need for human observer visualization promising near real-time assessment of lesions during procedures with minimal user interaction as a 'digital biopsy' enabled through advances in computer processing, artificial intelligence and broad cloud-based datasets.³⁹

Declarations

Author contributions: All the authors contributed to the design, implementation of the research, analysis of the results and to the writing of the manuscript.

Conflicts of Interest/Disclosures: Prof Cahill is named on a patent filed in relation to processes for visual determination of tissue biology, receives speaker fees from Stryker Corp, research funding from Intuitive Corp and holds research funding from the Irish Government in collaboration with IBM Research in Ireland. Drs Dalli and Hardy and are employed as researchers on the same collaboration. Dr Paul Huxel is employed by MathWorks®. Prof O Shea has a financial interest in patents filed and granted relating to NIR-fluorophores and processes for visual determination of tissue biology. Dr Khan, Dr Khokhar,

Mr Loughman and Ms Sarkar have no disclosures to report.

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Tables

Table One Table showing patient and case demographics overall and by analytic methodology. Dynamic intensity readings were made by the automated Fluorescence Tracker App. EUA, TAMIS and TART respectively denote Examination Under Anaesthesia, Transanal Minimally Invasive Surgery and Transanal Resection of Tumour.

	Dynamic Video Readings (MATLAB®)	Ex vivo spot readings (ImageJ)
n	20	10
Male:Female	16:4	7:3
Mean Age in years	68.6	62.6
Operation or Site	Diagnostic (EUA) = 9 Therapeutic (TAMIS/TART) = 11	Caecum/Ascending = 4 Sigmoid/Rectum = 6
Benign:Malignant	11:9	0:10
Tumour Stage by	Tx = 1 T1 = 1 T2 = 0 T3 = 6 T4 = 1	Tx = 0 T1 = 1 T2 = 1 T3 = 6 T4 = 2
TNM Classification	Nx = 1 N0 = 4 N1 = 2 N2 = 2	Nx = 0 N0 = 7 N1 = 2 N2 = 1
	Mx = 0 M0 = 7 M1 = 2	Mx = 0 M0 = 8 M1 = 2

Due to technical limitations, table 2,3 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Photograph of submucosal surface of ex vivo resected specimen in a patient with rectal adenocarcinoma being assessed for tumour static signal intensity using ImageJ. ICG was administered at the time of surgery commencement with the photograph being taken using the Pinpoint endoscopic NIR systems five hours later.

Figure 2

Screenshot of Fluorescence Tracker App (FTA, MATLAB®) showing automated classification of tumour (blue cluster) versus control tissue (orange cluster) once the regions of interest have been defined via initial annotation by a surgeon on the video frame corresponding to the time of fluorescence inflow.

Figure 3

Representative graph of NIR intensity patterns plotting fluorescence intensity versus time to show the variables selected for statistical comparison between malignant, benign and normal tissue in videos from patients in this series.

Figure 4

Diagram showing cohorts of patients in this study by their methods of fluorescence intensity analysis.

Figure 5

Mean fluorescence intensity measurements for cancers vs control tissue on still image analysis postoperatively using ImageJ in patients receiving ICG at operation commencement.

Figure 6

Graph of mean fluorescence intensity vs time for continuous (video) fluorescence signals using (a) manual Image J methodology (1 fps) and (b) the Fluorescence Tracker App with automated feature detection and tracker in the video recordings of patients with both benign and malignant rectal lesions.

Figure 7

Illustrative examples of dynamic time vs fluorescence signal intensity plots for both (a) a benign lesion using the manual (ImageJ) method above and (b) a cancer using the automated Fluorescence Tracker App.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table2.jpg](#)
- [Table3.jpg](#)