

EGF-IRDye™ 800CW: *In vitro* and *In vivo* Characterization As A Biomarker for Optical Fluorescent Imaging of Tumor Growth Kinetics

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ABSTRACT

Noninvasive imaging in the near-infrared spectral range offers the potential for highly sensitive tumor detection and tracking. Targeting a tumor-enriched cell surface receptor with a ligand-conjugated fluorescent probe specifically allows detection of the tumor relative to the negligible animal autofluorescence. In this study, we targeted EGF receptor, which is ~100-fold over-expressed in most solid tumors, using EGF conjugated to IRDye™ 800CW (maximal emission at 789 nm). To confirm specific binding and bioactivity of EGF-IRDye™ 800CW, two human prostate cancer cell lines, PC3M-LN4 and 22Rv1, were evaluated *in vitro* by In-Cell Western. Cells stimulated with 150 ng/mL labeled EGF (dye to protein ratio = 1.5) or unlabeled EGF showed equivalent efficacy in triggering downstream phosphorylation of ERK, indicating the label had no significant effect on biological activity of the probe. Specificity of labeled EGF for the EGF receptor was further demonstrated by competitive binding assays. Addition of unlabeled EGF or C225, a monoclonal antibody that blocks the EGF receptor, concurrently with EGF-IRDye™ 800CW effectively blocked at 1.25 or 9.4 µg/mL, respectively.

We then examined the imaging efficacy of EGF-IRDye™ 800CW in a longitudinal study of subcutaneous tumor growth in SCID mice injected with PC3M-LN4 (n=4) or 22Rv1 (n=4) cells. The EGF-IRDye™ 800CW biomarker was delivered by tail vein injection. Using a prototype 2D imaging system optimized for IRDye™ 800CW detection, we first determined systemic dye clearance kinetics. Calculations of tumor signal-to-noise ratio indicated the greatest sensitivity at 96 hours post-dye injection. Tumors of <1 mm in diameter were detected after 1 week. Tumor growth was tracked following weekly injections of EGF-IRDye™ 800CW for 5 weeks. Tumor signal-to-noise ratios were found to be representative of size as measured by wet weight at the endpoint analysis. This characterization of EGF-IRDye™ 800CW as a biomarker is the first study of tumor growth kinetics tracked by near-infrared imaging.

INTRODUCTION

EGF receptor expression is elevated approximately 100-fold over normal cellular levels in many tumor cell types, including prostate, making it a good general biomarker for tumor targeting. Its ligand, EGF, has been labeled successfully with FITC to characterize receptor binding and endocytosis (Carraway and Cerione, 1993), and with Cy5.5 to monitor molecular targeting therapy in breast cancer xenografts *in vivo* (Shi *et al.*, 2003).

Sensitive methods to detect and measure an internal target non-invasively would facilitate the analysis of responses to various therapies. Optical imaging within the near infrared (NIR) window (650-900 nm) is ideal, because tissue autofluorescence is very low and the penetration of this longer-wavelength light into tissues is significantly better than in other regions of the spectrum. IRDye™ 800CW (ex/em = 774/789 nm) emits directly in the “sweet spot” of the NIR window, where contributions from unwanted sources are minimal, so it has the potential to be an effective beacon if targeted to the tumor.

Here, we report the conjugation of EGF to IRDye™ 800CW and characterize its efficacy as a tumor biomarker using two human prostate carcinoma cell lines, PC3M-LN4 and 22Rv1. Following careful *in vitro* determination of binding specificity, bioactivity and fluorescence, the marker was tested in SCID mice. Parameters of probe clearance were evaluated in animals with and without tumors, and specificity of targeting was confirmed *in vivo*. Subsequently, the probe was successfully used to detect and monitor the growth of subcutaneous tumors over a 6-week period.

RESULTS AND DISCUSSION

Characterization of EGF-IRDye™ 800CW (*in vitro* assays)

EGF was labeled on free amine groups using an NHS ester derivative of IRDye™ 800CW. The specificity and activity of the EGF-IRDye™ 800CW were evaluated with confluent cultures of PC3M-LN4 and 22Rv1 cells using an *in vitro* microplate assay (In-cell Western; Chen *et al.*, 2005) scanned on Aeries™ (LI-COR® Biosciences, Lincoln, NE). Biopotency of the conjugate was verified by its ability to stimulate EGF receptor kinase activity. Figure 1 shows the outcome of specificity challenges performed *in vitro*. In panel A, binding of the EGF-IRDye™ 800CW probe to PC3M-LN4 and 22Rv1 cells is plotted. Panel B illustrates the low affinity of unconjugated IRDye™ 800CW and corresponds to the baseline level seen in Panel A. In panels C and D, binding of the labeled IRDye™ 800CW was effectively blocked by pre-treatment of cells with unlabeled EGF (C) or C225 (D) at 1.25 or 9.4 µg/mL, respectively.

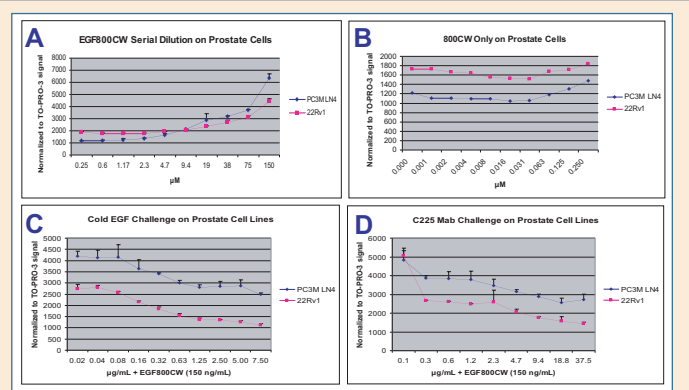


Figure 1. *In vitro* In-cell Western Analysis characterizing the effectiveness of EGF-IRDye™ 800 CW in PC3M LN4 and 22Rv1 cell lines.

In vivo animal imaging

PC3M-LN4 or 22Rv1 cells (10⁶) were implanted subcutaneously in the flank region of SCID mice (4 animals per cell type). Growth of tumors was monitored for 6 weeks by palpation, caliper measurement and optical imaging. For imaging, sterile-filtered EGF-IRDye™ 800CW probe (1–2 nmol per animal) was injected via the tail vein in isoflurane-anesthetized mice. Images were then collected at the indicated time points.

NIR fluorescence imaging of live animals was performed with a prototype LI-COR Biosciences small-animal imager. Images were acquired and analyzed with Wasabi software from Hamamatsu Photonics (Hamamatsu City, Japan).

Image Analysis

Images analyzed in a series were normalized to the same LUT (Look Up Table) with a common minimum and maximum value for visual presentation. Regions of interest (ROIs) of equal area were used for both tumor and background regions. ROIs were quantified for total pixel and mean pixel values. The standard deviation of mean backgrounds was calculated using 3-5 ROIs. This calculation yields the number of standard deviations over background a suspect tumor would represent.

$$SNR = \frac{(\text{Mean Intensity tumor}) - (\text{Mean Intensity background})}{\text{Standard deviation of mean background}}$$

Kinetics of probe clearance

Probe clearance from an animal with no tumor present was first evaluated. Figure 2 illustrates clearance of EGF-IRDye™ 800CW over 24 hours. Two ROIs were analyzed. The large ROI covered the whole body core, while the small ROI focused on the abdominal region only.

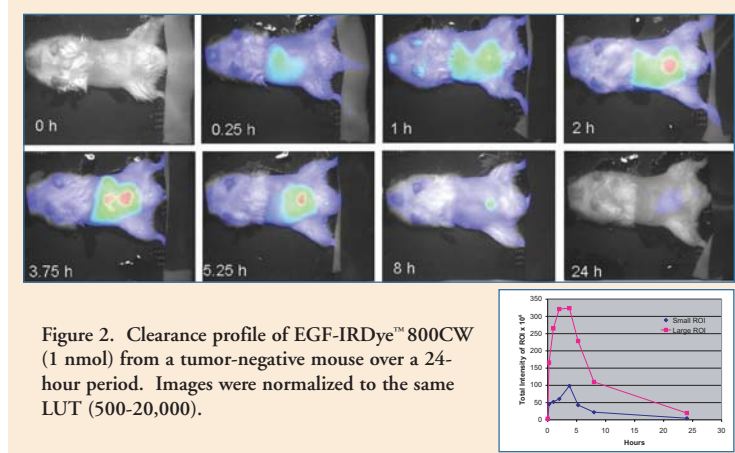


Figure 2. Clearance profile of EGF-IRDye™ 800CW (1 nmol) from a tumor-negative mouse over a 24-hour period. Images were normalized to the same LUT (500-20,000).

Results showed the peak signal for EGF-IRDye™ 800CW at 3.75 hours after injection, irrespective of ROI size. Accumulation in the bladder was clearly evident by 1 hour and 95% of total signal intensity had diminished by 24 hours.

A tumor-positive animal was then evaluated for probe clearance using SNR as a measure. A series of images was taken to determine the optimal imaging time post-injection for a longitudinal study. After injection via the tail vein, the mouse was imaged at 0.3, 24, 48, 72, and 96 hours (Figure 3) with the largest observed SNR achieved on day 4.

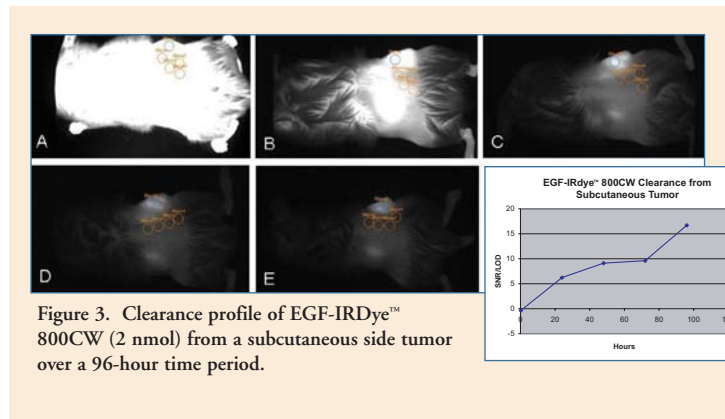


Figure 3. Clearance profile of EGF-IRDye™ 800CW (2 nmol) from a subcutaneous side tumor over a 96-hour time period.

Early detection of tumors

Imaging began two weeks after SCID mice were implanted with tumor cells. Limit of Detection was determined by evaluating an image that contained two small spots (< 0.5 mm) in the area of interest (arrows in Figure 4). SNR were determined to be 3.20 and 2.43 for A and B, respectively, on week 2. One week later, injection and imaging were repeated. The week 3 image in Figure 4 now shows that spot A is larger and confirmed as tumor signal by palpation, while spot B is no longer visible and determined to be an artifact. We suggest that a SNR of greater than 3 is necessary for any spot to be considered positive.

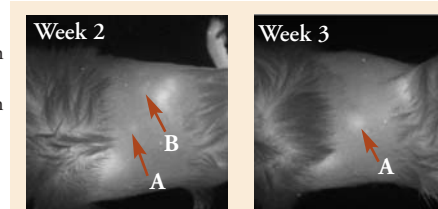
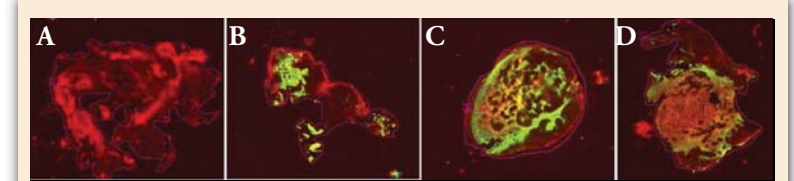


Figure 4. Limit of detection evaluation with two suspect subcutaneous tumors at week 2. Week 3 imaging identified spot A as a tumor (SNR = 3.2, at week 2). Spot B (SNR = 2.43) was false.

Tissue Section Analysis

A competitive challenge was conducted during week 5 with an animal receiving one of the following treatments: 1) EGF-IRDye™ 800CW (2 nmol); 2) IRDye™ 800CW only (3 nmol); 3) 2 mg/mL C225 plus EGF-IRDye™ 800CW (2 nmol) 24 hours after C225 injection, and 4) 0.9% saline mock injection. After final imaging (day 4 post-injection), animals were sacrificed. Tumors were weighed, measured, and frozen sections prepared (0.8 µm thickness) for analysis on the Odyssey® Infrared Imaging System (LI-COR).

Tissue section comparisons between EGF-IRDye™ 800CW and C225 + EGF-IRDye™ 800CW showed a 54% decrease in signal when C225 was given 24 hours prior to EGF-IRDye™ 800CW (Figure 5). When only IRDye™ 800CW was given, some signal was retained in the tumor, as also seen *in vitro*. The 24-hour interval between the C225 and EGF-IRDye™ 800CW injections may not be optimal for complete blocking. Wang *et al.* (2004) showed a similar phenomenon with a different probe, Cy5.5-cKRGDF, where a partial recovery of probe uptake was noted after a 24-hour interval between injection and imaging.



Treatment	ID	Fluor. Intensity 700	Fluor. Intensity 800	Area (mm ²)	Fluor. Intensity 800 /area (mm ²)	Avg
A) 0.9% Saline	C2P0	4.5	0.89	65.32	0.01	0.01
		3.5	0.34	65.33	0.01	
B) IRDye™ 800CW only	C2P2	1.45	18.7	30.06	0.62	0.56
		1.24	13.18	26.22	0.50	
C) EGF-IRDye™ 800CW	C1P0	1.08	14.08	21.73	0.65	0.70
		0.98	15.13	20.49	0.74	
D) C225+EGF-IRDye™ 800CW	C1P2	2.27	15.43	39.49	0.39	0.38
		1.99	13.71	37.86	0.36	

Figure 5. Duplicate tissue sections scanned on Odyssey. Red and green signals represent 700 and 800 channels, respectively. Signal per area (mm²).

CONCLUSIONS

We have shown that the EGF-IRDye™ 800CW probe is specific and sensitive with respect to PC3M-LN4 and 22Rv1 prostate cancer cell lines, both *in vitro* and *in vivo*. This probe may also be useful for other cancers, since EGF receptor is elevated in many tumor models. NIR optical imaging of live animals is a powerful technique that offers researchers the ability to monitor tumor growth non-invasively. The IRDye™ 800CW fluorophore, with its excellent spectral properties and straightforward labeling chemistry, should prove invaluable for such studies.

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