Poster # 3341

Development of a Multiplex Quantitative Immunofluorescence Assay to Determine DNA Damage Repair Deficient Models In Vitro and In Vivo and the Response to Cytotoxic Agents

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Introduction

The use of DNA damaging agents remains a mainstay in cancer therapy. Defects in a tumor cell, including deletions and mutations of key DNA repair proteins, can alter the response and dictate which of the DNA damage repair (DDR) processes will be critical in repair, enabling the ability to predict effectiveness of treatment by identifying sensitive populations (1,2). Our group is developing a quantitative multiplex immunofluorescence assay (qIFA) for biomarkers of active homologous recombination (HR) and nucleotide excision repair (NER) pathways. The complete DNA damage repair panel 1 (DDR1) consists of reporters for γH2AX, Nbs1 pS343, Rad51 and ERCC1. The assay in development uses highly specific antibody reporters, some of which have been directly conjugated to fluorophores, allowing the use of multiple antibodies from the same host species when following a properly controlled SOP.

Quantitation of repair pathway activation after drug treatment was originally built upon our validated qIFA for γH2AX, which scores individual nuclei as positive or negative for the biomarker and calculates the percent of nuclear area positive for the image field as a surrogate for nuclear counts (3). Addition of the Definiens analysis software has improved our analysis capacity of DNA damage markers, including enhanced ability to count the number of individual foci per nucleus over an image set that incorporates thousands of data points. This approach has proven utility in quantifying markers that occupy only a small intranuclear area, such as Rad51 and ERCC1, where total nuclear area measurements are less informative.

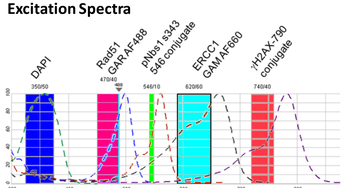
Initial experiments using the multiplex qIFA were performed on cell lines following treatment with a DNA damaging agent, proving utility of the markers in the DDR1 panel. The multiplex was next applied to an *in vivo* model of A375 xenografts treated with DNA damaging agent topotecan. These studies prove as proof of principle for using the multiplex on formalin-fixed, paraffin embedded tissue samples. An *in vitro* model of cell lines with varied BRCA1 expression exposed to DNA damaging IR was used as a platform for developing a Definiens software analysis algorithm, which accurately can measure focal counts per nucleus over a dataset including approximately 100,000 events. These results demonstrate the utility and feasibility of our DDR1 qIFA multiplex assay in monitoring DNA damage.

Materials and Methods

Formalin Fixed, Paraffin-Embedded Slide Preparation: ***In Vitro:*** A2780 ovarian cancer cells (NCI-Frederick Repository) were treated with vehicle or 20 uM Cisplatin (NSC 119875, Sigma) for 4 or 24 hours. Cells were prepared as FFPE blocks according to the standard protocol of the Pathology and Histotechnology Laboratory of NCI-Frederick. ***In Vivo:*** FFPE A375 xenograft samples obtained from a previous study were used for assay development of the DDR1 qIFA panel. Information about the tumor staging, drug dosing and biopsy collection can be found in the referenced source (3). Tumors biopsied 2 hours post-dose were used. Slides were cut to 5 um sections and stained using a Bond-max Autostainer (Leica Microsystems) with optimized antibody dilutions and antigen retrieval methods.
Image Acquisition and Analysis: Slides were observed on a wide-field fluorescent microscope (Nikon 90i, Andor Camera, NIS Elements Software) using either a 40 or 60X objective. Images of FFPE cell pellets were analyzed for nuclear area positive (gH2AX, pNbs1) or number of foci per nucleus (Rad51, ERCC1) using Definiens Tissue Studio IF software. Images are representative of the mean. For high-content screening data, cells were treated and fixed on a 96 well plate (Matrical MGB096-1-2-LG-PDL-L), fixed with 70% ethanol and stained for DDR1 markers. The GE InCell 2000 was used for image capture, analysis with Definiens software.
ERCC1 Antibody Competition Assay: Specificity of the ERCC1 antibody used was tested by competition of the antibody with full-length ERCC1 recombinant protein. ERCC1 protein was produced by Argonne National Lab (1 mg/ml in 50mM ammonium bicarbonate buffer, pH 8.0). UWB1.289 ovarian cancer cells (ATCC) were plated in chamber slides (Lab-Tek II coated glass, Nunc) one day prior to fixation with 70% ethanol. Overnight, the primary antibody was incubated with 20 fold molar excess of recombinant ERCC1 or equivalent volume of buffer at 4^{0}C with gentle rocking (total working volume, 100 ul). Chamber slides were stained using the DDR1 protocol. Images were captured under constant light exposures.

DDR1 qIFA Panel Background Information

Excitation Spectra



Plot from: Life Technologies Fluorescence SpectraViewer[4]

Antibody Description

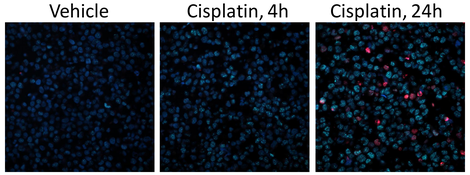
DDR1	Antigen Description	Reporter	Source
γH2AX	Histone H2AX phosphorylated at Ser139	790 direct conjugate	Millipore 05-636
Rad51	DNA repair and recombination protein	AlexaFluor 488	Novus NB100-148, Epitomics 3161-1
pNbs1	Nbs1 phosphorylated at Ser343	546 direct conjugate	Epitomics 2194-1
ERCC1	Excision Repair Cross Complement Group 1	AlexaFluor 660	Novus NB500-704
DAPI	Fluorescent stain for DNA	Blue channel	Invitrogen

Application of the DDR1 qIFA Assay

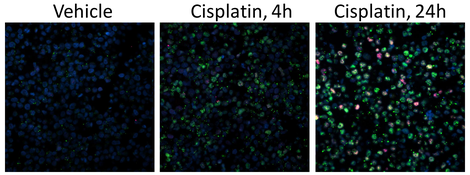
In Vitro Development of the DDR1 Multiplex and Definiens Analysis

1

FFPE A2780 cell pellet, ERCC1 and γH2AX



FFPE A2780 cell pellet, pNbs1 and Rad51



ERCC1-cyan; pNbs1-green; γH2AX-red; Rad51-pink

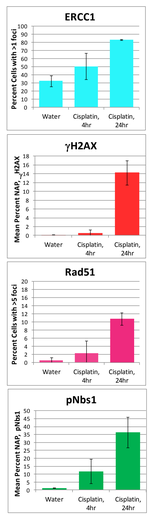


Figure 1: A2780 ovarian cancer cell lines were exposed to vehicle or 20 uM cisplatin (4, 24 hr). Cells were then formalin-fixed and paraffin embedded (FFPE). Sections of the FFPE block were stained with the markers of DDR1 split in two panels; ERCC1 and γH2AX (top row, cyan and red, respectively) or pNbs1 S343 and Rad51 (bottom row, green and pink, respectively). Representative 40x images are shown. Definiens analysis calculating nuclear area positive or percent of cells with foci are shown in bar graphs on right, error bars represents s.d. of two independent runs.

2

Application of Definiens analysis, ERCC1 foci counting

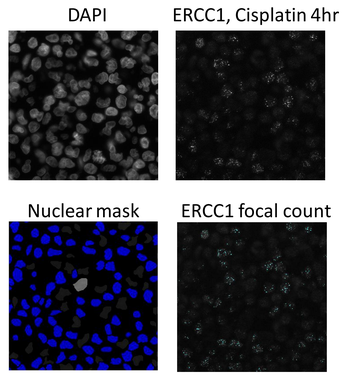


Figure 2: Definiens analysis of ERCC1 foci formation after exposure to cisplatin. Nuclei are classified using area and roundness with those passing criteria false colored blue, failing are gray (bottom left). Foci were counted per nucleus with high accuracy (focal count, bottom right). Images represent dataset used to produce bar graphs in Figure 1.

In Vivo Use of DDR1 qIFA Multiplex

3

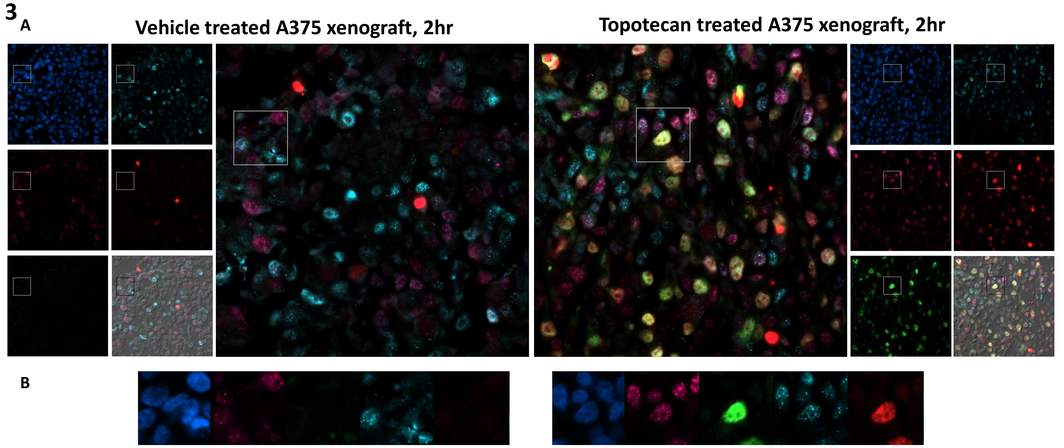


Figure 3: A375 xenografts were exposed to vehicle or 1.5 mg/kg topotecan (2 hr). Tumors were biopsied then formalin-fixed and paraffin embedded (FFPE). Sections of the FFPE block were stained with the DDR1 multiplex on a single slide; using unconjugated ERCC1 and Rad51 antibodies (cyan and pink) with custom conjugated antibodies to pNbs1 S343 and γH2AX (green and red). Representative 60x images are shown. ERCC1 is has baseline expression in the vehicle treated xenograft which shows modest change with topotecan exposure. pNbs1 and gH2AX are induced with topotecan exposure. **A:** Large merged image is 4 markers without DAPI. Small inserts are mono-channels as follows (DAPI, Rad51, pNbs1 left; ERCC1, γH2AX and merge plus pseudo-phase contrast right). **B:** Increased magnification of subset of cells. Cells expressing both pNbs1 (green) and gH2AX (red) will be yellow on merged image.

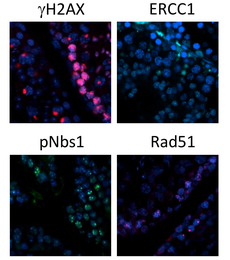
ERCC1-cyan; pNbs1-green; γH2AX-red; Rad51-pink

Antibody Validation

4

Antibody	Positive control tissue
γH2AX	Mouse testis
ERCC1	Rat testis
pNbs1	Mouse jejunum
Rad51	Mouse, Rat testis

Figure 4: Control tissue samples from mouse and rat as positive controls for DDR1 markers.



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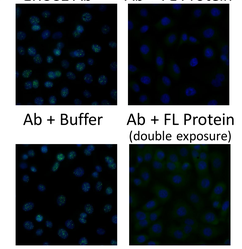
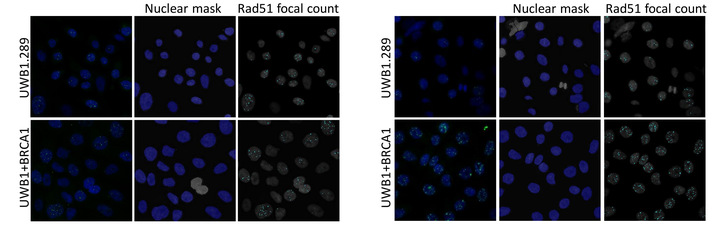


Figure 5: ERCC1 full length protein competition assay. Constant exposure, except bottom right (doubled).

Analytical Model: Use of Definiens Software for High-Content Data Analysis

An ovarian cancer cell line negative for BRCA1 expression (UWB1.289) and its isogenic, BRCA1 reconstituted line (UWB1.289+BRCA1) were exposed to IR, fixed and stained for Rad51 at 24 hours post-exposure. Differences in DNA damage repair pathway integrity altered Rad51 foci capacity.

6



Definiens analysis: Rad51 foci formation quantified in BRCA1 + and – cell lines, high content analysis

	UWB1.289		UWB1+BRCA1	
	24h control	24h post-IR	24h control	24h post-IR
Nuclei with >5 foci	2446	3212	4894	14648
Total # nuclei counted	20735	19223	33209	24560
Percent Rad51 positive	12	17	15	60

Figure 6: Definiens analysis of Rad51 foci formation 24 hrs after exposure to IR. The Definiens software enables quantification at a high-content scale. Nuclei were counted in 10 representative fields per group and sorted according to our developed mask. In total, the number of Rad51 foci per cell was counted across approximately 100,000 nuclei.

Conclusions

- We have developed a DNA damage repair immunofluorescent multiplex assay to assess DNA damage markers γH2AX, Nbs1 pS343, Rad51 and nucleotide excision repair protein ERCC1 on the same tissue section.
- Using multiple antibodies on the same cell population allows for characterization of drug treatment response and identification of sub-populations which respond differently to treatment.
- To achieve an assay with multiple markers, conjugation of primary antibodies directly to fluorophores was accomplished, further development of conjugated antibodies in the panel is required. Eliminating the use of anti-species secondaries will reduce background noise encountered when using an anti-mouse secondary on a xenograft sample.
- The Definiens Tissue Studio package was used to obtain quantitative results of both nuclear area positive and number of foci per nucleus from in vitro studies, including analysis of a large dataset containing approximately 100,000 nuclei. This software is being developed further for analysis of in vivo samples.

References

1. Wang XZ, Weaver DT. The ups and downs of DNA repair biomarkers for PARP inhibitor therapies. *Am J Cancer Res* 2011; 1(3): 301-327.
2. Plummer R. Prospective on the pipeline of drugs being developed with modulation of DNA damage as a target. *Clin Ca Res* 2010; 16(18): 4527-4531.
3. Kinders RJ, Hollingshead M, Lawrence S, Ji J et al. Development of a validated immunofluorescence assay for γH2AX as a pharmacodynamic marker of topoisomerase I inhibitor activity. *Clin Cancer Res* 2010; 16(22): 5447-5457.
4. http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Analysis/Labeling-Chemistry/Fluorescence-SpectraViewer.html

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NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. All studies were conducted under an approved Animal Care and Use Committee protocol.