Measuring tyrosine kinase inhibitor effects on cell signaling pathways

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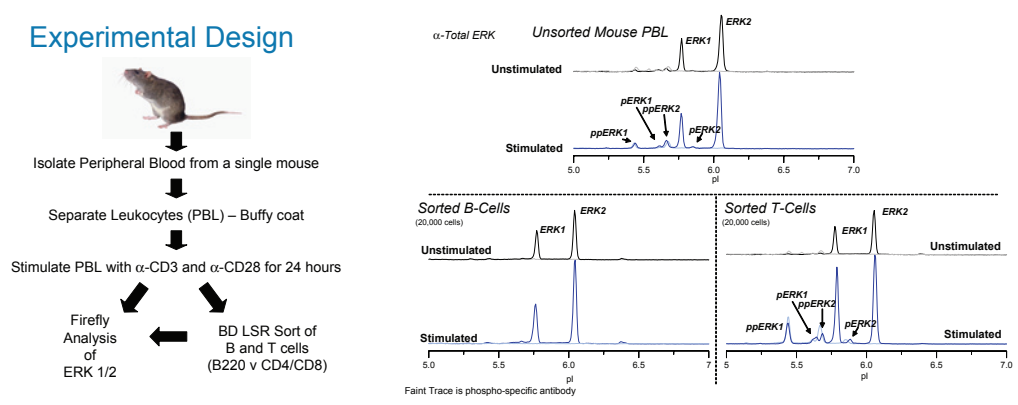
Abstract

A recent wave of anti-cancer compounds that target tyrosine kinases (TKIs) has been moving through the drug development pipeline. Assessment and screening of lead compounds in simple model systems is relatively straight forward. Until recently, however, determining the impact of these compounds in complex biology of patient-derived cells and tissues has been difficult. Proposed genetic or protein biomarkers can act as surrogates to a response, but, measuring the signaling pathway in both the target cells, and surrounding normal tissue will provide a more direct metric. This has proven difficult due to the limited nature of primary material and complexity of tissue structure. Here we describe a novel nano-immunoassay platform (Firefly™) that has two significant advantages over traditional immunoassay: (1) extremely sensitive protein detection, and (2) physical isoform separation which allows for quantitation of protein isoforms as well as post-translational modifications such as phosphorylation.

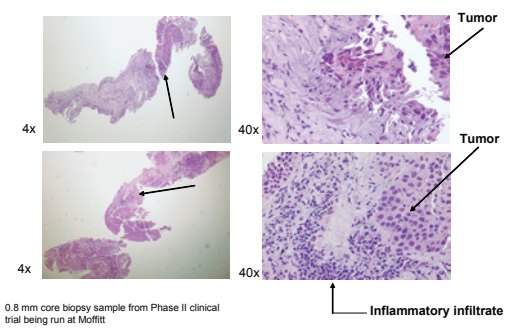
Applications of this technology that will be described include:

1. Effect of TKIs on signaling in punch biopsies of non-small cell lung cancer cells
2. Signaling pathway response from chronic myelogenous leukemia patients to therapies targeted to the bcr/abl translocation

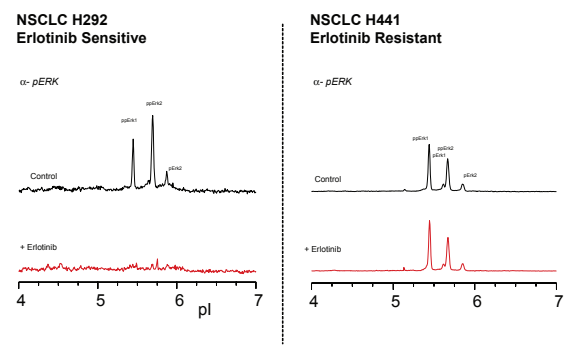
Sample heterogenity masks specific response



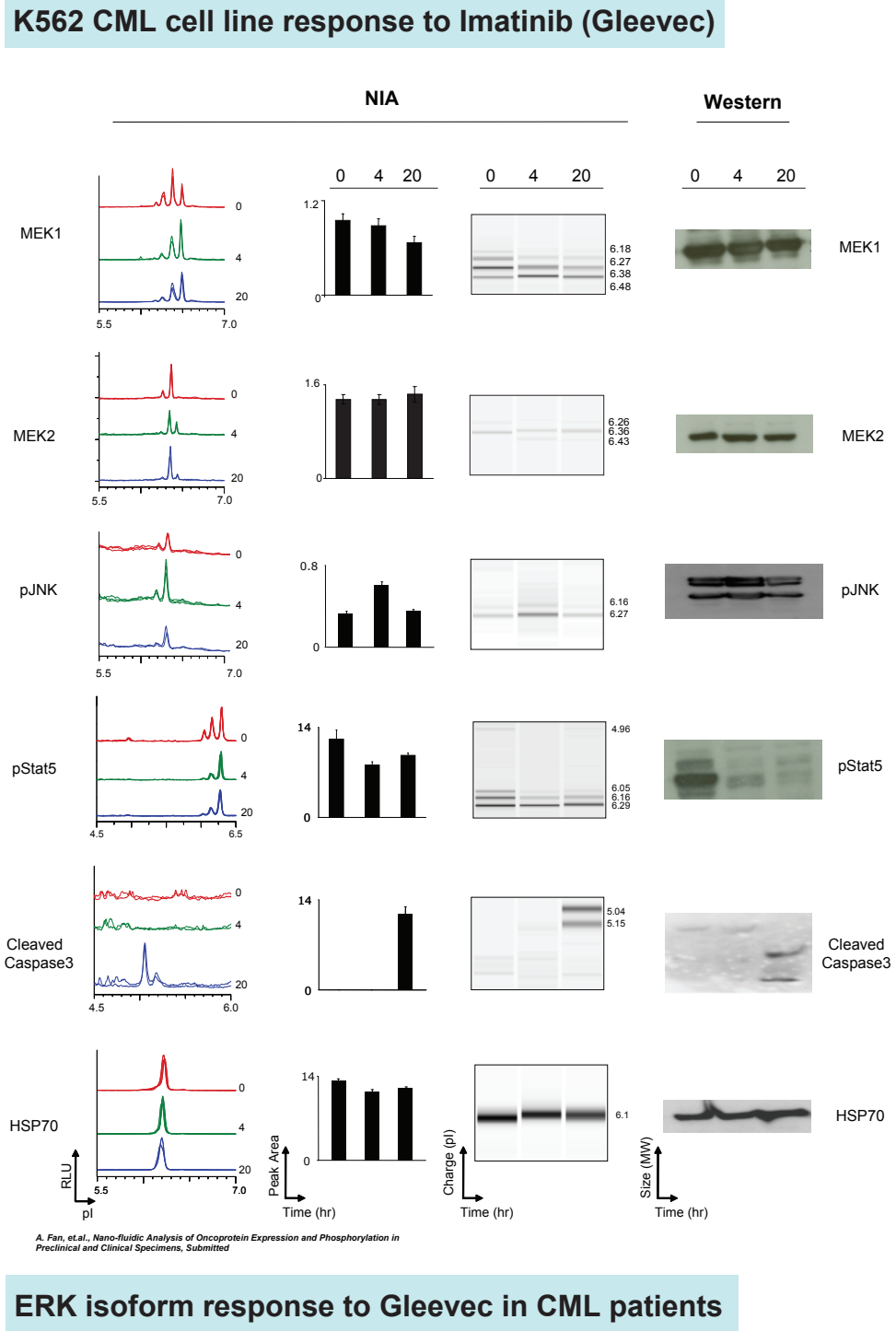
Heterogeneity of primary tissue: only a percentage of material is NSCLC



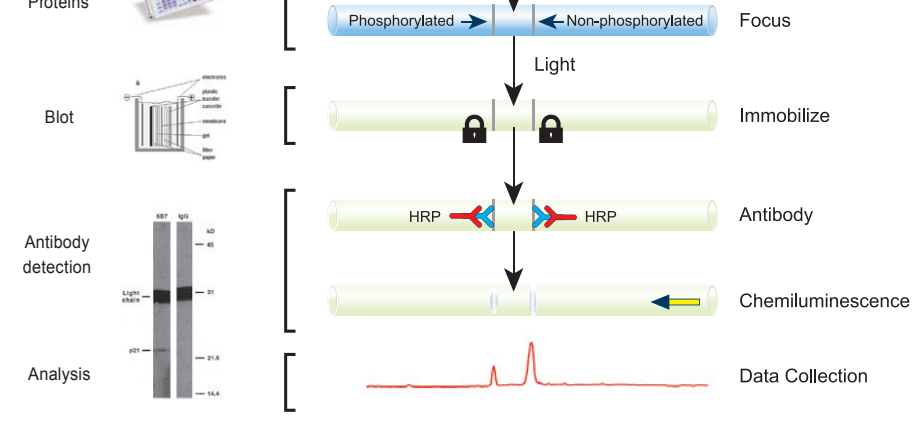
Cell lines give binary results because of their homogeneity



K562 CML cell line response to Imatinib (Gleevec)



The Firefly™ capillary nano-immunoassay



The Firefly™ Assay

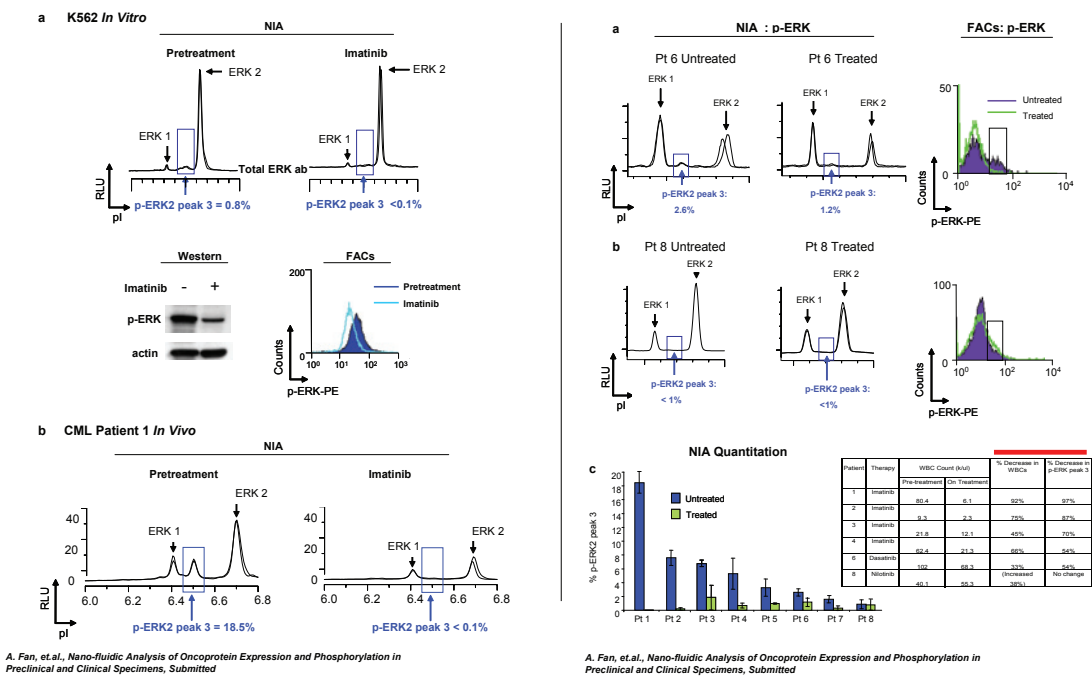
Figure 1. The Firefly nano-immunoassay:

- **Fill:** A 5 cm capillary is filled with a separation buffer containing sample proteins and fluorescent peptide standards.
- **Focus:** Isoelectric Focusing (IEF) is used to focus the proteins.
- **Immobilize:** The focused proteins are immobilized by exposing the capillary to the UV light source.
- **Antibody:** Labeled antibodies are flowed through the capillary to bind to the immobilized proteins.
- **Detection:** Whole capillary imaging is performed using chemiluminescence.
- **Data Collection:** Capillary images are converted to signal vs. pI graphs for analysis.

Detection of phosphorylated and non-phosphorylated proteins

Figure 1. Analysis of phosphorylated forms and isoforms of ERK protein. Cultured HT-29 human colorectal adenocarcinoma cells were treated with the cytokines insulin (500 ng/ml) and TNF-alpha (100 ng/ml) for times from 0 to 60 min prior to preparation of cell lysates. (A) Conventional SDS-PAGE western blot of 0 to 60 min samples, probed with antibodies specific for ERK1, ERK2, ERK1&2 (pan-specific ERK antibody), and phosphorylated ERK1&2 (pERK1&2). (B) Firefly analysis of these same samples, probed with the same antibodies allows peak identification as follows: 1 ppERK1, 2 pERK1, 3 ppERK2, 4 ERK1, 5 pERK2, and 6 ERK2. (C) Differences in phosphorylation upon stimulation are evident in the pan-specific Ab profiles obtained for 0 and 30 min lysates. Integration of the peaks corresponding to each identified species allows percent phosphorylation of ERK1 and ERK2 to be calculated. (D) Using percent phosphorylation data generated as in panel C allowed phosphorylation vs. time of cytokine stimulation to be plotted for both ERK1 and ERK2. Percent phosphorylation shown is the sum of peak integrations for mono- and di-phosphorylated species.

ERK isoform response to Gleevec in CML patients



Advantages of Firefly nano-immunoassay platform

- Single antibody detection of multiple protein variants.
- Small sample size: One drop can potentially fill the entire 96 capillary array.
- Quantification of cell signaling pathways.
- Completely automated

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