Introduction

The Zephyrus Biosciences Z1™ instrument and zWest™ chips enable users to simultaneously generate single-cell resolution western blots (scWesterns®) for over 1000 single cells, in a fraction of the time of a conventional western.

Why single-cell western blotting?

INCREASE RESOLUTION

Many cell populations, such as tumor cells or differentiating stem cells are heterogeneous. Protein expression measurements at the population level may therefore overlook significant differences in expression between individual cells and the existence of cell subpopulations (Figure 1). The Z1 combines single-cell resolution with the power and versatility of western blotting. This approach allows simultaneous measurement of multiple proteins in over 1000 single cells using commercially-available western-validated antibodies.

VALIDATE SINGLE-CELL RNA-SEQ TARGETS

The Z1 enables validation of newly-discovered transcripts from RNA-Seq. Up to 4 proteins can be probed simultaneously in single cells, and zWest chips can be stripped and reprobed to detect additional protein targets.

*Hughes et al., Single-cell western blotting, Nat. Methods, 2014

DETECT CHALLENGING FLOW TARGETS

The Z1 uses western-validated antibodies and is able to simultaneously detect both surface markers and internal markers. Phosphorylated proteins, transcription factors, cytoplasmic, and nuclear proteins can all be detected using the Z1. As a greater number of antibodies are available for western blotting than for flow cytometry, the Z1 presents a viable alternative to detect hard-to-identify intracellular proteins or targets for which flow antibodies are not readily available.

How does the Z1 work?

The Z1 system consists of the Z1 instrument, zWest chips, an antibody probing fixture, and accompanying buffers. Referring to Figure 2: a single-cell suspension (1) is settled onto a zWest chip - a polyacrylamide-coated glass microscope slide containing an array of microwells (2). About 1000-2000 single cells are captured and the zWest chip is placed in the Z1 instrument. The cells are then lysed, their contents are electrophoretically separated via SDS-PAGE, and the separated proteins are covalently bound in the gel (3). Protein targets are then probed in-gel using a custom antibody probing fixture and western-
validated primary and fluorescently-labeled secondaries (4). The probed chip is imaged in a microarray scanner (5), the resulting image is analyzed using Zephyrus software (6), and data is reported using standard visualization tools (7). Probed zWest chips can be stripped and reprobed for new targets or dried for long-term archiving (reprobing of archived chips is possible months later).

**Example Z1 measurements**

**PHOSPHOPROTEINS**

On-chip stimulation of cells can be used to study signaling pathways and probe for time-sensitive events such as phosphorylation. Figure 3 shows a scatter plot of pEGFR expression vs. GAPDH expression in stimulated and unstimulated cells. As in flow cytometry data, each point is a measurement from a single cell. However, unlike flow cytometry, each point is also associated with one or more separation images which can be inspected to verify that the signal is associated with a peak at the expected location.

**SUBPOPULATIONS AND HETEROGENEITY**

Conventional western blots show only average protein expression. The Z1 system reveals the existence of subpopulations and gives new insights into cell-cell heterogeneity (Figure 4). While flow cytometry measures single-cell heterogeneity, results are confounded by off-target binding or variants/isoforms that bind the same antibody. In contrast, single-cell westerns can distinguish off-target binding and protein variants/isoforms (Figure 4 and Figure 5).

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**Figure 3.** A 5-minute on-chip EGF stimulation induces pEGFR expression in A431 cells (blue). Unstimulated cells show no pEGFR expression (black). On the right, images of protein separations are shown (microwell at the top and electrophoresis in the downward direction). Characteristic peaks and representative intensity plots are displayed on the right.

**Figure 4.** The Z1 reveals three subpopulations within the same population of cells. A single antibody has affinity to two protein variants (X1 and X2) in HEK293 cells. While conventional western blotting can show only the population average expression for X1 and X2, single-cell western blotting reveals three subpopulations: cells expressing only the large molecular weight variant X1 (top left, red), only the small variant (top middle, blue), or both X1 and X2 (top right, black). Single-cell peak intensities plotted vs. separation distance (bottom) show the three populations and reveal cell-cell heterogeneity in protein expression.

**Figure 5.** Histogram of total peak area (all targets). This histogram shows the distribution of the total protein signal (X1 + X2) and represents what a flow cytometer would detect. The darker green portion of the histogram indicates the ~20% of measurements that contain signal from the smaller molecular weight species (X2). If X1 were the desired protein target, off-target binding of X2 in flow would lead to ~20% false measurements. Inset: fraction of the cell population that has protein species X1 only, X1 and X2, and X2 only.