APPLICATION NOTE

Rapid Single-Cell Cloning and Screening to Identify Superior Reporter Cell Lines Using the Lightning® Optofluidic System

APPLICATION SPOTLIGHT

- Rapidly screen heterogeneous cell pools to identify superior clones in 3 days instead of weeks
- Culture, visualize, and capture kinetics of reporter gene response across >1,000 single cells in real-time
- Select and export viable cells for further growth or analysis

INTRODUCTION

Reporter gene assays are powerful tools for studying a wide range of biological processes including signaling pathways that play critical roles in human biology and disease. However, reporter gene cell lines are often subject to noise due to multiple factors such as positional effects at different genomic integration sites,[1,2,3] copy number variations, and stability of the construct. Traditional clone selection for the generation of reporter cell lines requires labor-, time-, and material-intensive limiting dilution methods or FACS sorting.[4,5] While these methods can be used to sort or select cells into well plates, they still require further laborious and time-consuming downstream processing to grow and assay the cells in order to select and identify the single best clone.

The Lightning system allows selection and cloning of >1,000 single cells into individual NanoPen® chambers on a microfluidic chip. Clones can be cultured and assayed within several days. This bypasses weeks of culture that are required by traditional methods to yield a sufficient number of cells to perform assays. Cells with phenotypes of interest can then be selected and exported from the microfluidic chips for further expansion and characterization.

In this application note, we demonstrate that these capabilities allow the Lightning system to establish optimized reporter cell lines effectively and more rapidly than traditional methods. Using NF-κB reporter cell lines as a model system, we show that the Lightning system can be used to select reporter cell lines with the most desirable characteristics within days of single-cell selection saving at least 2 weeks compared to traditional subcloning approaches. Additionally, we demonstrate that the best clones can be selectively exported as stable clones that outperform the pool average.

MATERIALS AND METHODS

Development of TNFα-responsive reporter cells

K562 cells were transfected with an NF-κB reporter construct containing a green fluorescent protein (GFP)::puromycin selection cassette to identify and select for transfected clones. Red fluorescence protein (RFP) was placed under the control of the NF-κB promoter to induce RFP expression upon TNFα treatment. Cells were grown in 96-well plates with Iscove's Modified Dulbecco's Medium supplemented with 20% fetal bovine serum and penicillin/streptomycin, and integration of the construct was selected using puromycin (10 µM) for 6 weeks.

Rapid single-cell cloning and functional screening on the Lightning system

The generation of an efficient clonal cell line requires screening heterogeneous populations to find the best candidate clones. In this case study, a combination of desired growth characteristics in addition to a sensitive and consistent response to stimuli via the reporter gene was required. The Lightning system was used to characterize the activity of the individual clones to select the best clonal candidates.

Cells were collected at densities of approximately 5 x 10^5 cells/mL and pelleted for 4 minutes at 400 g. Cells were then resuspended at 1–2 x 10^6 cells/mL in media and allowed to recover for 30 minutes. A total of 25 µL of cells were imported in media onto an OptoSelect® chip using the Lightning system at a flow rate of 0.5 µL/sec. The opto-electropositioning (OEP™) and the Target Pen Selection (TPS) features of the Lightning system were used to identify and pen single GFP-positive cells into individual NanoPen chambers of the OptoSelect chip. A total of 2,272 cells were penned over three chips. Cells were cultured on-chip at 37°C with constant perfusion of 20% K562-conditioned media for 72 hours. TNFα (1 µg/mL) was then added to the media to activate the cells. The activation media was perfused at a flow rate of 0.5 µL/sec for 6 hours. After stimulation, cells were cultured with conditioned media. A series of time-lapse images were captured starting at the onset of TNFα stimulation and continuing for 30 hours (Figure 1).

Analysis and recovery of viable candidate reporter clones

To select NF-κB reporter clones, the red fluorescence of each penned cell over the assay period was assessed. Clones that reached a detected fluorescence threshold above unstimulated cells were identified and ranked. The homology of the response within the positive colonies was then observed. Clonal cell lines that exhibited the best response over threshold and provided a homogeneous response were ranked as positive hits. These clones were exported from the OptoSelect chip using the Lightning system into 96-well plates and cultured with conditioned media for 4 weeks before retesting to check for clonal stability.

Re-stimulation and validation of exported reporter clones

To validate the responsiveness of reporter cell lines developed from clones identified and exported on the Lightning system, the exported clonal cell lines and control cells were stimulated with TNFα (1 µg/mL) in media for 6 hours in 96-well plates. Stimulated cells were collected at densities of approximately 5 x10^5 cells/mL, pelleted (4 minutes at 400 g) and resuspended at 1–2 x10^6 cells/mL in media for 30 minutes prior to re-importing onto an OptoSelect chip. The fluorescence from each cell was then measured in the chip channel using TPS.

RESULTS AND DISCUSSION

NF-κB is a critical signaling pathway involved in inflammation, cancer, and embryonic development, and is known to be activated by the cell signaling protein tumor necrosis factor (TNFα).[4] Cell lines that report NF-κB pathway activity are useful for investigating specific functions within disease models and to understanding the efficacy and mechanism of action of potential therapeutic interventions.

To identify cell lines with a robust, homogeneous response, we exposed candidate cells to TNFα and characterized NF-κB reporter construct expression

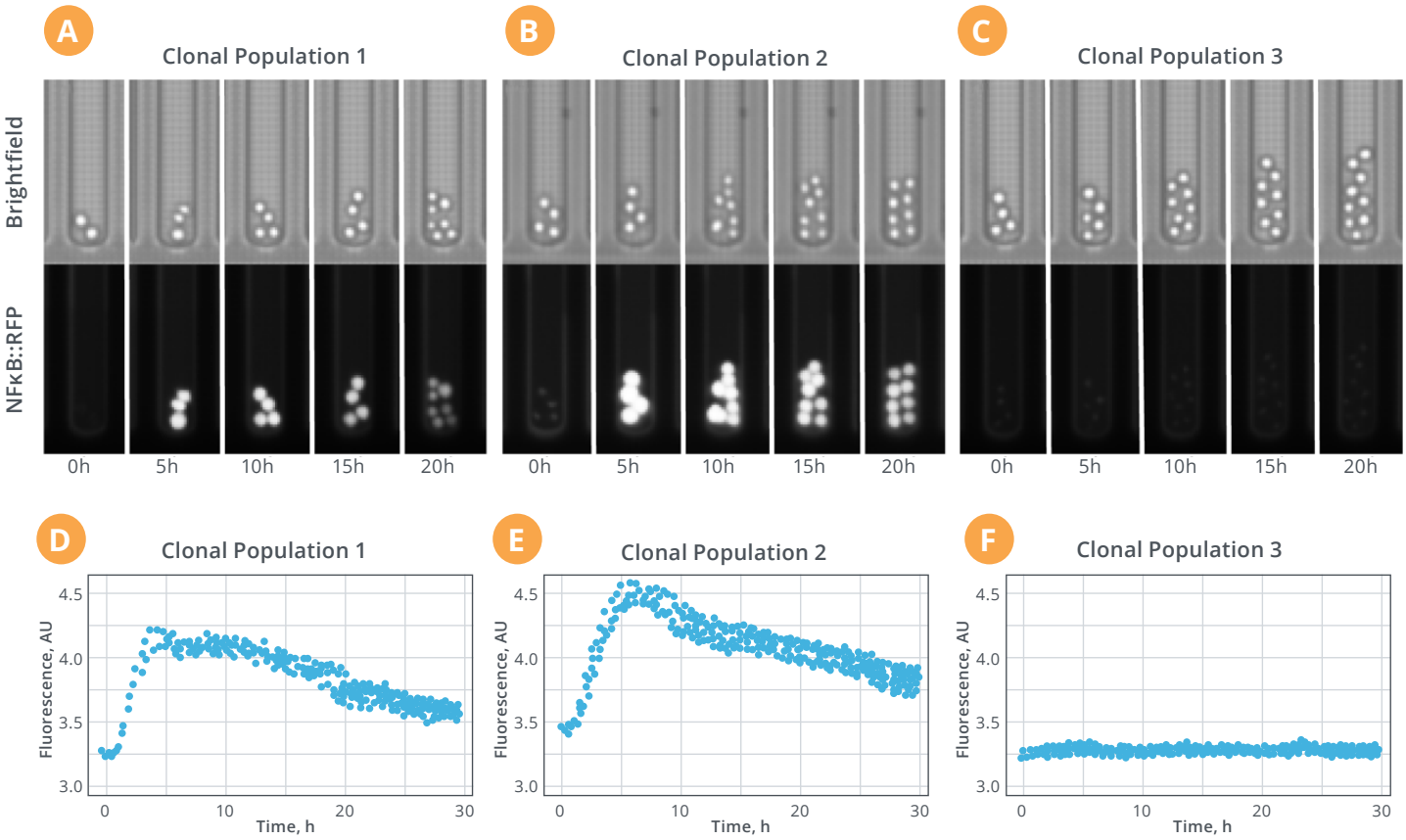

Figure 1. Identification of rare reporter cell lines with robust and homogeneous response on the Lightning system. A–C. Corresponding time-lapse brightfield and Texas Red images of three different pens culturing K562 cells on the Lightning system during stimulation with TNFα. Clones were generated by penning individual cells positive for a NF-κB reporter. TNFα activation started at t=0h and ended at t=6h. **D-F.** Scatterplots of red fluorescence (log mean brightness of individual cells) over a 30 hour period for each K562 cell in responsive (**D, E**) and unresponsive (**F**) clonal colonies (as shown in **A–C**).

as measured by RFP expression across thousands of cells. The Lightning system enabled the identification and isolation of rare clones (Clonal Population 1 and 2) that met the criteria for a reporter cell line – a robust and homogeneous response to TNFα across the clonal cell population as measured by RFP expression (Figure 1A, B, D, and E). Only 195 of the 2,272 assayed clones (less than 10%) met these criteria. In addition, we observed that the clones carrying the NF-κB reporter construct exhibited a broad range of RFP expression, presumably at least in part due to the influence of positional effects. Most reporter cell clones showed either high variability within the clonal cell population or little to no response to TNFα (Clonal Population 3, Figure 1C and F).

We next recovered clonal cell lines that were identified as positive hits and cultured these cells in 96-well plates with conditioned media for 4 weeks before retesting to evaluate clonal stability. Upon restimulation, the exported clones showed a superior response to TNFα stimulation compared to the original mixed starting population. The initial unenriched population of transfected cells exhibited a 1.36-fold average increase in fluorescence (Figure 2B). The clonal reporter cells exhibited a 3-fold average increase in RFP fluorescence after stimulation with TNFα compared to the unenriched pool of unstimulated cells ($p < 0.0001$, Mann-Whitney U test) (Figure 2A and 2C). These results indicate that the clonal cells outperformed the pool average with a superior reporter gene response to TNFα.

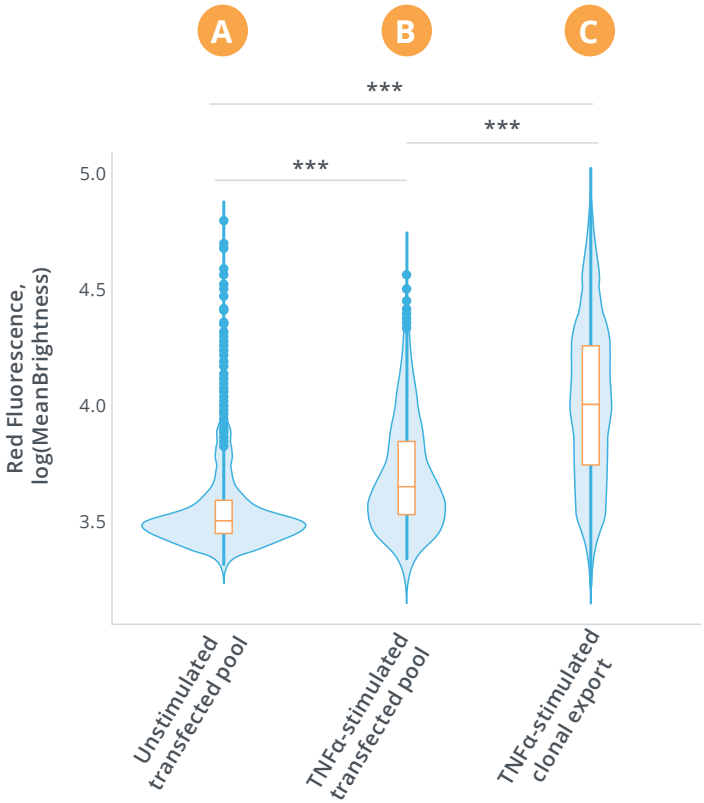

Figure 2. Improved reporter signal and stability of cell lines isolated on the Lightning system. A. The distribution of red fluorescence (log(MeanBrightness)) measured from the initial transfected pool of K562 cells prior to stimulation, **B.** the initial transfected pool after TNFα stimulation, and **C.** a clonal cell line identified and exported on the Lightning system (n=1,110, 447, and 1,457, respectively). Orange bars represent the population means, white boxes represent the upper and lower quartiles, and light vertical lines represent the standard deviation. Raw RFP signal induced by TNFα-stimulation is on average 3-fold brighter in a reporter clone identified on the Lightning system (*** $p < 0.0001$).

CONCLUSIONS

The Lightning system allows single-cell cloning and functional screening of thousands of cells. Here we have shown that the ability to clone and culture single cells and perform functional assays all on a single system allows identification of candidate cells more rapidly than traditional cell cloning methods where each step of the process is separated and generally performed on different platforms. On-instrument single-cell cloning enabled cell screening after only three days of culture while the ability to assay at any time point in the culture process provided insights into the heterogeneity of the clonal response across hundreds of clones during selection. This allowed us to screen a larger number of cells than is possible with traditional methods to identify and recover rare, high-performing, and stable reporter cell lines. This approach can be used to select reporter cell lines for a broad variety of biological characterization applications from basic research to drug discovery and development.

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