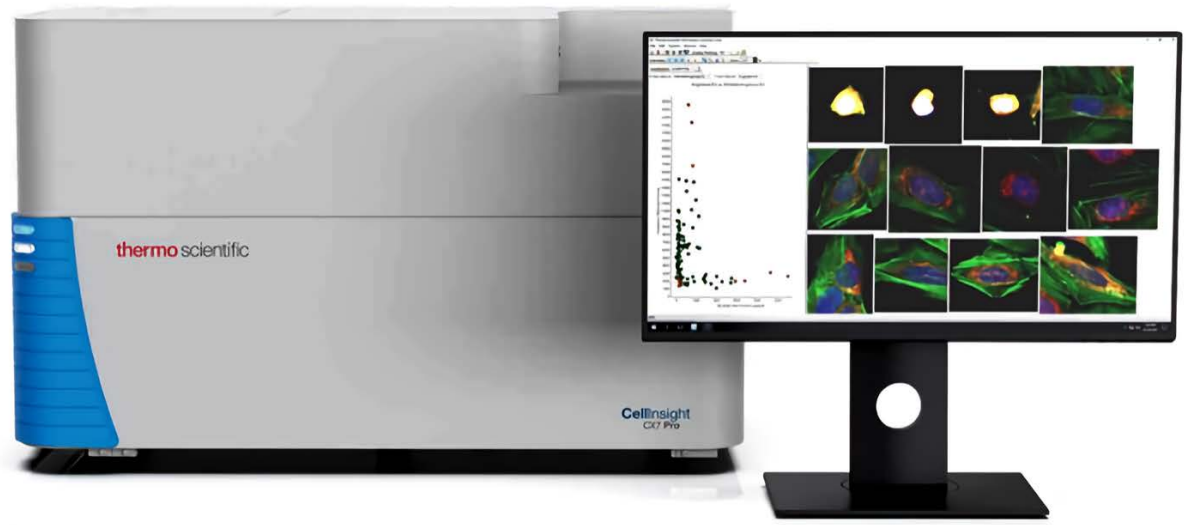

Cell analysis

Interrogating phenotypic changes in CRISPR p53 knockout A549 cells using an adapted cell painting assay on the Thermo Scientific™ CellInsight™ CX7 LZR Pro platform

Introduction

Tumor protein 53, also known as p53, is important for cellular health and maintenance. The p53 protein regulates the cell cycle, initiates DNA repair, and induces apoptosis if a cell's DNA has been damaged beyond repair (1). Mutations in the p53 gene, which lead to the cell's inability to make a functional p53 protein, generally result in cancer formation. Approximately 50% of cancers are the direct or partial result of mutations in the p53 gene (2).

The 2013 paper by Gustafsdottir S. M. *et al.* titled "Multiplex Cytological Profiling Assay to Measure Diverse Cellular States" and the subsequent 2016 paper by Carpenter et. al. in the journal Nature Protocols titled "Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes" demonstrated foundational use of the cell painting technique for multiple cell imaging applications. Since that time, the cell painting assay has been an important tool for interrogating phenotypic changes in cell culture models resulting from factors such as gene knockout, drug treatment, or environmental changes (3).

Although the use of this assay has grown significantly since it was first published, the spectral overlap of some of the dyes used in this assay has limited the ability to accurately quantify the targets of certain dyes on high-content analysis platforms. Specifically, the overlap of SYTO 14™ and Concanavalin A - Alexa Fluor™ 488 conjugates disrupt the quantification of RNA, nucleoli, and endoplasmic reticulum (ER) in the green channel. The overlap of Wheat Germ Agglutinin-Alexa Fluor™ 555 and Alexa Fluor™ 568 Phalloidin also disrupts the quantification of the plasma membrane, Golgi apparatus, and actin in the orange channel. Advances

in near-infrared (NIR) reagents and imaging platforms, paired with platform-specific reagent optimization, can eliminate or significantly reduce the spectral interference of these targets.

Herein, we show how cell painting, with advanced NIR reagents optimized for the Thermo Scientific™ CellInsight™ CX7 LZR Pro, can be used to interrogate the differences in phenotype between A549 wild-type cells and p53 knockout (KO) A549 cells, and how these changes can alter the cells' reaction and sensitivity to certain drugs and compounds.

Materials and methods

CRISPR/Cas9 for p53 knockout

- A549 cells were transduced with Invitrogen™ LentiArray™ Cas9 Lentivirus and treated with blasticidin to remove cells that were not positive for Cas9.
- 4 total gRNAs targeted at the 2nd, 4th, and 5th exons of the p53 gene were electroporated into the cells using the Invitrogen™ Neon™ Transfection System. A second population of cells was electroporated with an untargeted, scramble gRNA.
- Single-cell dispersal was used to generate homogeneous cell populations.
- Knockout was confirmed using a p53 primary antibody and Alexa Fluor™ 488 conjugated secondary antibody on the Thermo Scientific™ CellIInsight™ CX7 LZR Pro system.
- Populations that had no statistically significant difference in signal from the wild-type without p53 labeled were confirmed as knocked out.

Cell painting assay

- A549 p53 KO cells were plated at a density of 5,000 cells per well on the top 4 rows of a 96-well plate. A549 wild–type (scramble) cells were plated at a density of 5,000 cells per well on the bottom 4 rows of a 96-well plate.
- Cells were placed in an incubator under standard cell culture conditions overnight.
- The following day, ½ log dose treatments of the compounds cytochalasin D, etoposide, and staurosporine were added to the plates and incubated for 3 hours, 20 hours, and 4 hours respectively.
- The Invitrogen™ Image-iT™ Cell Painting Kit was adapted to take advantage of the near-infrared detection capabilities of the CellInsight™ CX7 LZR Pro, helping to minimize spectral overlap and improve channel separation. The assay was performed following drug incubation, using the same core components as the original kit with two targeted adjustments:
  - Alexa Fluor™ 594 Phalloidin was used instead of Alexa Fluor™ 568 Phalloidin.
  - Concanavalin A-Alexa Fluor™ 750 Plus conjugate was used instead of concanavalin A-Alexa Fluor™ 488 conjugate.

Results

Cell painting results in A549 p53 KO cells

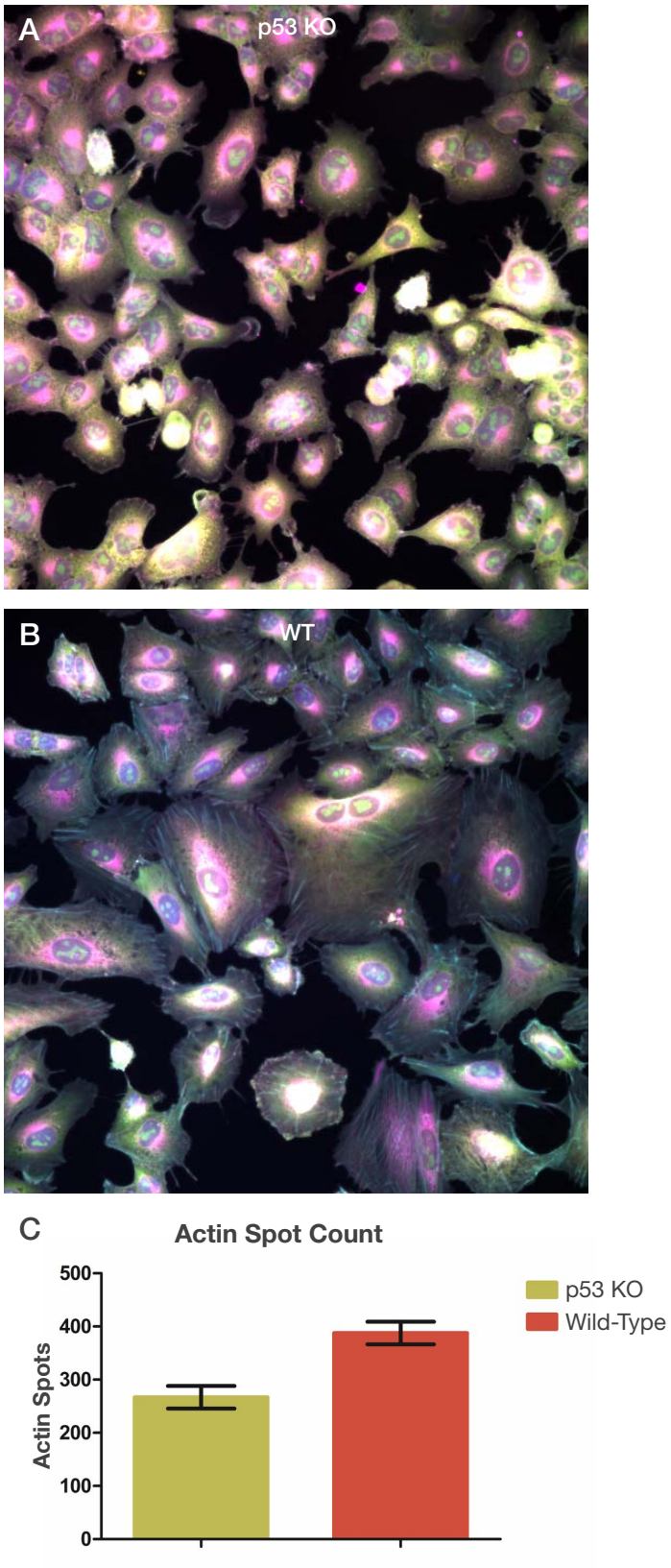

Figure 1. p53 knockout cells (A) showed statistically relevant, phenotypic changes to all the cell painting targets compared to wild-type cells (B). In addition, p53 knockout cells show a significant reduction in the number of actin filaments in the cell (C).

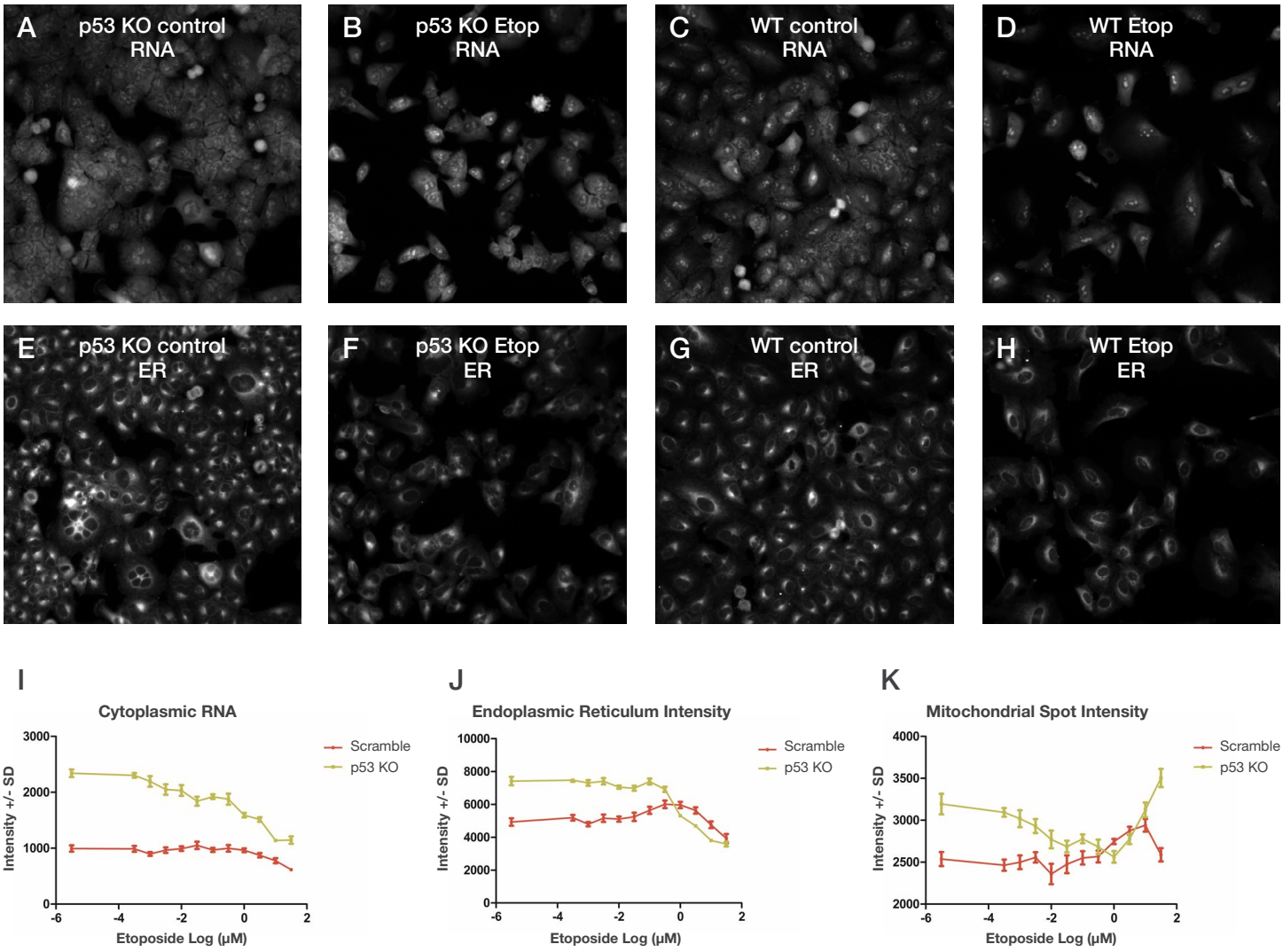

Figure 2. In response to etoposide (Etop) treatment, p53 knockout cells showed accelerated decrease in cytoplasmic RNA (A and B) and endoplasmic reticulum (E and F) compared to wild-type cells (C, D, G, and H). Quantification of cytoplasmic RNA (I) and endoplasmic reticulum (J) using the NIR-adapted version of cell painting assay resulted in overall better resolution because Concanavalin A was moved to the NIR channel. In addition, mitochondrial membrane potential (K) shows an initial decrease followed by an increase for p53 knockout cells whereas the opposite is true for the wild-type cells.

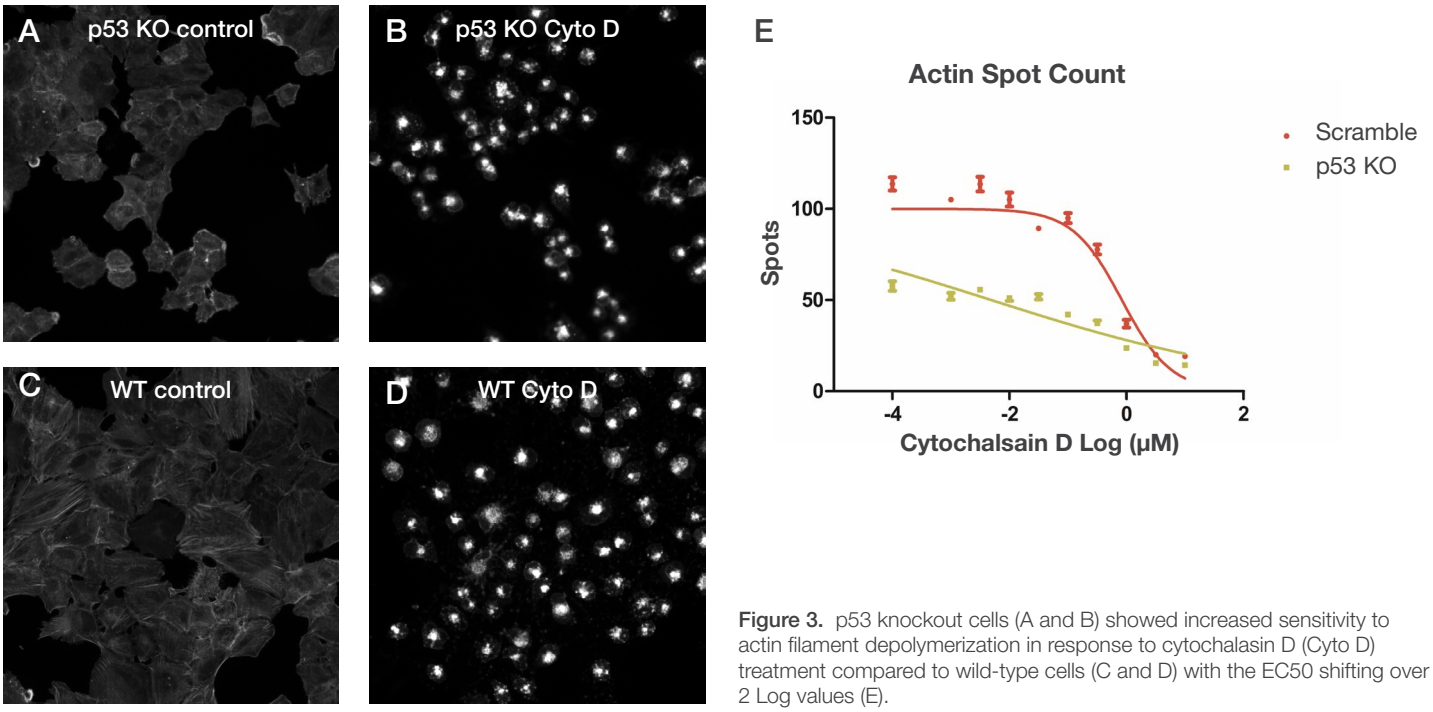

Figure 3. p53 knockout cells (A and B) showed increased sensitivity to actin filament depolymerization in response to cytochalasin D (Cyto D) treatment compared to wild-type cells (C and D) with the EC50 shifting over 2 Log values (E).

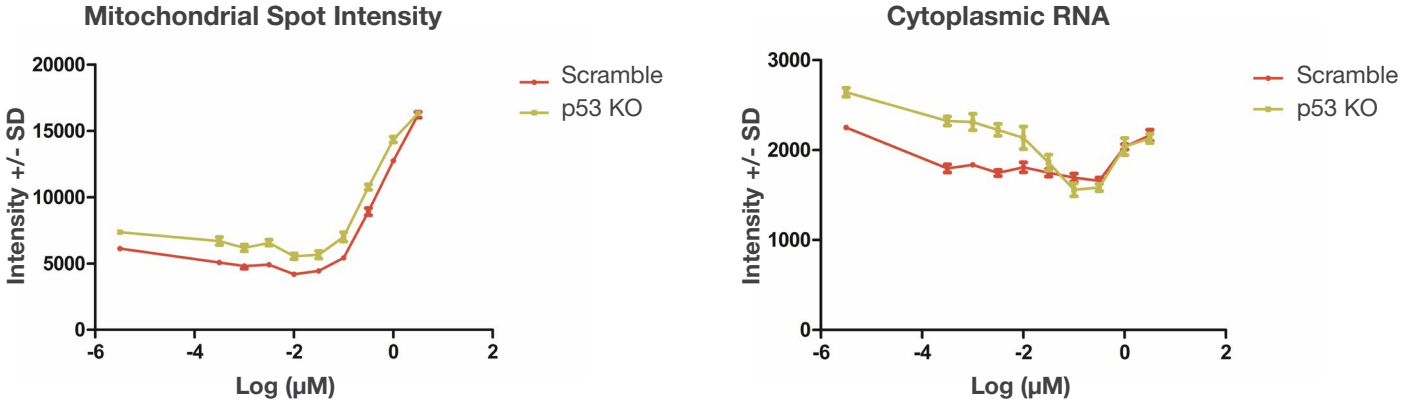

Figure 4. p53 knockout cells react the same as wild-type cells for staurosporine drug treatments and cell painting targets.

Conclusions

The cell painting assay is a useful tool for interrogating phenotypic changes in p53 knockout cells compared to wild type. Our study demonstrates the significant phenotypic differences between p53 knockout and wild-type A549 cells, particularly in response to drug treatments with cytochalasin D, etoposide, and staurosporine. These findings highlight the importance of p53 in maintaining cellular integrity and its role in modulating cellular responses to external stressors.

By tailoring the cell painting assay to better leverage the NIR detection capabilities of the platform used — the CellInsight CX7 LZR Pro— researchers can eliminate or reduce the need for dual labeling in certain channels and thereby significantly enhance quantification. Such optimization allows for more accurate and reliable data, which is crucial for understanding the phenotypic consequences of p53 knockout and the cellular response to various treatments.

Ordering Information

Description	Quantity	Cat. No
CellInsight CX7 LZR Pro HCS Platform	1 instrument	HCSDCX7LZRPRO
Neon NxT Electroporation System	1 instrument	NEON1S
Image-iT Cell Painting Kit	1 kit	I65000
Alexa Fluor 594 Phalloidin	300 units	A12381
Concanavalin A-Alexa Fluor Plus 750 conjugate	1 mg x 5 vials	C56127
LentiArray Cas9 Lentivirus	100 µL	A32064
Neon Transfection System 100 µL Kit	1 kit	MPK10025

References

1. Surget S, Khoury MP, Bourdon JC (2013). Uncovering the role of p53 splice variants in human malignancy: a clinical perspective. *OncoTargets and Therapy.* **7**: 57–68. doi: 10.2147/OTT.S53876

2. Janic A, Abad E, Amelio I (2025). Decoding p53 tumor suppression: a crosstalk between genomic stability and epigenetic control?. *Cell Death and Differentiation.* **32**(1): 1–8. doi: 10.2147/OTT.S53876

3. Bray MA et al. (2016). Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes. *Nature Protocols.* **11**(9): 1757–1774. doi: 10.1371/journal.pone.0080999

Learn more at thermofisher.com/cellpainting