APPLICATION NOTE

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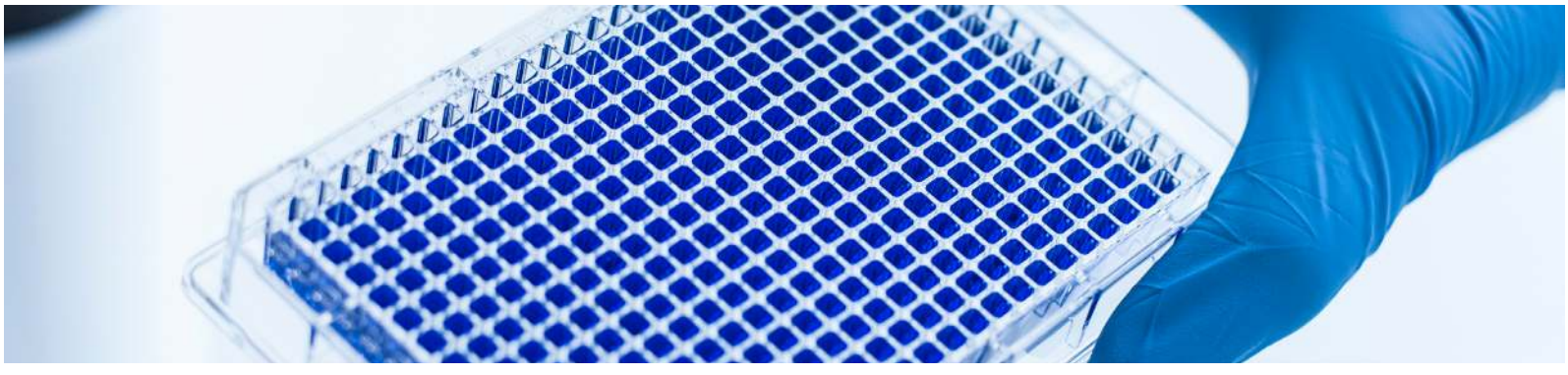
Deconvoluting clonal complexity of barcoded cell populations

Lukas Beumers[1], Katja Bauer[2], Sara Burmeister[1], Stefan Wiemann[1], Rainer Will[2]

[1] Division of Molecular Genome Analysis, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany

[2] Cellular Tools Core Facility, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany



Abstract

Identifying and selecting individual cells in a complex mixture of cells in space and/or time has opened new opportunities in a wide range of research fields, including cancer research. Many complex cellular screening approaches apply selection pressure like drugs, gene knockout/editing or overexpression and often require enrichment of the desired candidate cell populations. However, these approaches may compromise clones that carry different states of the desired alterations. Accordingly, the total populations are ideally deconvoluted to the single-cell level, to then connect the respective cellular modifications directly with biological effects. Here, we outline a reliable process of isolating and verifying monoclonal single-cell clones from pools of differently-barcoded subpopulations.

Introduction

Traditionally thought of as homogenous masses, it is now widely accepted that tumors may harbor different subpopulations with specific characteristics that influence therapy outcomes [1, 2]. To identify and determine the fate of individual cells or subpopulations, diverse barcoding approaches have been developed using the lentiviral integration of over a million different DNA sequences into cells of interest [3, 4]. Isolation of individual cells or clones from complex pools allows to further elaborate the differences of individual subpopulations while simultaneously propagating the subclones of interest. Here, we describe the isolation of individualized cell clones derived from a pool of eight differently-barcoded subpopulations for the luminal breast cancer cell line T47D.

Deconvolution experiment

Eight differently-barcoded T47D cell lines (Table 1) were exponentially grown to 70% confluence and cell density was then measured for each cell line using the CytoSMART Exact FL cell counter. Equal cell numbers for every cell line (5x105 cell/mL) were pooled and single cells were dispensed using the CYTENA F.SIGHT™ FL spotter. Proof of monoclonality and outgrowth control were performed using the NYONE high-throughput microscope (Figure 1). In total, 20 96-well plates were seeded for further deconvolution analysis.

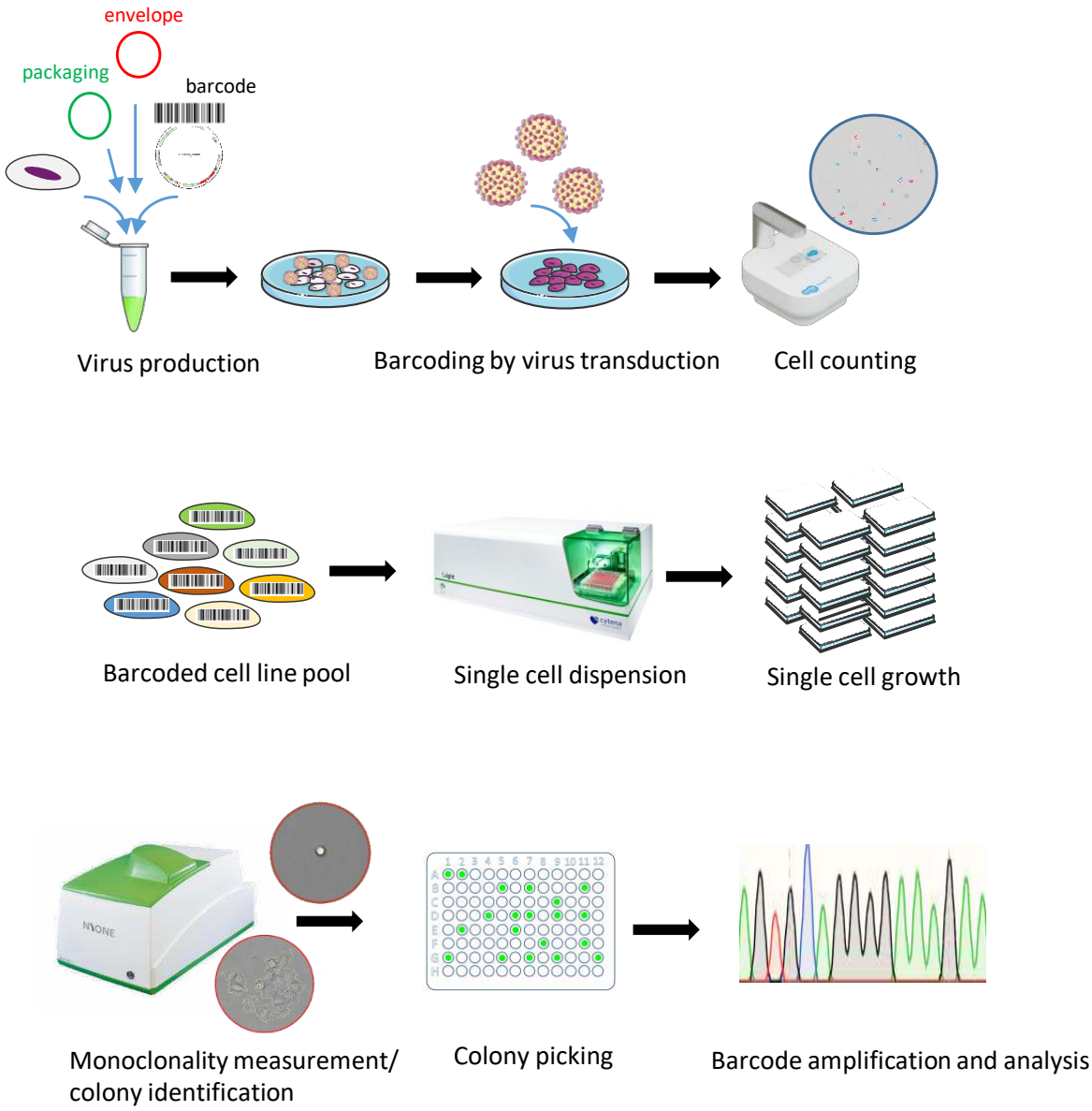


Figure 1. *Schematic workflow of the deconvolution experiment. Eight different batches of barcoded viral particles were produced in HEK293FT cells and separately used to generate stable barcoded target cell lines (T47D). The cell lines were counted in quadruplicates using an image-based cytometer (CytoSMART FL) and equal amounts of each barcoded cell lines were pooled. Single cells were dispensed in 20 x 96-well plates (CYTENA F.SIGHT FL). Monoclonality and colony outgrowth were measured over time (SYNENTEC NYONE). Growing colonies were selected and the barcodes identified by PCR amplification and Sanger sequencing.*

Materials and methods

Isolation of single lentiviral ClonTracer library plasmids

NEB® Stable Competent E.coli (New England Biolabs GmbH, Germany) were chemically transformed with 100 ng plasmid DNA of the ClonTracer library [4] pool and then grown overnight at 30°C on Ampicillin (100 µg/mL) selection plates. For the isolation of the single library plasmids, 20 E.coli colonies were picked, inoculated in 2YT media supplemented with Ampicillin (100 µg/mL) and grown overnight at 30°C. Plasmids were isolated using the NucleoSpin Plasmid Mini kit (MACHEREY-NAGEL, Germany) according to the manufacturer's instructions. The resulting barcode sequences of the isolated plasmids were identified by Sanger sequencing (Eurofins Genomics Germany GmbH, Germany) and analyzed with the SnapGene software (Insightful Science, snapgene.com).
For deconvolution, eight different sequence-verified barcode plasmids were chosen and transfection-grade plasmid DNA was isolated with the NucleoBond Xtra Midi Kit (MACHEREY-NAGEL, Germany) following the manufacturer´s instructions.

Generation of stably-labeled barcode cell lines

Lentiviral particles were produced in HEK293FT cells (Thermo Fischer Scientific, Germany). HEK293FT cells were co-transfected with the lentiviral barcode plasmids, second generation viral packaging plasmids VSV.G (Addgene #14888), and psPAX2 (Addgene #12260). Virus-containing supernatant was removed and cleared by centrifugation (5 min/500 x g) 48 hours after transfection. The supernatant was passed through a 0.45 µm filter to remove remaining cellular debris. T47D target cells were transduced with lentiviral particles at 70% confluence in the presence of 10 µg/mL polybrene (Merck, Germany). Virus-containing medium was replaced with selection medium for the expression constructs (1 µg/mL puromycin; Merck, Germany) 24 hours after transduction. The stably-transduced T47D cell line pool of each construct served as the basis for the deconvolution experiment.

Table 1. *Sequences of chosen E.coli colonies as well as stably-transduced and selected T47D cell pools.*

E. coli clone	T47D cell pool	Barcode sequence
21	1783	TGTGTGACACACTGACTCTCTCTCTGTGTC
26	1784	AGACTCTGTCACAGTGACTGTCAGTCAGAG
31	1785	TGTGAGTGAGTGTCTCACTCAGAGTGAGAC
41	1786	TGTGACTGAGTCTGTGTCTGAGACAGAGTG ACTGACTGTGTGTGACTGTGTCACACAGAG
56	1787	ACAGACAGTCTGAGTGTCACTCAGTCAGTC
	1788	AGAGAGACACTGACTCAGACTCTGTGAGAG
70	1789	ACTCACTGAGAGAGAGTGAGTCTGTGACTG
71	1790	TCAGACTGTGTGTGAGAGAGACTCACTGTC

Isolation of single-cell clones

The selected stably-transduced T47D cell line pools for each construct were exponentially grown to 70% confluence and the cells were detached and separated by trypsinization. Trypsin was inactivated by the addition of complete medium containing 10% FCS. Cells were pelleted by centrifugation (5 min at 300 x g and RT), medium supernatant was removed and the resulting cell pellet was resuspended in serum-free, chemically-defined CD OptiCHO medium (Gibco BRL, Germany).

Cell density and average cell size were then determined using the image-based CytoSMART Exact FL cell counter (CytoSMART). For single-cell dispensing, the cell concentration was adjusted

to 5x105 cell /mL by dilution with OptiCHO medium. Forty µL of the final cell suspension was loaded in the CYTENA single-cell dispensing cartridge (CYTENA, Germany) and single cells were dispensed (size 13-25 µm, roundness 0.3-1) into Poly L-Lysin-coated (Merck, Germany) 96-well plates (Corning, USA) prefilled with 150 µL complete medium (DMEM REF 41966-029 LOT 2209304 + 10% FCS + 1% P/S, Gibco BRL, Germany + 10-8 M 17-ß-estradiol Merck, Germany). Dispensed plates were incubated at standard cell culture conditions (37°C / 5% CO2).

Proof of monoclonality and colony outgrowth

The monoclonality during single-cell dispensing with the F.SIGHT was documented in a series of nozzle images for each cell and further proven after 12 hours by the whole-well, imaging using the automated cell culture microscope NYONE® (SYNENTEC GmbH, Germany) equipped with a 4x objective and the single-cell cloning application of the YT-SOFTWARE (SYNENTEC GmbH, Germany). Monoclonal outgrowth was documented by whole-well imaging at least once a week with NYONE® (Figure 2). Monoclonal T47D barcode clones were expanded for five weeks until the clones covered about one tenth of the well.

Cell lysis and PCR-amplification of barcode region

Growth media was aspirated from the 96-well plates and 60 µL of lysis buffer **(Table 2)** was added to wells containing clones of interest. Afterwards, the samples were incubated at 56°C for 30 min and then at 96°C for 5 min. Samples were stored at 4°C until further processing. The barcode region was PCR amplified as described in **Table 3** and **Table 4** using Phusion Hot Start II DNA Polymerase 2 U/µL (Thermo Fisher Scientific) and the following primers: Fw: GCTGTGCCTTGGAATGCTAGTTGG, Rev: TCTGCTGTCCCTGTAATAAACCCG. PCR purification and Sanger sequencing was performed and the barcode sequences were analyzed with the SnapGene software.

Table 2. *Lysis buffer.*

Component	Manufacturer	Amount
5xHF buffer	Thermo Fisher Scientific (F518L)	12 µL
Proteinase K (20 mg/mL)	Thermo Fisher Scientific (25530049)	3 µL
RNase A (100 mg/mL)	Qiagen (19101)	0.3 µL
Nuclease-free Water	Thermo Fisher Scientific(AM9937)	44.7 µL
Total		60 µL

Table 3. *PCR Master mix. All components were obtained from Thermo Fisher Scientific.*

Component	Amount
5xHF buffer	10 µL
dNTP Mix (10mM each)	1 µL
Fw primer (10mM)	2 µL
Rev primer (10mM)	2 µL
MgCl2 (50mM)	0.5 µL
Polymerase	0.5 µL
H2O	34 µL
Cell lysat	10 µL

Table 4. *Thermocycler program.*

98 °C	2 min	
98 °C	10 sec	32x
71 °C	20 sec	
72 °C	70 sec	
72 °C	10 min	
4 °C	∞	

Results

In total, 279 clones were obtained that contained at least 20 cells after five weeks of clonal outgrowth. The rate of clonal outgrowth was determined as 15% (279 clones/20x94 spotted cells). 104 clones were chosen for further analysis and 97 PCR products could be generated from the lysed clones as visualized, after agarose gel electrophoresis (1% Agarose in 1% TAE buffer) and ethidium bromide staining. Finally, high quality Sanger sequencing data were obtained for 94 out of 96 submitted PCR products. On average (mean), 11.75 clones were isolated per barcode from the complex barcoded cell pool **(Figure 3)**. This finding is in line with the expected clonal outgrowth (11.75 per barcode) of 94 isolated clones from the pool consisting of eight differently-barcoded subpopulations.

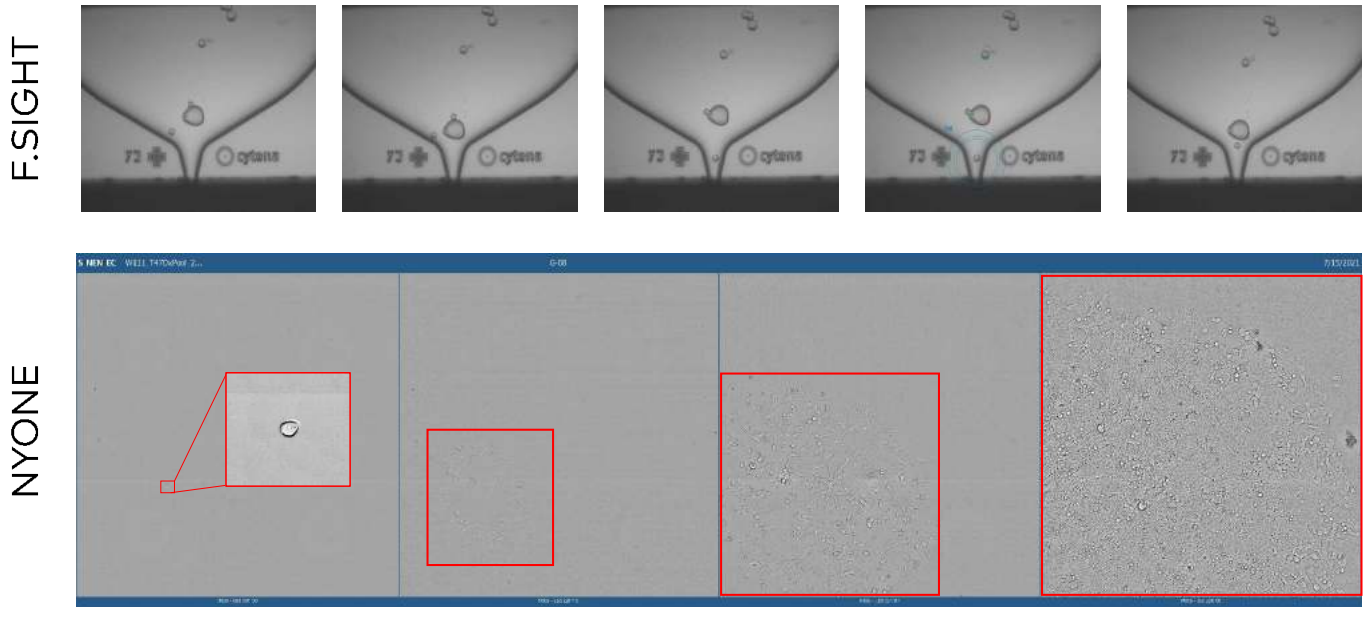


Figure 2. *Clonal outgrowth of single clones spotted (plate 5, well G8). Upper panel: Single cell spotting images from F.SIGHT. Lower panel: Automatic outgrowth analysis from low-contrast single cell to final clone with NYONE, summarized in the automatically generated clone gallery of YT-SOFTWARE.*

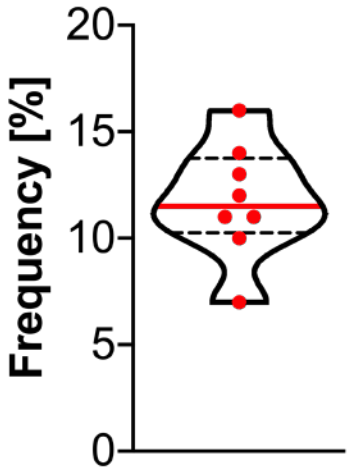


Figure 3.*Distribution of identified sequences. 94 clones were analyzed for sequencing. Expected frequency per barcode: 11.75. Range: 7-16. Mean: 11.75. Median: 11.5.*

Conclusion

Identifying and tracking individual subpopulations by barcoding libraries has become a powerful tool in cell selection. We describe the reproducible isolation and clonal outgrowth of individual cells from a pool of eight differently-barcoded subpopulations at the theoretically expected clonal outgrowth rate.

This approach of deconvolution experiments using barcoded cell line pools and employing high-throughput devices such as the single cell dispenser f-sight and the automated cell microscope NYONE provides a focused, reliable method for identifying and selecting individual cells from a complex population of cells.

References

[1] Dagogo-Jack I, Shaw A. Tumour heterogeneity and resistance to cancer therapies. Nature Reviews Clinical Oncology. 2018; 15(2): 81-94. DOI: 10.1038/nrclinonc.2017.166

[2] Marusyk A, Janiszewska M, Polyak K. Intratumor heterogeneity: the rosetta stone of therapy resistance. Cancer Cell. 2020; 37(4): 471-484. DOI: 10.1016/j.ccell.2020.03.007

[3] Naik S.H., Schumacher T, Perié L. Cellular barcoding: a technical appraisal. Experimental Hematology. 2014; 42(8): 598-608. DOI: 10.1016/j.exphem.2014.05.003

[4] Bhang H-e.C., Ruddy D, Radhakrishna V.K., et al. Studying clonal dynamics in response to cancer therapy using high-complexity barcoding. Nature Medicine. 2015; 21: 440-448. DOI: 10.1038/nm.3841

CONTACT US

CYTENA
Neuer Messplatz 3,
D-79108 Freiburg,
Germany

Phone: +49 761 70 88 90-0
E-mail: info@cytena.com
www.cytena.com

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