A MICROFLUIDIC APPROACH TO PERSONALIZED DRUG SENSITIVITY PROFILING IN HIGH-RISK NEUROBLASTOMA

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Abbreviation list

Abbreviation	Definition
ACK	Ammonium-Chloride-Potassium
BF	Brightfield
BT chips	Big trap chips
CTG	Cell-Titer Glo
CY2/TRITC	Cyanine2/Tetramethylrhodamine
DAPI	4’,6-diamidino-2-phenylindole
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
ECM	Extracellular Matrix
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
FNA	Fine needle aspiration
HR	High-risk
NB	Neuroblastoma
PBS	Phosphate-buffered saline
PDX	Patient-derived xenograft
PI	Propidium Iodide
RPMI medium	Roswell Park Memorial Institute medium
SNP	Single nucleotide polymorphism
ST chips	Small trap chips
ULA	Ultra-low attachment

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Laymen's summary

Neuroblastoma (NB) is a neuroendocrine tumor that occurs in early childhood. There is a huge variety in tumor progression, in which high-risk NB (HR NB) affects survival rates the most. Around half of the HR patients will have relapse, despite multiple treatment strategies. These treatment burdens have reached their limits, therefore there is an unmet need for new innovative treatment strategies. Focusing on molecular characterization of NB tumors can help to find new biomarkers. The heterogeneity of each tumor makes it very difficult to find new treatment strategies, as tumors with the same morphology will react completely differently to certain treatments. The study MAPPYACTS tries to identify new biomarkers based on high-throughput drug screenings, leading to individual molecular profiles. The results of this study showed that in 15% of pediatric cancer patients in general, molecular profiling led to a new proposed treatment. Innovative strategies are still required to develop more accurate (individual) predictive biomarkers. A predictive preclinical model such as patient-derived xenografts (PDXs) may solve this issue since this is personalized patient tissue, grown further in immunodeficient mice. Patient-derived tissue contains the genetic make-up of an individual patient and large-scale drug screening can be performed to obtain more accurate individual molecular profiles.

In the COMPASS project, the aim is to build an international platform for drug testing where response to drugs is classified by molecular and image analyses. This drug sensitivity testing platform can give information about (i) drug efficacies, (ii) mechanisms and new biomarkers and (iii) create a large-scale online data resource of drug efficacies. This project already demonstrated the feasibility of implementing ex vivo drug sensitivity profiling using molecular profiles of PDX material of childhood cancers. However, high-throughput screening of primary patients 'material is not yet standardized and the invasive manner of taking biopsies from patients makes it difficult to perform high-throughput screenings for clinical application.
In this context, the aim of this project is to build a microfluidics platform for high-throughput screening and thus individual drug sensitivity profiling in pediatric cancer patients. This may increase predictive power and only a small amount of tumor material is needed to perform a high-throughput drug screen. Primary tumor material of NB patients will be screened on a drug library of approved cancer drugs of drugs included in clinical trials. This study is in collaboration with Okomera, a company that provides microfluidic chips and Biophenics, a platform within Institut Curie which is specialized in high-throughput screening. Given the fact of the limited amount of available patient material from needle biopsies, PDX models will be used to validate technical methods and obtain 3D spheroids. These spheroids recapitulate individual patient tumor characteristics, such as cellular heterogeneity and organization, physical barriers, growth kinetics, and drug response mechanisms. This report focuses on validation experiments of a workflow from PDX material to the formation of spheroids.

The workflow and protocol are optimized and standardized by testing spheroid formation on microfluidic chips within Okomera and on 384 ULA plates within Biophenics. Serum-enriched medium (10% FBS), low Collagen I concentrations, and use of Miltenyi mouse cell depletion kit help in spheroid formation and viability over time. An optimal number of seeded cells depends on the PDX sample and the use of chips or plates. A proof of concept shows that a single chemotherapy reduces spheroid viability after 72h hours of exposure, which is in line with results for 2D cultures. When further optimizing the workflow and protocol and screening NB PDX spheroids on selected drug libraries, new predictive biomarkers may be found and this is an important step for individual therapeutic decisions based on molecular profiles and drug sensitivity characterization, ultimately leading to individualized patient care strategies in clinical settings for NB patients.

Abstract

In Neuroblastoma (NB), treatment burden reaches its limit, especially in high-risk (HR) patients with poor prognoses. Molecular characterization may help to identify new predictive biomarkers. Study MAPPYACTS showed that 15% of pediatric cancer patients at relapse proposed a targeted treatment matched to molecular profiling, however the overall gain in survival is limited. In the COMPASS project, the aim is to build an international, standardized and validated drug sensitivity platform based on image and molecular analysis with the ultimate goal of discovering new biomarkers and increase predictive power. This ex vivo high throughput drug screening and profiling is performed by using patient-derived xenografts (PDXs), with the ultimate goal to use fine needle aspiration (FNA) directly from patients and test whole drug libraries within a short timeframe.

In this context, the ultimate aim of this study is to establish a drug sensitivity and resistance microfluidics platform for drug sensitivity profiling in NB patients by using primary patients derived tumor cultures. The validation of workflow, protocols and optimization of best spheroid conditions for drug screening in microfluidic chips (Okomera) and 384 ULA well plates (Biophenics) is performed in NB PDX spheroid cultures. Different parameters are tested; optimal medium, extracellular matrices (ECM), number of cells, mouse cell depletion kits. Finally, a proof of concept with a single chemotherapy is performed to test workflow.

Results show that the use of serum-enriched medium (10% FBS) and low concentrations of Collagen I in culture, show better viability over time. Optimal number of cells is established at 40 cells/trap (Okomera) and varies for Biophenics. For the Biophenics part the mouse cell depletion kits help in spheroid formation and use lower amount of cells effectively. When optimizing the workflow and testing drug libraries on NB PDX spheroids, this procedure will be applied to study drug response directly on patients' material and then help to discover new predictive biomarkers. This is an important step to personalized therapeutic care strategies in clinical settings for NB patients.

1. Introduction

Neuroblastoma (NB) is a neuroendocrine pediatric tumor that occurs in early childhood. It affects around 150 patients per year in France, with a median age of diagnosis of 18 months [1]. There is a high variability in tumor progression, in which high-risk (HR) disease, occurs in about half of the patients, and affects survival rates (40-50%) the most. Half of the HR HB patients, and 70% of all pediatric cancer patients, will have relapse despite different treatment strategies [1,2]. However, in HR NB patients, treatment burden of multiple standard treatment strategies reaches its limit and little modifications in these treatments only lead to very little improvements in overall survival rates [3]. This raises an unmet need for innovative therapeutic strategies to increase survival rates and reduce complications of current treatments in children with HR NB.

To overcome this need, HR NB research is focusing more on molecular characterizations, intending to find predictive biomarkers. At diagnosis, copy number alterations are the main source of genetic alterations, whereas *MYCN* amplification is related to poor prognosis and outcome [4]. This defines HR NB independently of other clinical symptoms and segmental alterations [5]. In total, only a few recurrent driver gene alterations have been described, such as genomic alterations in the *ALK* gene and telomere mechanism genes *TERT* and *ARTX* [6]. The molecular heterogeneity displays clinical challenge, since individual tumors that have the same morphology will respond completely different to the exact same treatment.

To identify new targetable genetic alterations following a relapse in patients, the precision medicine program MAPPYACTS performed high throughput screening techniques to obtain molecular profiles of individual pediatric cancer patients. Recent results showed that at least one genetic alteration that may lead to a new targetable treatment can be identified at relapse [7]. However, only 10% of the identified treatments are drugs that are immediately ready to use. In total, in 15% of pediatric patients at relapse, molecular profiling led to a proposed targetable treatment, as discussed in clinical molecular tumor board meetings [8,9]. Despite these steps giving the possibility of personalized medicine, a big group of patients cannot benefit from this study and if so, the expectation is that this has only a little influence on overall survival rates. Further strategies are required to develop more accurate predictive biomarkers. A predictive preclinical model is therefore of high importance.

Patient-derived material can give a more accurate indication of drug response since this contains the genetic make-up, and in the case of NB, the complex heterogeneity of a patient. In this way, large-scale drug sensitivity profiles can be made from tumor tissue when performing high-throughput drug screenings [10]. However, obtaining primary tumor tissue from patients is invasive. A model such as patient-derived mouse xenograft (PDX) may solve this issue, as little amount of primary patient tumor tissue can be grown further in immunodeficient mice and later screened on drug libraries [11,12].

In the COMPASS project (Clinical implementation Of Multidimenstion PhenotypicAl Drug SenSitivities in Pediatric Oncology), the aim is to build an international, standardized, and validated platform for drug testing where drug response of different types of tumors is classified by molecular and image analyses. The ultimate aim is building a globally standardized *ex vivo* so-called drug sensitivity testing platform to discover (i) drug efficacies and drug re-positioning opportunities, (ii) mechanisms and molecular new biomarkers for drug efficacies and to (iii) build a large-scale online data resource of drug efficacies with integrated data providing a basis for novel precision therapies for incurable pediatric tumors. This project already demonstrated the feasibility of implementing *ex vivo* drug sensitivity profiling using molecular profiles of PDX material of childhood cancers, including NB. However, high-throughput screening of primary patients 'material is not yet standardized in pediatric precision medicine trials and clinical decision-making. Concerning the invasive manner of collecting

primary patients' material, it remains a challenge to perform high-throughput screening to direct clinical application.

In this context, the ultimate goal of this study is to build a miniaturized microfluidics platform for high-throughput screenings and personalized drug sensitivity profiling in HR NB-derived tissue. Drug sensitivity combined with molecular profile data, may add a new functional component and increase predictive power. When using a microfluidic platform, only very little amount of tumor material (fine needle aspiration, FNA) is needed to perform a personalized high-throughput drug screen and this may provide a robust drug sensitivity profile comparable to other drug testing ways, if standardized. Also, using patient-derived material reflects HR-NB tumor biology, is easy to reproduce in a microfluidic system, is cost-effective, and provides robust results in a short time manner [13]. Recent *ex vivo* data within Institut Curie indicated that HR NB might be sensitive to HDACi, of which early clinical trials are ongoing. This is a first step in targeted treatments in NB based on molecular profiling.
This study is in collaboration with Okomera, a company that designs microfluidic chips and Biophenics, a platform within Institut Curie which is specialized in high-throughput cell-based screening. Given the fact of the limited amount of available patient material from needle biopsies, PDX models will be used to validate technical methods and obtain 3D spheroids. These spheroids recapitulate individual patient tumor characteristics, such as cellular heterogeneity and organization, physical barriers, growth kinetics, and drug response mechanisms [10,14]. The first step is the optimization of a microfluidics protocol by standardization of tumor shipment and tissue viability and on the other hand the optimization of 3D tumor spheroid cultures and image acquisition in microfluidics chips. In this way, both chips and drug screening protocol can be validated. In the second step, 3D spheroids will be treated with a large library of either approved oncogenic drugs or drugs included in clinical trials. In this way, individual drug sensitivity profiles can be generated. The final step is the clinical translation by reporting results to clinical molecular tumor board meetings. The total workflow of this study is shown in ***Figure 1***.

This report will focus on the first step, which is optimizing and standardizing the microfluidics protocol of *ex vivo* 3D PDX spheroid cultures, to obtain the best conditions to perform high-throughput drug screening. Optimization experiments at either Okomera or Biophenics are performed to obtain healthy spheroids over a period of approximately 7 days, as needed to get a readout after drug screening from fresh PDX tissue. A small proof of concept is performed with a single chemotherapy to test the workflow and protocol.

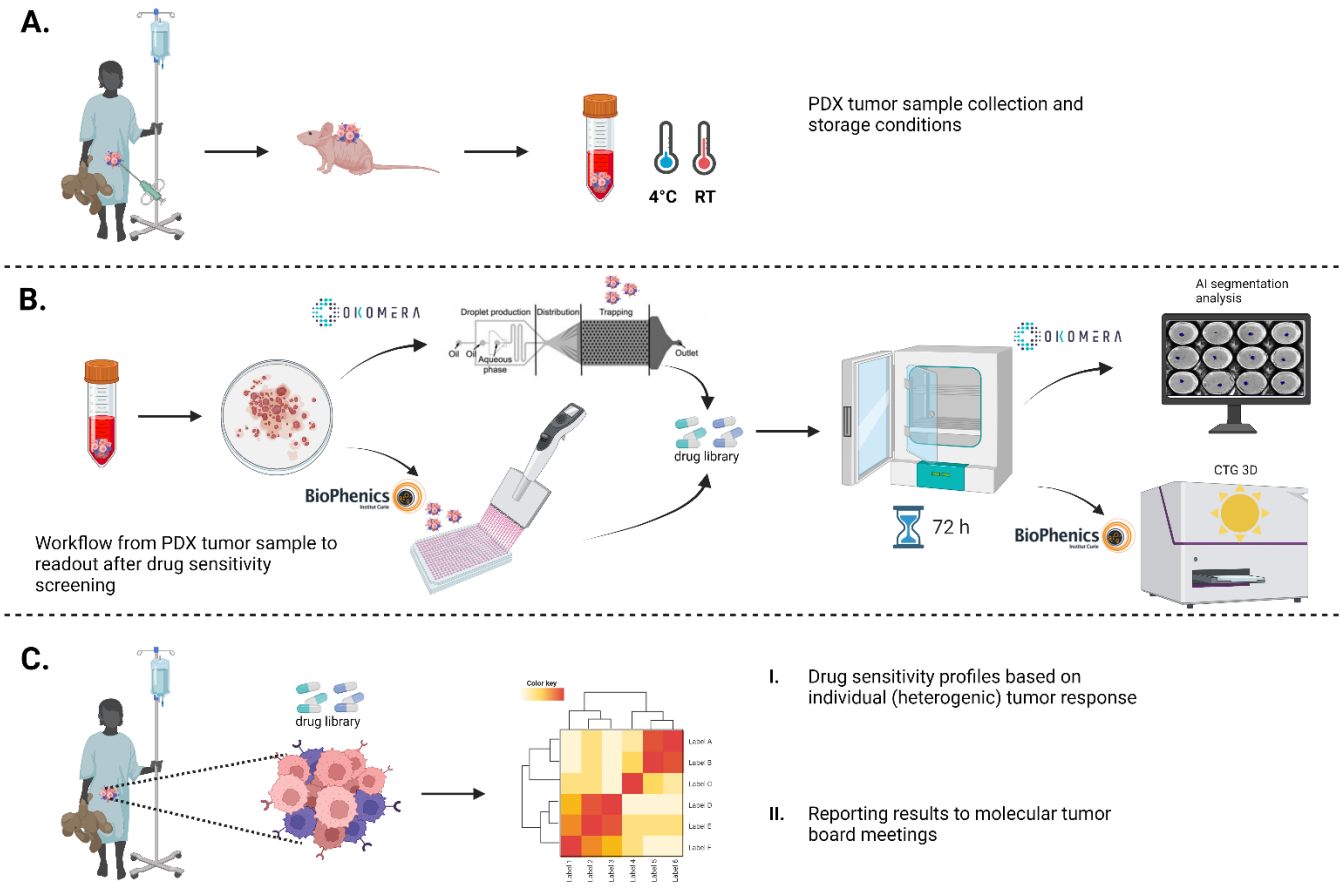


Figure 1. Total workflow drug sensitivity screening. ***A.*** *PDX tumor sample collection and storage conditions.* ***B.*** *Generation of PDX 3D spheroid cultures on either microfluidic chips (Okomera) and 384 well plates (Biophenics), followed by drug screening and either AI segmentation analysis (Okomera) and metabolic activity (CTG 3D, Biophenics) as readout.* ***C.*** *Generation of drug sensitivity profiles based on individual (heterogenic) tumor response and reporting of results to molecular tumor board meetings. Figure created in BioRender and pictures adapted from* [15].

2. Material and methods

Ethical approval for patient-derived tumor tissue

Primary tumor tissue from HR NB patients was collected at Institut Curie according to informed consent and standard protocols. PDXs were established from primary NB tumors and grown in Female Swiss nude immune-deficient mice, according to institutional and ethical guidelines. Mice were sacrificed and PDX material was collected and stored in 50 mL tubes at -4°C or RT containing RPMI medium. Relevant clinical and molecular characteristics of used PDX samples in this study are shown in ***Table S1.***

Cell dissociation and processing of NB PDX tumor samples

Upon receipt of fresh PDX models, medium was discarded and tumor with little amount of left medium was transferred to a sterile 10 cm culture dish. Frozen PDX samples, stored in 10% DMSO (VWR) cryotubes in liquid nitrogen, were thawed and washed in PBS (Fisher Scientific) twice to remove DMSO. Tumor tissue was cut as small possible with sterile scalpels and all collected in a 50 mL tube. The pieces of tumor were resuspended in 10 mL of standard DMEM/F12 stem cell medium (Gibco, Thermo Fisher) supplemented with 2% B27, 1% pen-strep (Gibco), 20µg EGF (Gibco), and 10µg FGF2 (Gibco), and cells were mechanically dissociated with a P1000 pipet. Enzymatic digestion was started by adding 120 µL of trypsin (10 mg/mL, Gibco) and 100 µl of Collagenase type II (100 mg/mL, Merck). After incubation at 37°C for 30-45 minutes, dissociation was stopped by adding 120 µL of soybean trypsin inhibitor (10mg/mL, Merck sigma). Released DNA from dead cells was removed by adding 100 µL DNase (2mg/mL, Thermo Fisher), and 100 µL magnesium chloride (1M, Merck) was added to activate DNase. After tumor fragments settled down and cell solution was centrifuged at 300g for 5 minutes, the supernatant was excluded and the pellet was resuspended in 10 mL PBS (Gibco, Biophenics) or 10 mL DPBS without magnesium and calcium (Gibco, Okomera). Cell solution was filtered through a 30 µm filter (Miltenyi Biotec) to obtain single cells or small aggregates. Cell suspension was centrifuged at 300g for 5 minutes again and the pellet was dissolved in 10 mL standard 2% B27 medium or serum-enriched medium, which contains 10% or 20% FBS instead of the 2% B27 supplement. Optionally, extracellular matrices Collagen I (rat tail, Corning) or Matrigel (Corning) were added to test optimal spheroid viability over time. Cells were counted by using a ViCell XR Beckman Counter. To deplete red blood cells and mouse cells, Ammonium-Chloride-Potassium (ACK) lysis buffer (Thermo Fisher) and Miltenyi kit columns (Miltenyi Biotec) were used respectively, according to protocols, to test whether this improves spheroid viability. Cells were injected in microfluidic chips with small traps (ST) or big traps (BT) for Okomera experiments or 384 Ultra-Low-Attachment plates (ULA, Corning) for Biophenics experiments. Cells were imaged in brightfield (BF), 10x magnification. 3D spheroid viability was followed over time by adding fluorescent dyes Propidum Iodide (PI) to follow dead cells, and DAPI (Thermo Fisher) to mark living cells (nucleus). The metabolic activity of cells was tested by using Cell Titer Glow 3D (CTG 3D), according to standard protocol (Promega). More detailed experimental design for both sites are stated in the separate sections below. All tested conditions are shown in ***Table 1***.

	Okomera	Biophenics
Design	Chip: Small Trap (ST) and Big Trap (BT)	Plate: 384 Well ULA plate
Cell amount per trap or well	20, 40, 80, 100	250, 500, 1000,2500, 5000, 10000
Medium conditions	2% B27, 10% FBS, 20% FBS	2% B27, 10% FBS
Extracellular Matrix (ECM)	Collagen I , Matrigel	Collagen I
Cell isolation reagents	ACK lysis buffer	Miltenyi kit
Viability	Fluorescent dye: PI	Fluorescent dye: PI, DAPI Metabolic activity: CTG 3D

Table 1. Overview of tested conditions for spheroid optimization experiments. *Conditions are divided in type of condition and performed at Okomera or Biophenics.*

2.1 Okomera

Chip design

Okomera's proprietaty chips have previously been described in [15,16]. Cells are encapsulated in aqueous droplets surrounded by oil, the droplets are distributed and anchored to the strong regions of the anchors (***Figure 2A***). Cells can then sediment at the bottom of the droplet to aggregate and form a spheroid (***Figure 2B***). A second droplet solution, for example containing drugs, media or extracellular matrices, can then be produced, injected and trapped in the triangular part of the anchor, followed by merging of the spheroid and additional droplet, enabling secondary content to the spheroid. To differentiate the drugs used on a chip during a drug screening, fluorescent dyes are used to barcode the drugs in the secondary droplet(s) (***Figure 2C***). This report will focus up until the point of adding a second droplet with drugs.

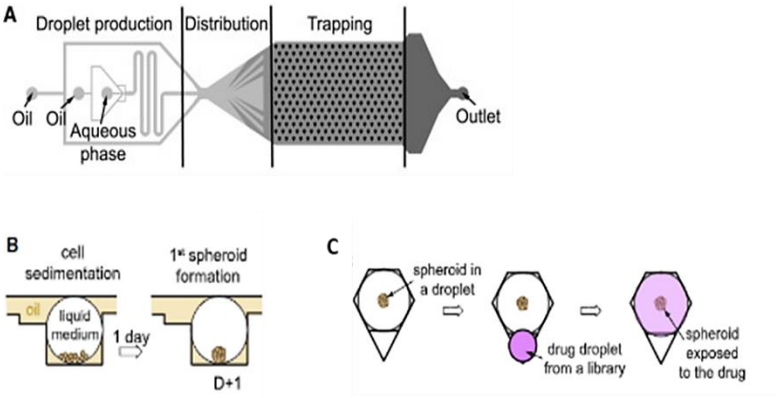


Figure 2. Overview microfluidic chip design. ***A****. Composition of chip from injection to outlet region.* ***B.*** *Spheroid formation after injection of liquid medium containing cells.* ***C.*** *Drug screening with fluorescent second droplets. Pictures adapted and retrieved from [15].*

Workflow spheroid formation

After tumor digestion, cells were counted manually by using Kova slides (Fisher), according to standard procedure. Droplets of oil containing required number of cells and 0,01% Pluronic F-127 (Sigma) are distributed and trapped on two different kind of chips. The ST chips (400 µm of diameter, 154 traps and drops of 50 nL) are loaded manually using syringe pumps and the BT chips (800 µm of diameter, 78 traps and drops of 500 nL) are loaded using Okomera's automated machine. Cells are imaged over a period of 7 days by a Zeiss Axio Observer in Brightfield (BF) and CY2/TRITC and further analyzed by live microscopy and addition of fluorescent dyes PI (3µM, Invitrogen) and DAPI (Thermo Fisher). The images were analyzed by AI driven methods developed by Okomera to extract diameter and viability of each spheroid at each time point. The workflow for the Okomera technology from single cell suspension to spheroid formation is shown in ***Figure 3.***

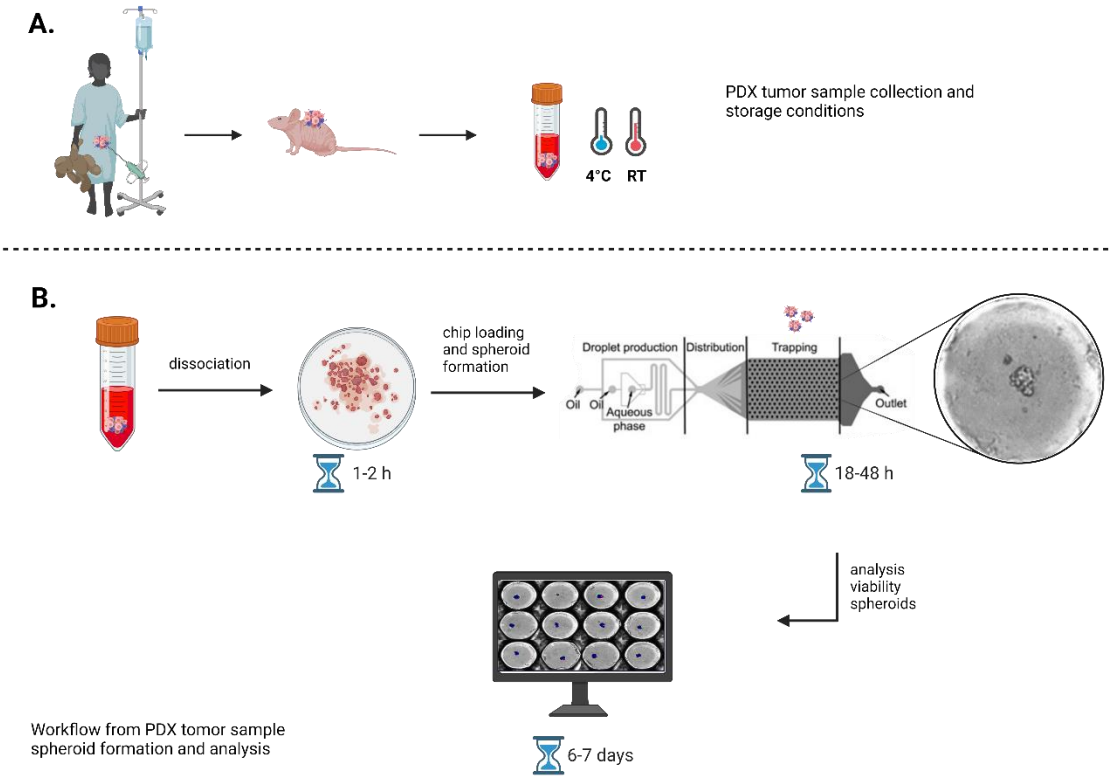


Figure 3. Workflow of Okomera spheroid formation from PDX tumor samples in microfluidic chips. ***A****. PDX models are generated by growing biopsies from patients in Female Swiss nude mice. PDX samples are stored at 4 degrees or at RT in RPMI medium.* ***B.*** *Workflow from PDX sample to spheroid formation. PDX is dissociated in single cells and loaded in microfluidic chips, where cells form a spheroid. Viability followed over 6-7 days by cell imaging and AI analysis. Picture created in BioRender.com and some images retrieved and adapted from [15].*

2.2 Biophenics

Workflow spheroid formation

After dissociation and cell counting, required number of cells were resuspended in required medium, supplemented with Collagen I (stock concentration 3 mg/mL, different concentrations) if tested and seeded in 40-50µl/well in 384 well ULA plates. Plate was centrifugated at 300g for 3 minutes and cells were imaged daily in BF at 10x magnification by using an IN-Cell analyzer 6500HS imaging microscope. Additional channels were used for imaging fluorescent dyes, PI (3 µM) and DAPI (1 µM), which were added at different timepoints to image cell viability. CTG 3D was used to measure metabolic activity at different timepoints using a CLARIOstar plus plate reader luminescence program, according to protocol (BMG Labtech) with a minimum of three biological replicates per condition. A workflow of Biophenics is shown in ***Figure 4***.

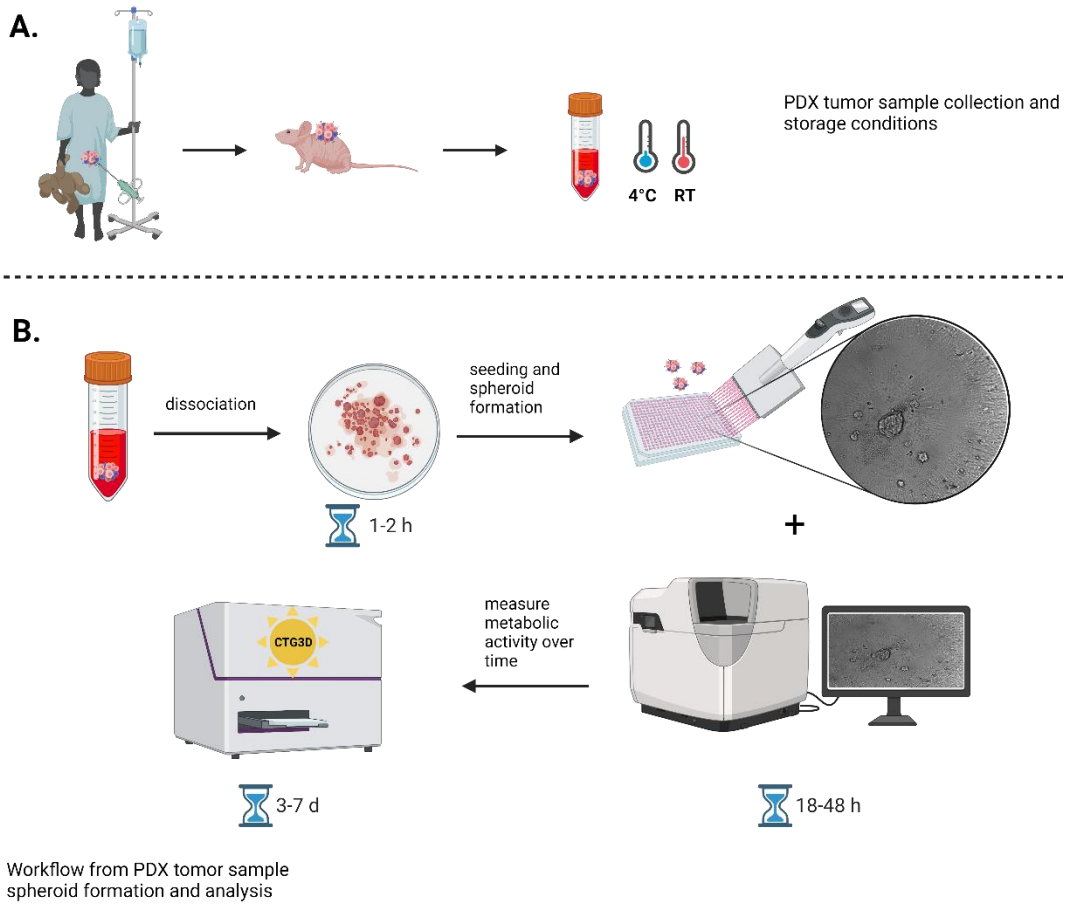


Figure 4. Workflow of Biophenics spheroid formation from PDX tumor samples in 384 well ULA plates. ***A.*** *PDX models are generated by growing biopsies from patients in Female Swiss nude mice. PDX samples are stored at 4 degrees or at RT in RPMI medium.* ***B.*** *Workflow from PDX sample to spheroid formation to metabolic activity as readout for viability. PDX is dissociated in single cells and seeded in 384 well ULA plates, where cells form spheroid(s). Spheroid were imaged over time. Metabolic activity is measured by using CTG 3D. Picture created in BioRender.com.*

Proof of concept drug screen

To test the workflow and protocol, spheroids were exposed to a single chemotherapy, doxorubicin (1µM and dissolved in 10mM DMSO, Selleckchem) at D2 after seeding, for 72h. Metabolic activity was measured by using CTG to compare control spheroids versus treated spheroids.

2.3 Statistical analysis

To perform statistical analysis, GraphPad Prism 9 was used to calculate standard deviation (SD) and performing analyses. A p-value of < 0,05 is considered as significant.

3. Results

In this study, validation experiments are performed to establish a protocol for drug sensitivity profiling on microfluidic chips in HB PDX samples. Chip design, cell depletion kits ACK, cell density, medium conditions and extracellular matrices are tested for Okomera validation. Cell density, medium conditions, extracellular matrices and mouse cell depletion kit Miltenyi are tested within Biophenics validation experiments. Lastly, the Biophenics workflow is tested with a proof of concept single drug screen.

3.1 Okomera

Chip design selection for optimal spheroid viability

First, both small trapped chips (ST) and big trapped chips (BT) were compared in two independent experiments to choose the best chip design. Two PDX models, HSJD-NB-009 and HSJD-NB-011, were tested in ST chips and BT chips in a cell density of 80 cells/trap and dissolved in general 2% B27 medium. PI was added in both experiments to the solution to follow viability over time. Images of a representative well of the comparison between ST and BT in 2% B27 medium with 80 cells/trap in two different tested models is shown in ***Figure 5***. In general, we observed no systemic aggregation of cells and no equal drop size per trap in the BT designed chips. ST chip design was then selected for further experiments, whereas the BT chips were further optimized by Okomera. Results on different media and number of cells is described later.

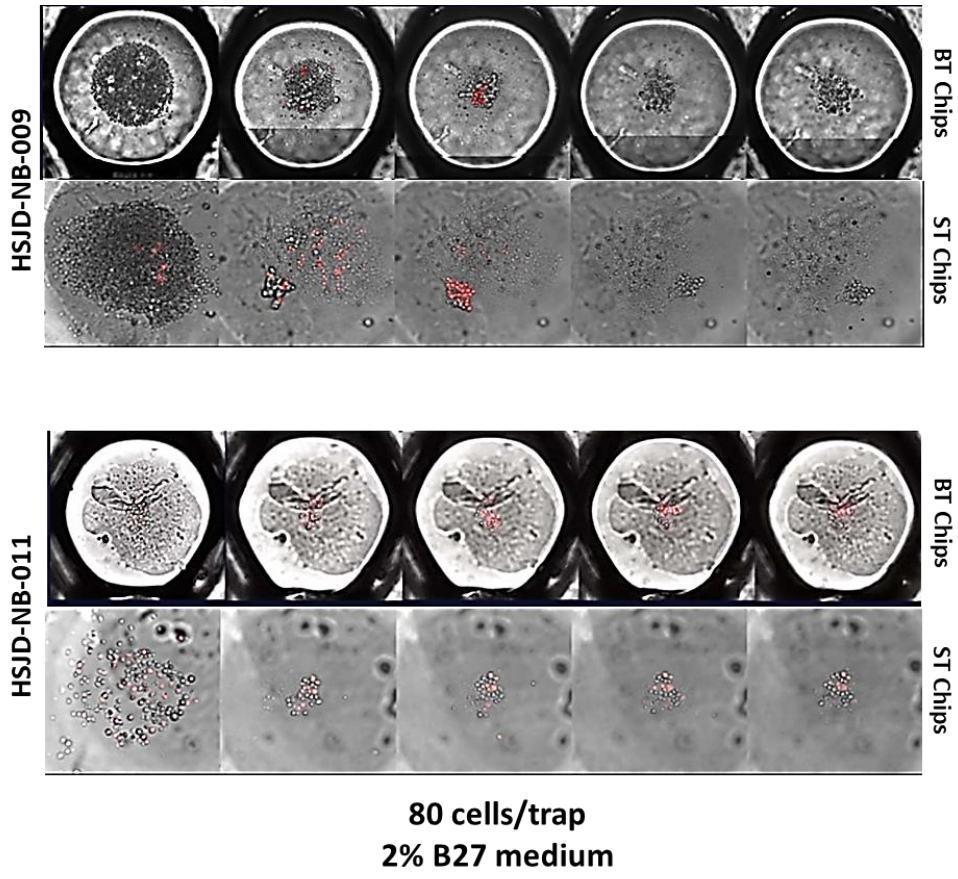


Figure 5. Images of PDX spheroid formation over time in chip design selection experiment*. NSJD-NB-009 and HSJD-NB-011 PDX models were tested in BT chips and ST chips in two independent experiments, containing 80 cells/trap dissolved in 2% B27 medium. Red dots on cells is PI, marker of dead cells. Cells were imaged in BF, 10x magnification.*

Presence of different type of cells emphasize need of cell depletion kits

When looking at images of seeded cells, we observed different type of cells, as shown in zoom of NSJB-NB-009 model in ***Figure 6.*** White big cells are likely neuroblasts, whereas the smaller cells may be immature red blood cells, immune cells or other cell types, which may have influence on readout after drug screening. To purify the sample, ACK buffer is added right after cell dissociation to deplete blood cells. Images of three independent experiments with different PDX models in 2% B27 medium with or without using ACK buffer in ST chips is shown in ***Figure 6.*** We observe less aggregation or spheroid formation after performing ACK buffer. Further experiments were performed without using ACK buffer.

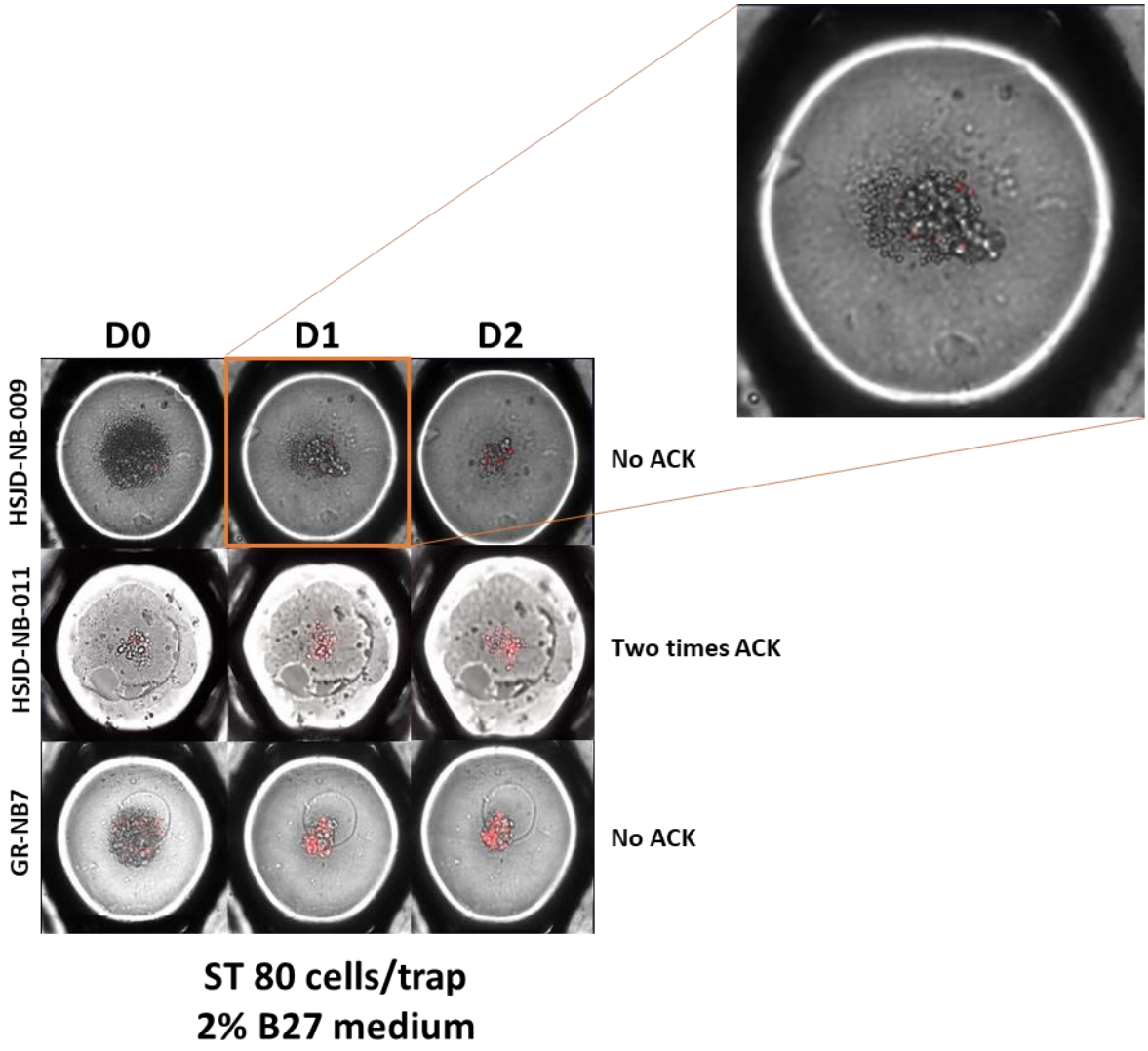


Figure 6. Images of PDX spheroid formation over time in cell depletion kit experiment. *Images of HSJB-NB-009, HSJB-NB-011 and GR-NB7 PDX models in ST chips containing 2% B27 medium with or without addition of red blood cell depletion ACK buffer. Red dots on cells is PI, marking dead cells. Zoom in on HSJD-NB-009 D1 shows different type of cells, as the bigger white cells are neuroblasts and smaller cells red blood cells or other type of cells. Cells are imaged in BF, 10x magnification.*

Obtaining optimal cell density for best viability

In the next step, the optimal cell density for obtaining the best viability over time is investigated. Two independent experiments are performed on HSJB-NB-009 and GR-NB7 NB PDX models in 2% B27 medium on ST chips containing 20, 40 or 80 cells/trap (***Figure 7***). We observe different viability profiles for both PDX samples. For HSJB-NB-009 we observe in general lower PI signal, indicating fewer dead cells, and disaggregation of spheroids around D5. Spheroids are not visible after D2 for 80 cells/trap. For the GR-NB7 model, we observe in general higher compatibility and higher PI signal, which indicates more dead cells, and no disaggregation until D6. For both samples, 40 cells/trap present good compactness and viability.

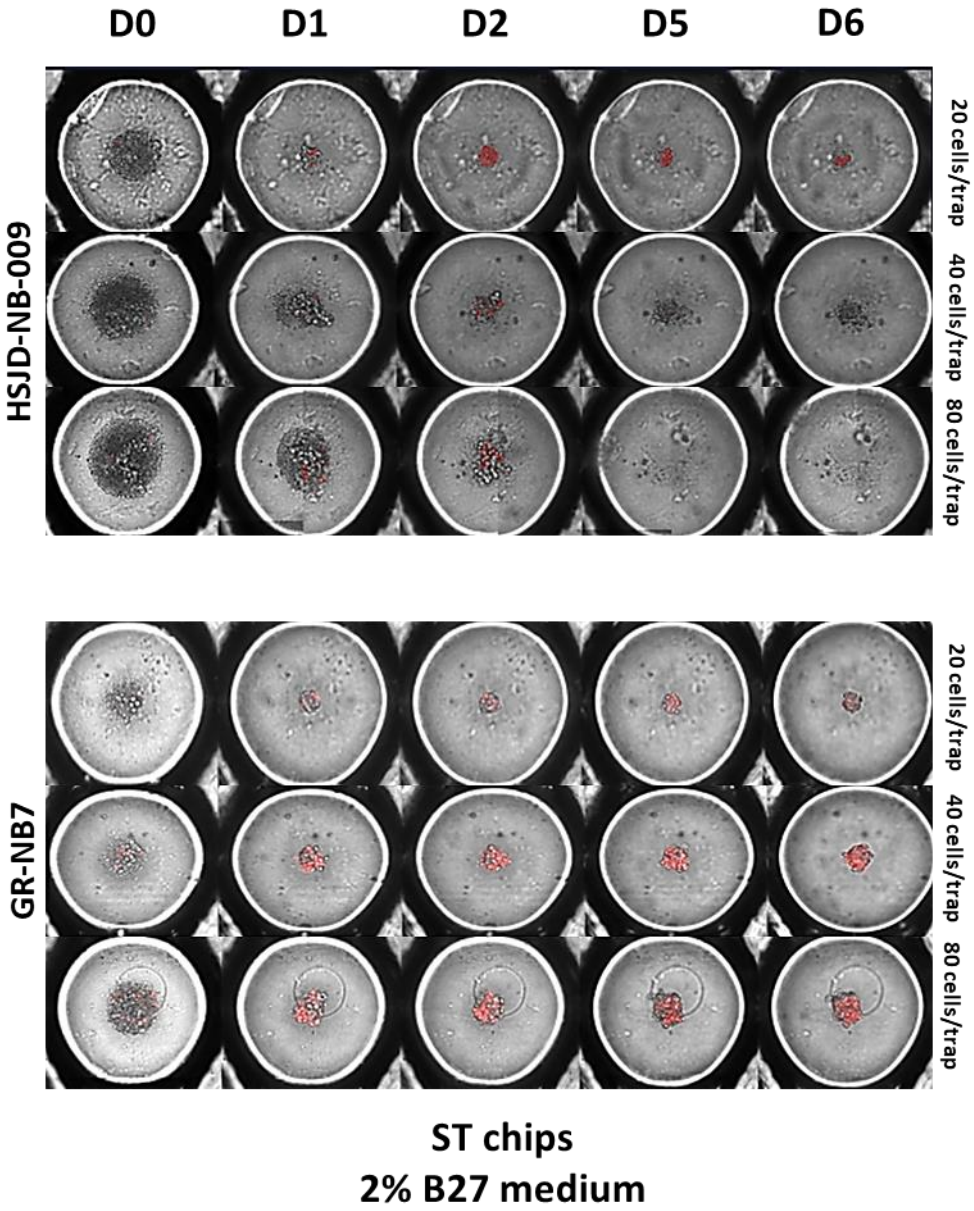


Figure 7. Images of PDX spheroid formation in cell density experiment. *HSJD-NB-009 and GR-NB7 PDX models are imaged over time in ST chips containing 20, 40 or 80 cells/trap dissolved in 2% B27 medium. Red dots on cells is PI, marking dead cells. Cells are imaged in BF, 10x magnification.*

Serum enriched medium promotes higher viability and differentiation over time

Different type of media were tested in this study, the regular 2% B27 NB medium and serum enriched medium, containing 10% FBS or 20% FBS. Media were tested on GR-NB7 and IC-pPDX-112 PDX models in independent experiments on three ST chips containing 80 cells/trap. Spheroid formation was followed over time and cell viability tested by PI addition. A representative well over time for each condition is shown in ***Figure 8A,B*** . We observe lower PI signal , more homogeneous spheroids and more surrounded cells (indicating cell differentiation) in serum-enriched medium in both PDX models.

Extracellular matrices affect spheroid viability

To increase spheroid viability across time (after D5-D6), extracellular matrices (ECM) are added to media when seeding cells. Matrigel (0,2mg/mL) and Collagen I (5µg/mL) were tested in two independent experiments in PDX models GR-NB7 (80 cells/trap) and IC-p-PDX-112 (100 cells/trap). A second droplet of media was added to the chips at D2 for the Collagen I experiment. A representative well over time for each condition is shown in ***Figure 8C,D*** We observe more PI signal and less homogenous spheroids when adding Matrigel, compared to non-Matrigel containing cell solutions. We observe more and earlier homogeneous spheroid formation in Collagen I enriched medium conditions and high PI signal in 2% B27 conditions.

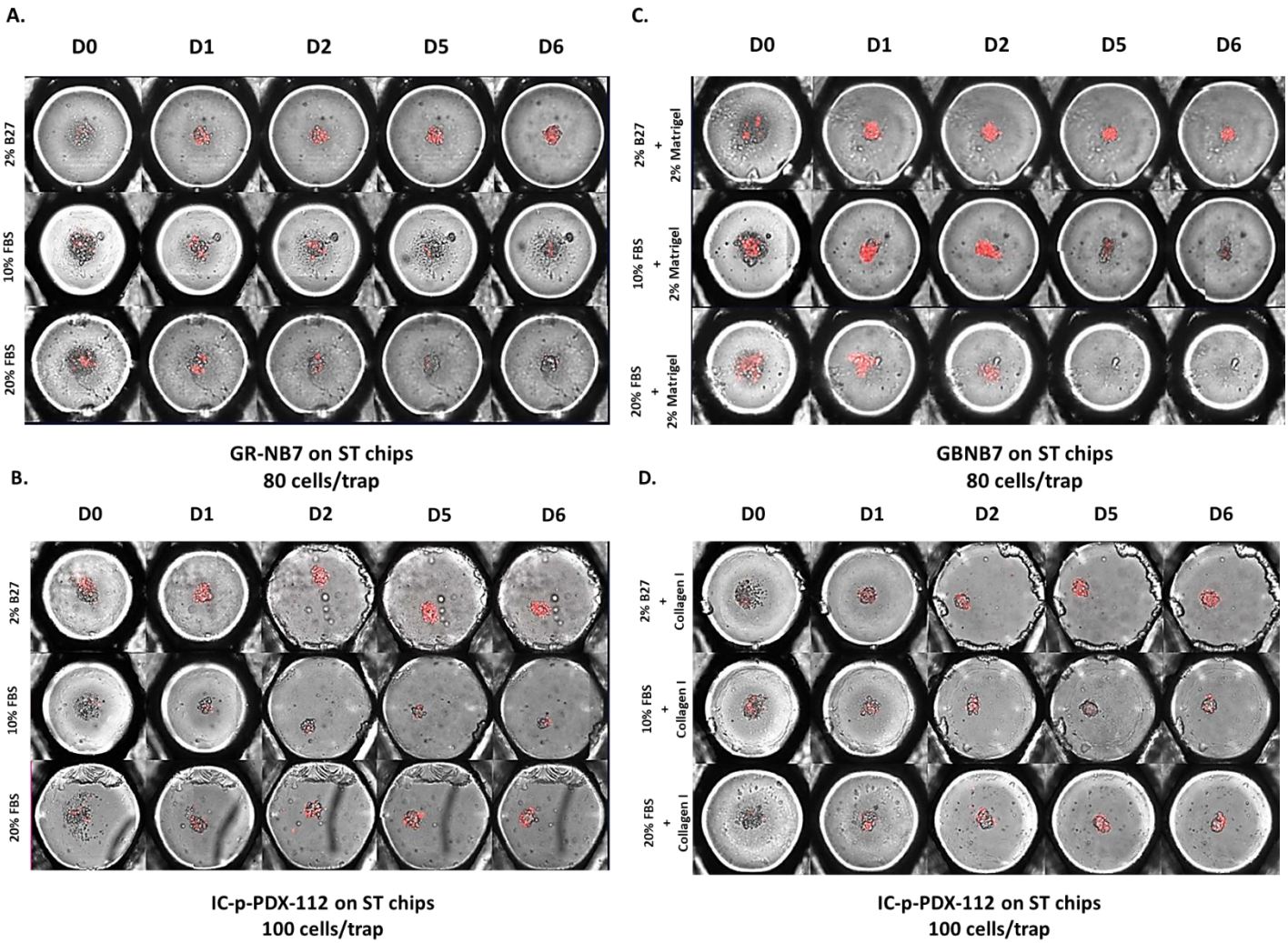


Figure 8. Images of PDX spheroid formation in experiments of selecting optimal media and ECM. *Experiment performed in ST chips using three different media with and without ECM.* ***A,B.*** *GR-NB7 Matrigel experiment with 80 cells/trap and* ***C,D*** *IC-p-PDX-112 Collagen I experiment with 100 cells/trap. Red dots on cells is PI, marking dead cells. Matrigel (0,2mg/mL) and Collagen I (5µg/mL) are used as extracellular matrices. Cells are imaged in BF 10x magnification.*

3.2 Biophenics

Spheroid(s) formation in high number of seeded cells

To test whether spheroids are forming in 384 ULA plates, a high number of cells (10000 cells/well) were seeded with different concentrations of Collagen I (3µg/mL). Fresh IC-pPDX-17 sample is used as model. 2% B27 and 10% FBS medium are tested in a total volume/well of 50 µl. Cells are followed until D7. We observe (multiple) spheroid formation in all tested conditions. In higher Collagen I enriched conditions, spheroids are not in the bottom of the well and cells seem to start migrating. Also, no optical difference in viability is found between spheroids in 2% B27 and 10% FBS medium. More spheroids are observed in lower collagen I concentrations. Results of a representative well per tested condition is shown in ***Figure 9***.

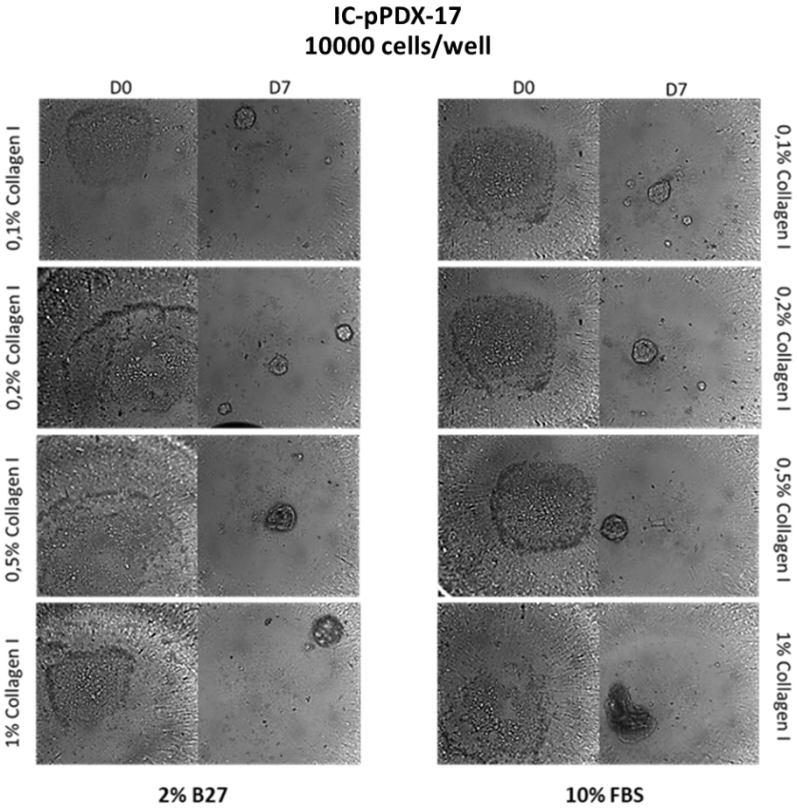


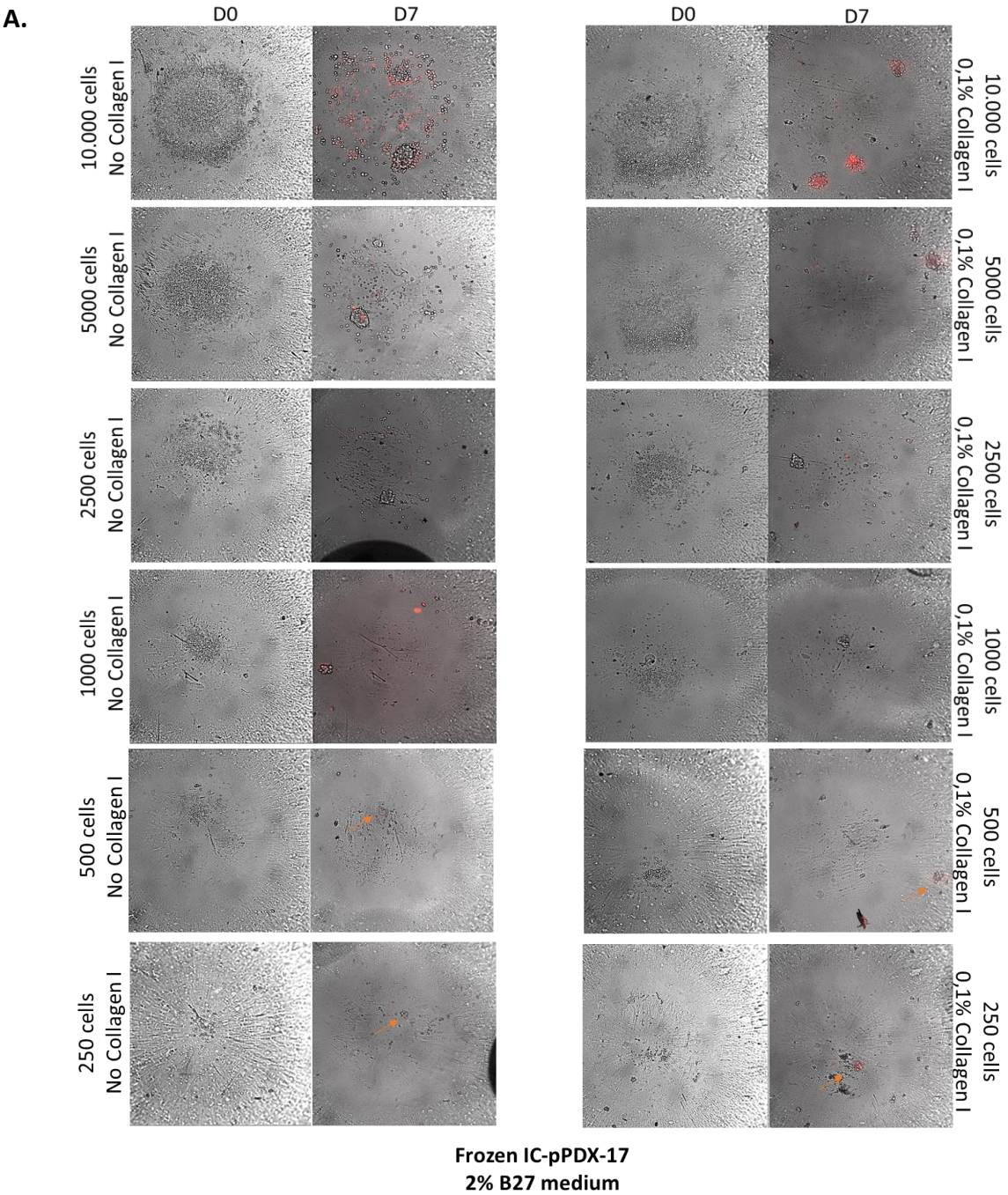
Figure 9. Images of spheroid formation in 384 well ULA plates (10000 cells/well) using range of Collagen I. *Fresh IC-pPDX-17 model used. General 2% B27 medium and serum enriched medium (10% FBS) tested in different Collagen I concentrations (3µg/mL). Images from D0 and D7 in BF and 10x magnification.*

Different number of cells show spheroid formation and presence of different cells

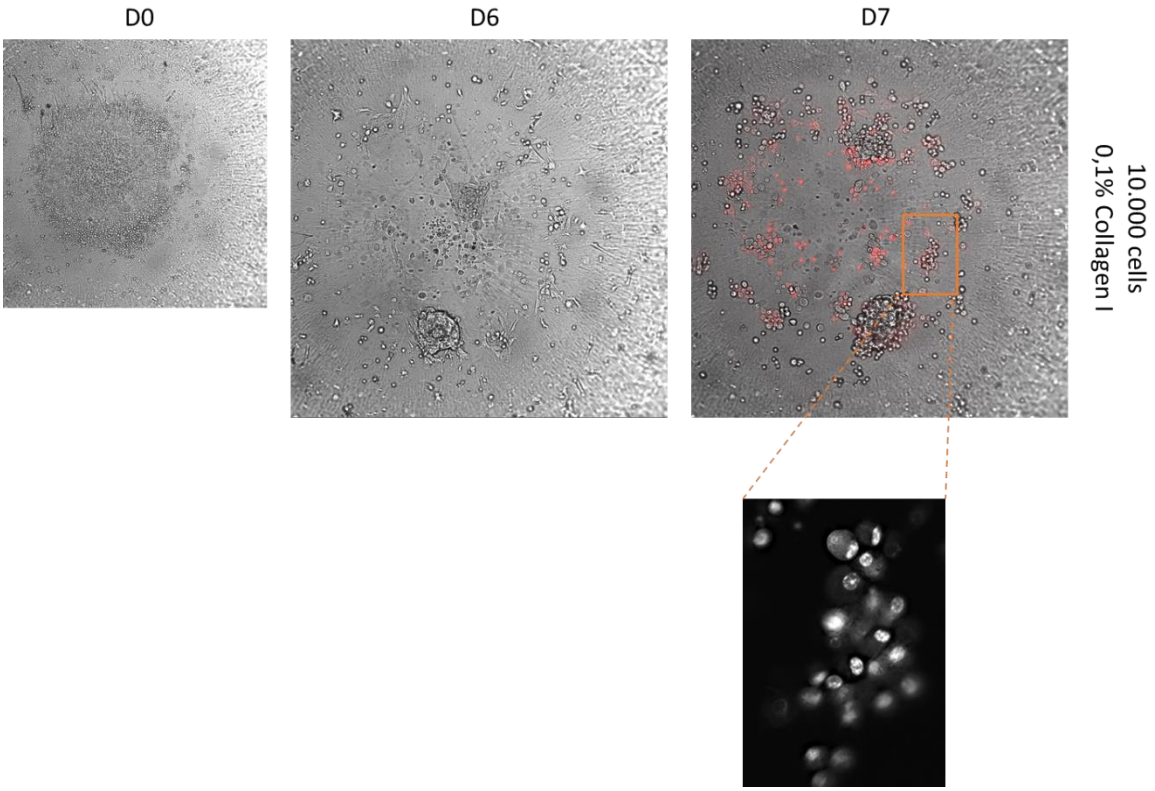
In the next step, the goal was to optimize conditions to obtain viable spheroid(s) over time with as less cells as possible. A broad range of seeded cells (250, 500, 1000, 2500, 5000 and 10000) and a low Collagen I concentration (0,1%) is used as results of observations in previous experiment in a new frozen IC-pPDX-17 model. Standard 2% B27 medium is used to prevent cell differentiation, in a total volume/well of 50 µl. A representative well of all tested conditions is shown in ***Figure 10A.***

After D3, we observe attached cells. PI and DAPI were added at D7 (3µM and 1µM in both 5 µL of 2% B27 medium respectively), to follow different type of cells. Metabolic activity is measured using CTG

3D. After adding fluorescent dyes, we observe dispatchment of cells. Attached and detachment of cells after adding fluorescent dyes and DAPI stained cells in 384 well ULA plate is shown in ***Figure 10B***. The DAPI stained channel showed dot-like nucleus structures. At D3 and after addition of dyes at D7, CTG is added and metabolic activity is measured (***Figure 10C***). Overall, we observe no clear difference in spheroid formation between non-collagen and collagen treated conditions on images. Lower number of seeded cells show small or no visible spheroid formation. CTG values show no difference in metabolic activity between tested conditions (two-tailed t-test gives ns p = 0,656).



B.



C.

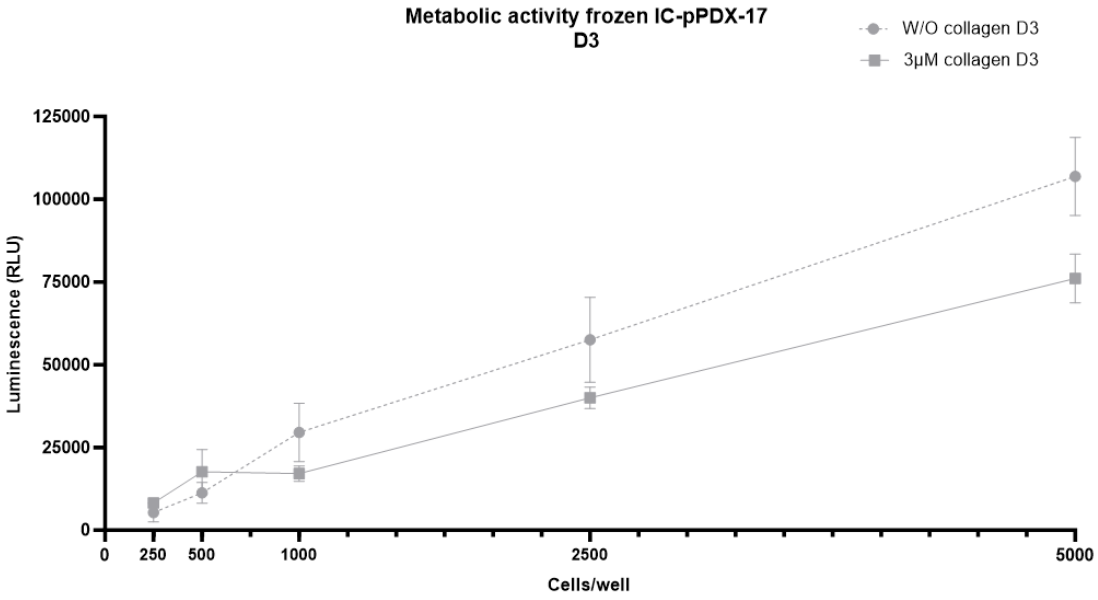
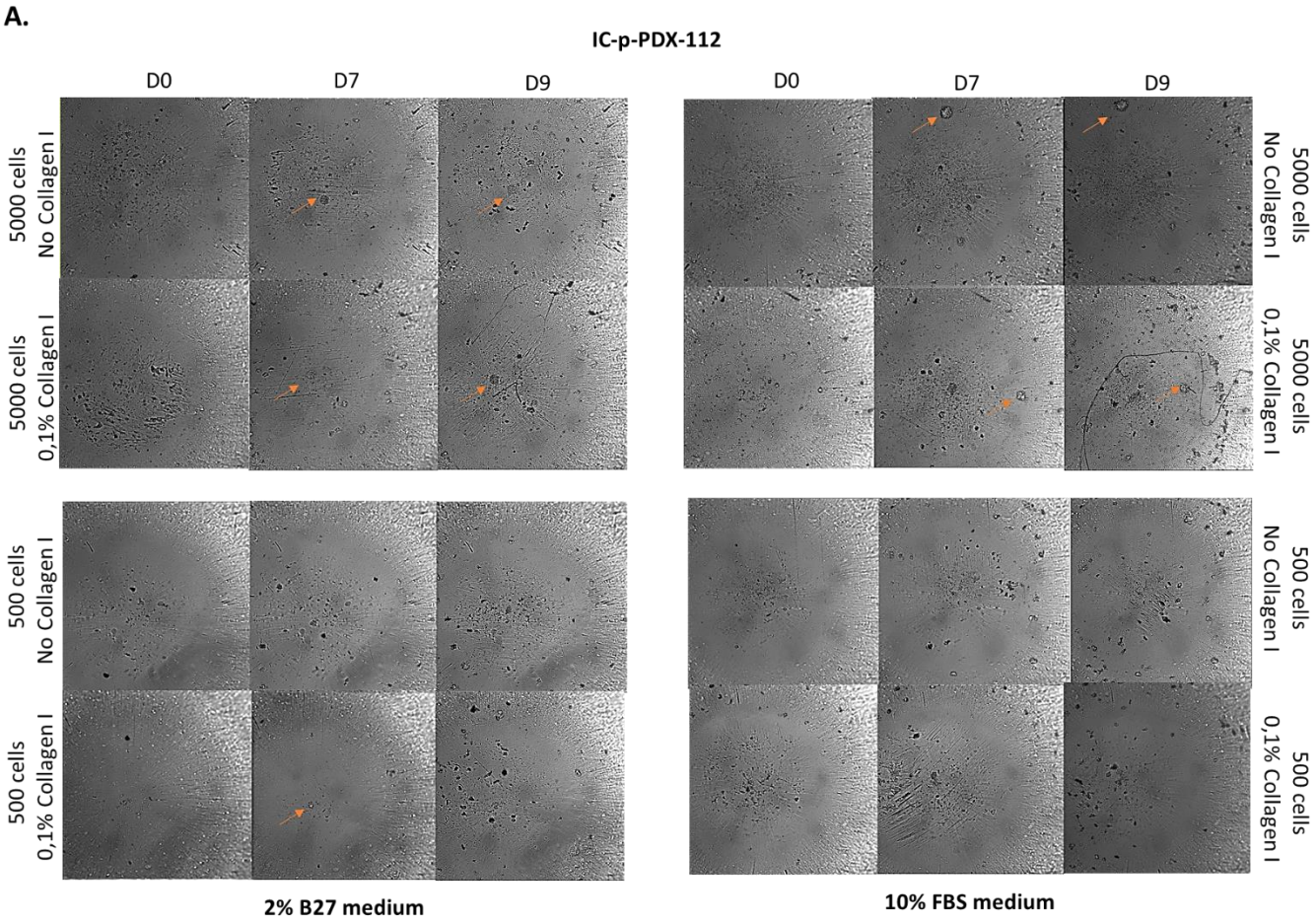


Figure 10. Results spheroid optimization experiment using different cell densities. *Frozen IC-pPDX-17 sample in 2% B27 medium.* ***A****. Images of different number of seeded cells (10000, 5000, 2500, 1000, 500, 250) at D0 and D7 with or without addition of Collagen I (3µg/mL 0,1%). PI added at D7 and marked in red.* ***B****. Example of well with attached cells (D6) and detachment at D7. Marked area at D7 is area stained with DAPI which is stated below. PI added at D7.* ***C****. Results metabolic activity after adding CTG 3D at D3 in Collagen I versus no Collagen I. Number of cells/well on x-axis and luminescence (RLU) on y-axis, measured in biological triplicates. Error bars indicate SD. A two-tailed t-test gives ns p = 0,656.*

Different PDX model display disparate spheroid growth and growth over time

As the previous experiment also show spheroid formation in low number of cells, the aim of the next experiment was to have viable spheroids over a long time period, as the timeline of drug screening last approximately 7 days. Here, a frozen IC-p-PDX112 sample is tested in different number of cells (5000, 2500, 1000, 500 and 250) and serum free and serum enriched medium, supplemented with no Collagen I or 0,1% in a total volume of 40 µl/well. Results from a high number of cells (5000) and low number of cells (500) for all conditions is shown in ***Figure 11A***. We observe growth in some spheroids after D7. Lower number of cells show no or small spheroid formation. Metabolic activity is measured at D5 (***Figure 11B***). Serum enriched medium (10% FBS) show higher metabolic activity than standard 2% B27 medium, however not significant (p = 0,4496 , one-way ANOVA). In general, spheroids are less visible than previous IC-pPDX-17 model and overall range in RLU is also lower than previous IC-pPDX-17 model.



B.

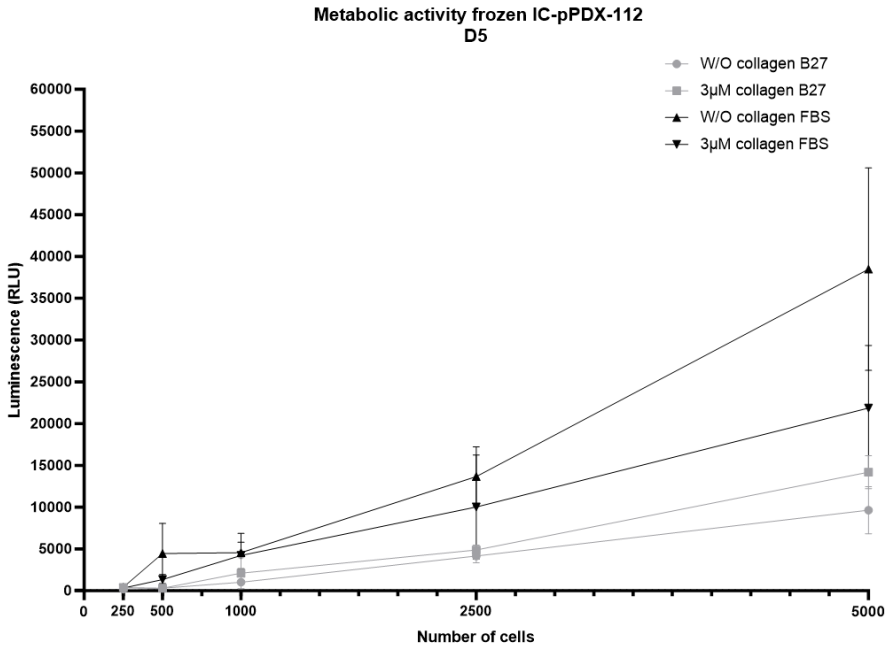
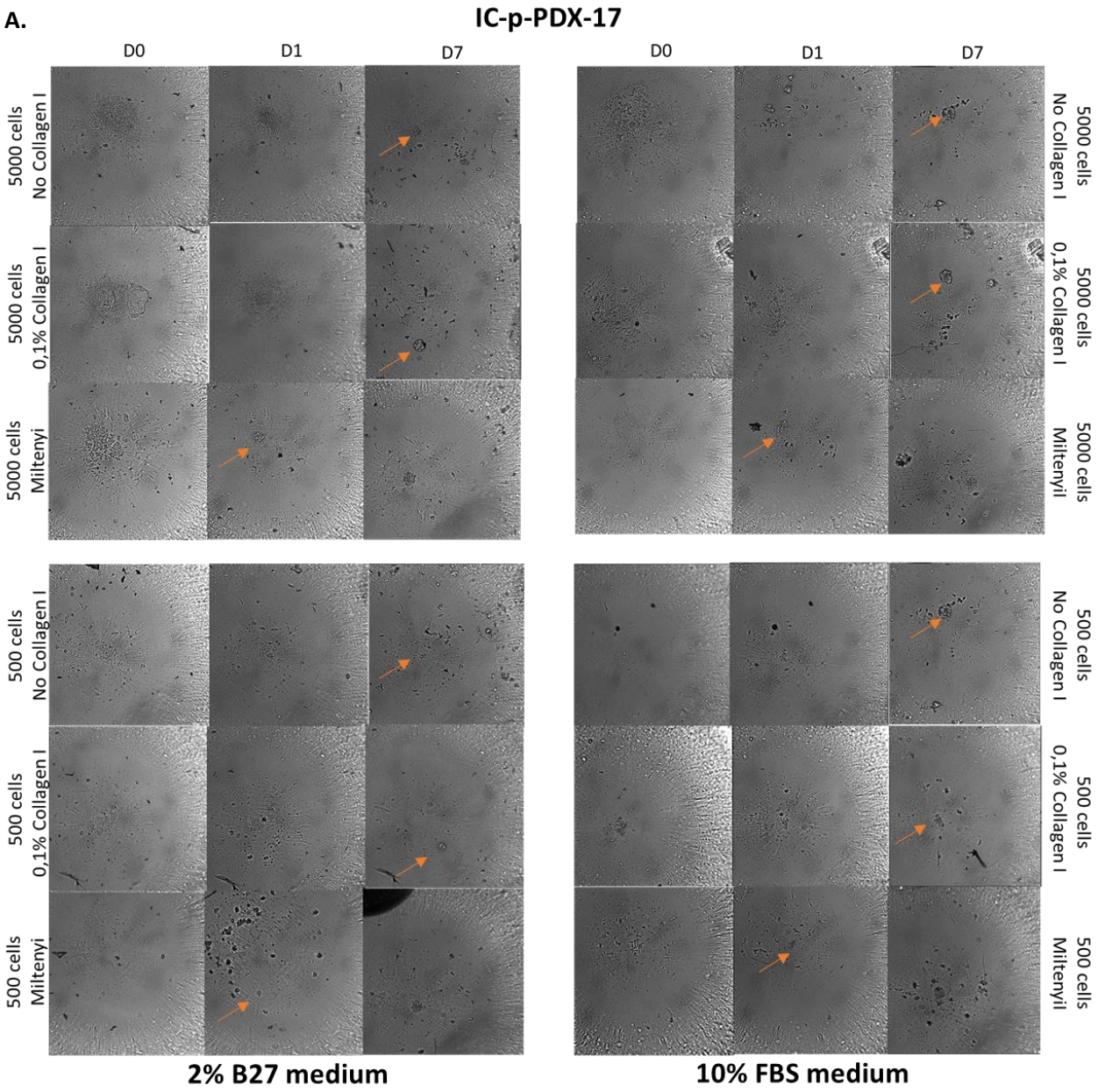


Figure 11. Results spheroid optimization experiment using different cell densities in frozen PDX sample. ***A****. Images of different number of seeded cells (5000 and 500) at D0, D7 and D9 with or without addition of Collagen I (3µg/mL 0,1%). Arrows indicate spheroids.* ***B****. Metabolic activity (RLU) at D5 for all conditions. Error bars indicate SD. A one-way ANOVA gives ns p = 0,4496.*

Viable spheroids in lower number of cells after introducing mouse cell depletion kit

Since the ultimate goal of this project is drug screening of primary patient-derived NB cells by FNA, a low number of cells is required. To isolate primary NB cells from other type of cells, a Miltenyi mouse depletion column is used to deplete mouse cells. A fresh IC-pPDX-17 sample is tested in both serum free and serum enriched medium (10% FBS), supplemented with 0,1% Collagen I and no Collagen I in a total volume of 40 µl/well . Miltenyi kit is performed right after dissociation and different number of cells/well (5000, 1000, 500 and 250) are seeded. Results of a representative well over time for a high number of cells (5000) and low number of cells (500) is shown in ***Figure 12A***. We observe earlier spheroid formation and more viable spheroids, also in low number of cells after performing Miltenyi kit. Difference in cell consistency between Miltenyi and non-Miltenyi treated cells is shown in ***Figure 12B***. Metabolic activity at D7 display that Miltenyi kit helps to establish more viable spheroids, especially when comparing lower number of cells from non-treated versus Miltenyi treated samples (***Figure 12C***). No signification is found between the tested conditions in both 2% B27 medium (one-way ANOVA gives ns p = 0,0551) and 10% FBS medium (one-way ANOVA gives ns = 0,5932).


A.
IC-p-PDX-17
D0
D1
D7
5000 cells No Collagen I
5000 cells 0,1% Collagen I
5000 cells Miltenyi
500 cells No Collagen I
500 cells 0,1% Collagen I
500 cells Miltenyi
2% B27 medium
10% FBS medium

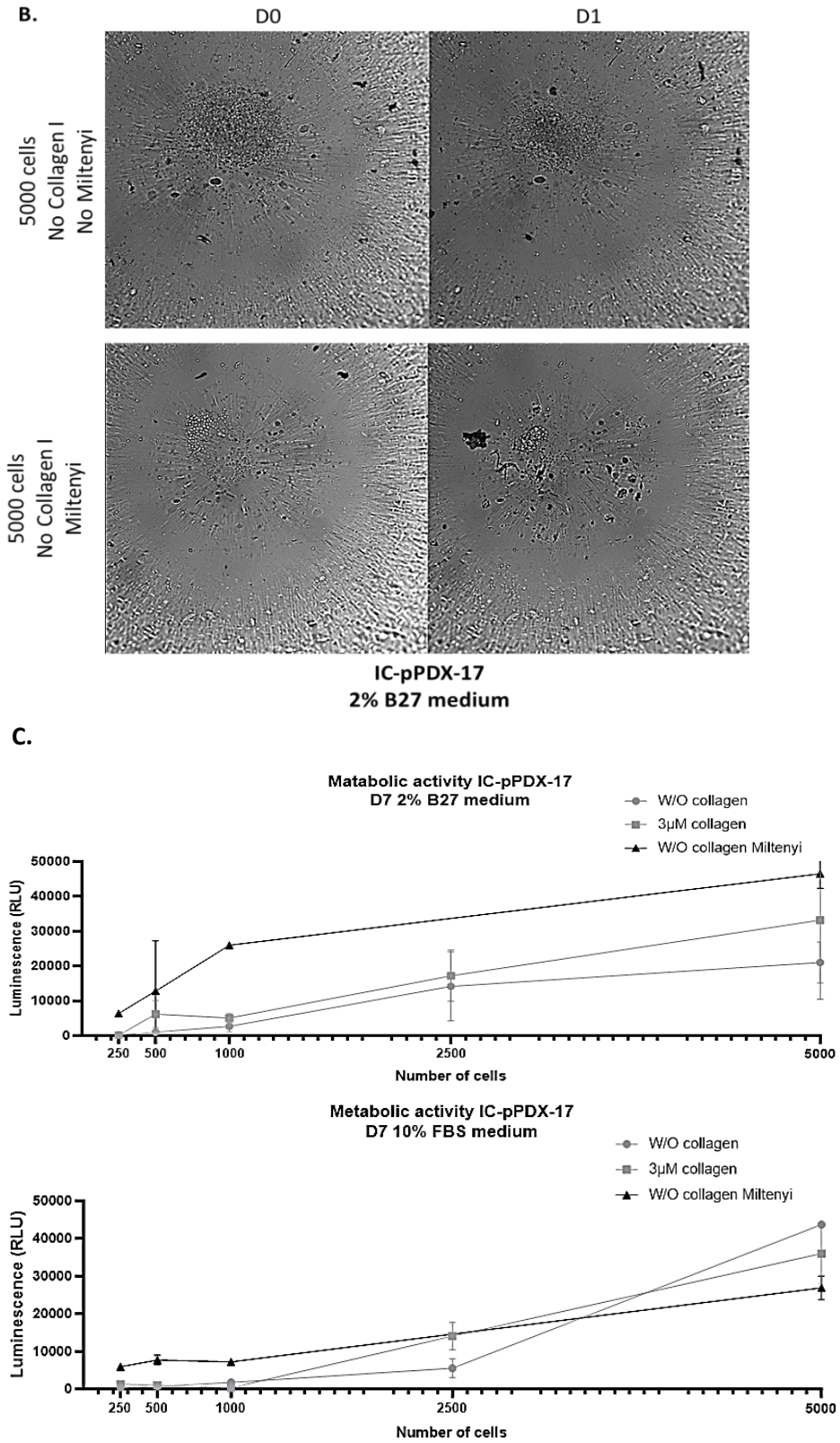


Figure 12. Results spheroid optimization experiment using Miltenyi mouse cell depletion kit. IC-pPDX-17 model used. ***A.*** *Images of different number of seeded cells (5000 and 500) at D0, D1 and D7 with or without addition of Collagen I (3µg/mL 0,1%), with or without Miltenyi treatment in both 2% B27 medium and serum enriched (10% FBS) medium.* ***B.*** *Difference in cell composition between non-treated cells versus Miltenyi-treated cells at D0 and D1. Seeded cells (5000) dissolved in 2% B27 medium.* ***C.*** *Metabolic activity (RLU) at D7 for different number of seeded cells w/o Collagen I, Collagen I and Miltenyi treated cells in 2% B27 and 10% FBS medium. Error bars indicate SD (biological triplicates). A one-way ANOVA gives ns p = 0.0551 (2% B27 medium) and ns p = 0,5932 (10% FBS medium).*

Proof of concept of single drug sensitivity effect on spheroid viability

Previous experiments display healthy spheroid formation over time. A small proof of concept is performed to test the workflow until now. According to results for optimized conditions so far, a new frozen IC-pPDX-17 is seeded in either 1000, 500 and 250 cells, with and without Miltenyi column in serum enriched medium (10% FBS), supplemented with 0,2% Collagen I (3µg/mL) in a total volume of 40 µl/well. 1 µM of a single chemotherapy, doxorubicin (dissolved in 10 mM DMSO), is added at D2. 72h later, metabolic activity is measured using CTG 3D and compared to non-treated samples (biological quadruplicates). Results show a decrease after doxorubicin treatment for both non-treated and Miltenyi treated spheroids, wheras Miltenyi treated spheroids show more activity in general (***Figure 13***). So statistical significance is found between control and treatment of non-treated cells (p = 0,2324, one-way ANOVA) and Miltenyi treated cells (p = 0,0782, one-way ANOVA).

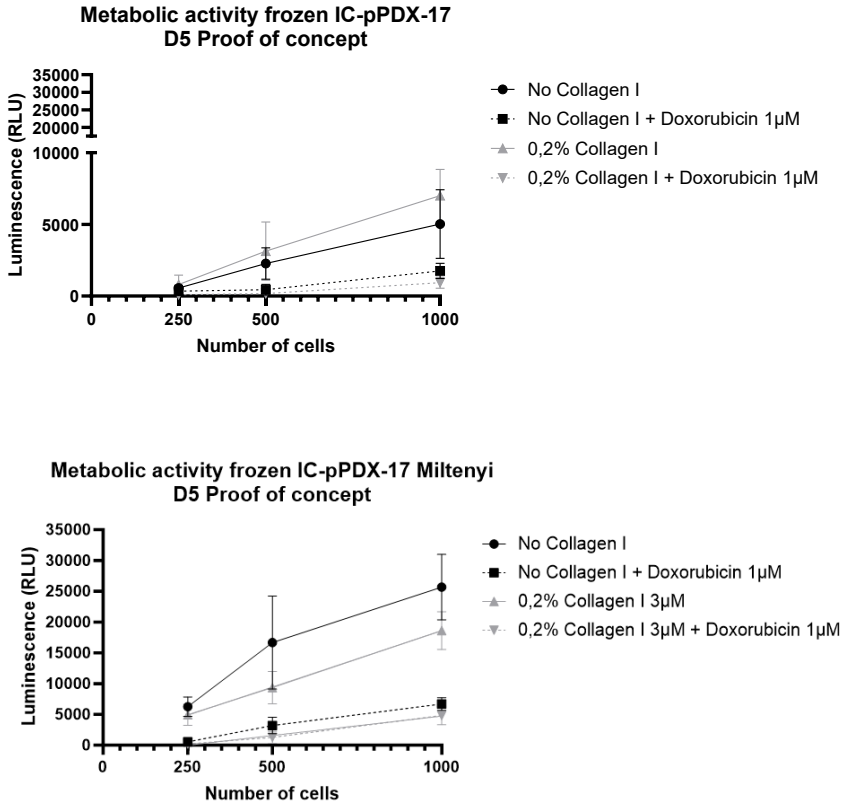


Figure 13. Results spheroid optimization using Miltenyi mouse cell depletion kit and proof of concept experiment. *Metabolic activity (RLU) of IC-pPDX-17 at D5 after 72h doxorubicin (1µM) exposure or control, with or without Collagen I (3µg/mL 0,2%) and comparing non-treated cells versus Miltenyi treated cells. Serum enriched medium (10% FBS) used. Error bars indicate SD (biological quadruplicates). A one-way ANOVA gives ns p-value = 0,2324 (non-treated cells) and ns p-value = 0,0782 (Miltenyi treated cells).*

5. Discussion

In this study, the ultimate goal is to establish a drug sensitivity and resistance microfluidics platform for personalized drug sensitivity profiling in HR NB derived primary tumor tissue. This report focusses on the first step, optimizing and standardizing protocols and workflow for 3D PDX cultures, with the goal of obtaining best conditions for performing high-throughput screenings. Experiments are performed at two different sites, where (i) Okomera provide the microfluidic chips and aims to validate the workflow when using NB PDX tissue and (ii) Biophenics for validating workflow on 384 well ULA plates. A proof of concept with a single chemotherapy is performed to test optimized conditions and workflow.

Okomera developed microfluidic chips which allows to pair two droplets at any moment over a period of time, using trapped anchors. Considering this, the primary droplet can trapped irreversibly while a second droplet can be trapped later, making the chips effective for testing mono or multi drug compounds [15]. The chip design used within Okomera (ST and BT) display better spheroid formation in ST chips. However, BT chips are optimized further and should be tested to obtain a proper comparison between the different size chips. Also, chips are loaded with manual syringe pump loading, which is sensitive to human technical mistakes, leading to small differences in loaded cells/trap. The in-house automatic machine loading may solve this problem and give more accurate results. A drawback from the chip design for validation experiments is that only one condition per chip can be loaded, in this way a lot of chips are needed to test different parameters within the same PDX sample.

Experiments with different amount of seeded cells are performed to determine the optimal cell density for obtaining healthy spheroids over time. To determine the optimal number of seeded cells, various aspects should be considered. Every tumor, in this case PDX sample, has its own tumor cell content. When a sample is more vascularized, this may lead to less neuroblasts and therefore slower spheroid formation or no spheroid formation for lower amount of seeded cells. Within Okomera, two different models are tested for this experiment, resulting in different results concerning optimal number of cells. Both models show healthy spheroid formation within 48h in a minimum of 40 cells/trap. In Biophenics experiments, optimal number of cells depends on applying the Miltenyi kit. For non-treated cells, a higher number of seeded cells is needed to achieve rapid spheroid formation after D1, as Miltenyi treated cells achieve rapid spheroid formation at D1 for lower amount of cells (500 cells). Metabolic activity is more accurate when seeding high number of cells, depending on the use of Miltenyi kit. For the proof of concept drug test, different number of seeded cells is used, starting from 250 of to 1000. Ultimately, drug screening for pediatric cancer patients is performed after obtaining a FNA, thus a low amount of cells/well is required. More validation experiments on different PDX models are needed to select the optimal number of seeded cells.

Using different kind of media display different results between tested sites. Within Okomera experiments, serum-enriched medium show lower PI signal and more homogenous spheroids, which indicate higher viability. This can be explained by the facts that serum contain nutrient supply, growth factors, anti-apoptotic factors, factors involved in cell-cell communication and serum can help in metabolite exchange in cells. However, earlier research display that serum-enriched medium enhance cell differentiation, which may influence drug responses and differentiation is seen in serum enriched tested conditions within Okomera. Avoiding serum appears to be a strategy to avoid maturation of NB cells[17]. Following PDX spheroids in long term culture flask may give more information about effects of serum-enriched medium on genetic profiles of PDX tissue, for example by performing SNP array's. Within Biophenics, serum-enriched medium shows higher metabolic activity in PDX model IC-p-PDX-112. In IC-pPDX-17, no significant difference in spheroids formation is

observed when different media were tested. Serum-enriched medium may help mouse related fibroblasts and other type of cells to grow, giving an inaccurate readout when measuring metabolic activity. Based on metabolic activity readout and spheroid viability within Okomera, 10% FBS medium is chosen for the proof of concept experiments. However, more detailed considerations need to be conducted about choosing optimal medium for drug screening.

In this study, two ECM's are used to improve spheroid formation and viability. Within Okomera experiments, Collagen I seems to help in spheroid formation comparing to other ECM Matrigel or no ECM. Collagen I is a major component of the ECM, providing structural scaffold, cell adhesion and migration, cell-cell communication, and tumor-stromal interactions, all mimicking an accurate complex 3D tumor microenvironment [18] . Within Biophenics experiments, high concentrations of Collagen I sometimes lead to migration of tumor cells and disformation of spheroids. Low concentrations lead to no significant difference in optical viability and CTG levels, comparing to non-collagen containing spheroids. A possible reason for the differences between the two sites is that within Okomera, no mouse cell depletion kits are used. As Collagen I used in this study is mouse derived collagen, this may explain why no difference in optical viability and metabolic activity is observed in Biophenics experiments, especially after introducing Miltenyi kit experiments. However, no difference is observed in Biophenics non-Miltenyi treated cells and different final concentrations Collagen I are used between Okomera and Biophenics site. For proof of concept drug testing, Collagen I is used as optimized condition (0,2%) , as it seemed to help spheroid formation within Okomera.

Cell depletion kits display different effects on spheroid viability. Within Okomera experiments, using ACK lysis buffer to deplete red blood cells lead to less viable spheroids. ACK lysis buffer might be toxic on PDX models used. Testing different concentrations may avoid spheroids to die.
Images of spheroids after using Miltenyi kit in Biophenics experiments show that depleting mouse cells help in earlier formation of spheroids and spheroids show higher CTG values in few amount of cells. When considering a rapid workflow from biopsy to readout after drug screening, rapid spheroid formation is required. By eliminating mouse cells, spheroids may better mimic primary tumor biopsies obtained from patients. This is crucial when studying drug response of human cancer cells more accurate. Standardizing the PDX tissue composition when forming spheroids is also important for achieving consistent results in high-throughput drug screenings. Moreover, mouse stromal cells may interfere with analyses, make them less interpretable. Lastly, when testing drug efficacy, interactions between mouse cells and human cells may interfere actual drug response on human cancer cells. The proof of concept experiment show more accurate CTG values for Miltenyi treated cells, this lead to a more robust comparison between tested conditions.
In the frozen IC-PDX-17 experiment, attached cells are shown after D3. After staining with PI and DAPI at D7, we observed a cells detachment. DAPI staining show dots within cells, indicating presence of mouse cells, probably fibroblasts [19]. Interestingly, cells were attached when using ULA plates. Using mouse cell depletion kits may avoid this problem. However, fibroblasts are a crucial part of the tumor microenvironment, so PDX spheroids may need them to survive[20]. This is something which can be tested by culturing purified PDX tumor cells after performing Miltenyi kit and follow survival over time. Also, when comparing experiments within Okomera and Biophenics, Miltenyi columns should also be tested in Okomera experiments. Ultimately, the goal is to use FNA's from primary patient cancer cells, making the use of pure human cells in PDX tissue a reliable way of testing and standardizing these drug efficacy workflow and protocols.

For the proof of concept, doxorubicin is added after D2 on IC-pPDX-17 model. CTG values show a proper decrease in metabolic activity after 72h of drug exposure, however not significant. More biological or technical replicates may increase statistic power. However, the reduction of metabolic activity is in line with a previous experiment within Institut Curie on 2D PDX cultures, where doxorubicin show a reduction of approximately 60%. Doxorubicin was dissolved in DMSO, whereas control samples were not. This is something which must be tested next to more replicates to make a more robust comparison between 2D and 3D cultures. When testing drug compounds or combinations, genetic profiles of specific PDX models should be investigated to individualize drug libraries based on expected responses, which may help in discovering trends in response when having specific genomic alterations.

6. Conclusion

In this study, validation experiments are performed on NB PDX spheroids to optimize conditions for microfluidic drug sensitivity screenings in NB. The workflow and protocol are optimized and standardized by testing spheroid formation on microfluidic chips within Okomera and on 384 ULA plates within Biophenics. Serum-enriched medium (10% FBS), low Collagen I concentrations and use of Miltenyi mouse cell depletion kit help in spheroid formation and viability over time. Optimal number of seeded cells depends on PDX sample and using chips or plates. More optimization experiments must be performed on different PDX samples to determine optimal conditions for a 7-days workflow from biopsy to readout after drug screening. A proof of concept show that doxorubicin reduce spheroid viability after 72h hours of exposure and this is in line with results for 2D cultures. When further optimizing the workflow and protocol and screening NB PDX spheroids and ultimately primary NB patients' tumor material on individually selected drug libraries, new predictive biomarkers may be found and this is an important step for individual therapeutic decisions based on molecular profiles and drug sensitivity characterization, ultimately lead to individualize patient care strategies in clinical settings for HR NB patients.

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Supplementary material

IC-pPDX-17			
Primary tumor	Suprarenal	**Age at diagnosis**	30 months
Stage	3	**Age at biopsy for PDX**	50 months
Gender	F	**Status at biopsy for PDX**	Relapse
Site of biopsy (patient)	Peritoneum	**Molecular abnormalities**	ATRX c.5242G>A (100%) , p.(Gly1748Arg) (MAF:100%), CDK4amp, MDM2 amp, GLI3 c.1607A>T, (25%), ETV1 c.492C>G (35%), PDGFB c.246C>A (38%), ARID1B c.4034A>T (12%)

IC-pPDX-112			
Primary tumor	Suprarenal	**Age at diagnosis**	42 months
Stage	4	**Age at biopsy for PDX**	42 months
Gender	M	**Status at biopsy for PDX**	Diagnosis
Site of biopsy (patient)	Primary tumor	**Molecular abnormalities**	Hypermutated, high mutational load

HSJD-NB-009			
Primary tumor	Unknown	**Age at diagnosis**	41 months
Stage	4	**Age at biopsy for PDX**	72 months
Gender	M	**Status at biopsy for PDX**	Relapse
Site of biopsy (patient)	Bone	**Molecular abnormalities**	ALK E1419K (57%), BARD1 c.1518_1519delTGinsCA (65%), APOBEC3 c.76G>C (40%), MYC c.164T>C (43%), APC2 c.6560G>A (99%)

HSJD-NB-011			
Primary tumor	Retroperit	**Age at diagnosis**	19 months
Stage	4	**Age at biopsy for PDX**	34 months
Gender	M	**Status at biopsy for PDX**	Relapse
Site of biopsy (patient)	Primary tumor	**Molecular abnormalities**	ALK I1171N (87%), NF1 c.1223A>G 75%; CHK2 c.573+1G>A 42%

GR-NB7			
Primary tumor	Thor-abd	**Age at diagnosis**	17 months
Stage	4	**Age at biopsy for PDX**	35 months
Gender	M	**Status at biopsy for PDX**	Relapse
Site of biopsy (patient)	Bone	**Molecular abnormalities**	RAS/MAPK NF1 mut+loss (c.4537C>T, 100%), PALB2 mut (c.3383C>T, 20%), SMARCA4 (c.2467G>A, 40%), PTEN mut+loss (c.968del, 100%), PIK3R2 (c.1331C>T, 50%), DNMT3B (c.416G>A, 50%), MSH6 c.3950A>G (50%)

Table S1. Clinical and molecular characteristics of used PDX samples.