A microfluidics platform for combinatorial drug screening on cancer biopsies

Federica Eduati1,2,8, Ramesh Utharala2, Dharanija Madhavan2, Ulf Peter Neumann3,4, Thomas Longerich5,9, Thorsten Cramer4,6, Julio Saez-Rodriguez1,7,10 & Christoph A. Merten2

Screening drugs on patient biopsies from solid tumours has immense potential, but is challenging due to the small amount of available material. To address this, we present here a plug-based microfluidics platform for functional screening of drug combinations. Integrated Braille valves allow changing the plug composition on demand and enable collecting >1200 data points (56 different conditions with at least 20 replicates each) per biopsy. After deriving and validating efficient and specific drug combinations for two genetically different pancreatic cancer cell lines and xenograft mouse models, we additionally screen live cells from human solid tumours with no need for ex vivo culturing steps, and obtain highly specific sensitivity profiles. The entire workflow can be completed within 48 h at assay costs of less than US$ 150 per patient. We believe this can pave the way for rapid determination of optimal personalized cancer therapies.
Most efforts in personalized medicine have been focusing on tailoring the treatment to the specific patient based on genomic data, which are increasingly available due to advances in sequencing technologies. While there have been some impressively successful examples, cancer genomics is generally very complex and, despite the increasing knowledge on occurring mutations, there is still limited understanding on how they affect drug response. Multiple efforts have been devoted to the large-scale in vitro screening of drugs across cell lines that have proven useful to identify some genomic markers associated with drug response. However, molecular data alone has not proven sufficient to predict the efficacy or toxicity of a drug on an individual cell line in a reliable way. This predictability is likely to be even lower in patients, given the additional complexities when compared to cell lines.

Due to these limitations, genomics data have to be supplemented with other information in order to optimally guide the treatment for each patient, and systems for phenotypic stratification are urgently needed. The need for new approaches is even more acute for the application of drug combinations. Combinatorial targeted therapy has been shown to be a powerful tool to overcome drug resistance mechanisms, which can be due to tumour heterogeneity, clonal selection or adaptive feedback loops, and seems to be a particularly promising approach for treatment of pancreatic cancer. However, strategies to identify effective combinations are still in their infancy.

A powerful means to overcome these difficulties would be to test the drug compound directly on patient samples. Despite recent progress toward functional testing of live patient tumour cells, drug screening technologies are limited by the need of large numbers of cells. Therefore, large-scale drug screening of patient tumours has been so far limited to blood tumours (where a much larger amount of malignant cells is easily accessible) or requires ex vivo culturing steps (e.g., patient derived cell lines, PDX models and organoids) that require long times to grow the cells and can cause changes in the phenotype of the cells.

In contrast, microfluidic technology allows to carry out cell-based assays in tiny volumes, thus opening the way for screens on very limited material such as primary cells or patient biopsies. In line with this, microfluidics has recently been applied successfully to the testing of a few individual drugs on cancer cells, drug screening technologies are limited by the need of large numbers of cells. Therefore, large-scale drug screening of patient tumours has been so far limited to blood tumours (where a much larger amount of malignant cells is easily accessible) or requires ex vivo culturing steps (e.g., patient derived cell lines, PDX models and organoids) that require long times to grow the cells and can cause changes in the phenotype of the cells.

To avoid plug breakup and/or the adhesion of plug contents to the channel walls (generally termed “wetting”—an effect that can cause significant cross contamination) we integrated an additional oil phase serve as independent reaction vessels. Conventional targeted therapy has been shown to be a powerful tool to overcome drug resistance mechanisms, which can be due to tumour heterogeneity, clonal selection or adaptive feedback loops, and seems to be a particularly promising approach for treatment of pancreatic cancer. However, strategies to identify effective combinations are still in their infancy.

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Results

Microfluidic platform. Our platform (Fig. 1a) is based on a combination of plug technology with microfluidic Braille valves controlling individual fluid inlets of the microfluidic chip (Fig. 1a–d, Supplementary Fig. 1 and Supplementary Movie 1). To facilitate the alignment of the chip with the Braille display below, we designed additional channels that can be filled with dye (top and bottom left corner of the chip shown in Fig. 1a) and easily brought into line with particular Braille pins. All reagents (including the cell suspension, drugs and assay components) are permanently injected into the device and, depending on the valve configuration, sent either to the waste (each two inlets share one waste outlet; Fig. 1a) or to a droplet maker with a T-junction geometry. This approach allows rapid switching (~300 ms) between 16 liquid streams and, upon injection of fluorinated oil at the T junction, the generation of combinatorial plugs at high throughput. If necessary, the chemical diversity can be increased further by connecting an autosampler to one of the inlets (sequentially loading compounds from microtiter plates; Supplementary Figs 2 and 3, and Online Methods), but this procedure also requires a higher number of cells. All plug generation steps are executed in a fully automated fashion using an in-house LabVIEW program (see Online Methods).

To avoid plug breakup and/or the adhesion of plug contents to the channel walls (generally termed “wetting”—an effect that can cause significant cross contamination) we integrated an additional mineral oil inlet into our chip design, enabling the insertion of mineral oil droplets in between all (aqueous) plugs (Supplementary Movie 2). Such three-phase systems are particularly efficient in keeping plugs separated, even under conditions that normally cause wetting. A further crucial factor for reducing wetting was the use of special, protein-free media. It is well known that protein adsorption can render Polydimethylsiloxane (PDMS) surfaces hydrophilic, which in turn increases the tendency of plugs wetting the channel walls. Therefore we chose FreeStyle Media (Gibco) for all experiments, as it does not contain proteins and allows the culturing of cells in absence of fetal bovine sera (FBS, which is protein-rich itself). However, while a combination of these measures significantly improved reliability, plug integrity could still not be ensured for all plugs. Hence an identification system that would still work if individual plugs break or fuse had to be implemented. Therefore, we introduced sequential barcodes to separate and identify plugs belonging to different experimental conditions, based on sequences of plugs with binary (high/low) concentrations of the blue fluorescent dye cascade blue (Fig. 1e, g and Supplementary Movie 3). This way numbers assigned to each tested condition, can be encoded (e.g., high-low = binary number “10” = decimal number “2”). Plugs were stored in PTFE tubes (Fig. 1f) and the readout was performed by flushing the plugs through the
detection module. In our case, we used an optical setup (Supplementary Fig. 4) with three different excitation lasers (375, 488 and 561 nm) and performed a multiplexed readout at three different wavelengths (450, 521 and >580 nm) to allow highly multiplexed assays in the blue, green and red spectrum, using photomultiplier tubes (PMTs). To demonstrate the power of our barcoding approach, we converted a simplified EMBL logo (Supplementary Fig. 5) into a binary black and white image and translated all 2808 pixels into a sequence of plugs with two different fluorescence intensities. These barcodes were then...
detected using our laser spectroscopy setup (Supplementary Fig. 4) and converted back into the initial image, which did not show a single mistake. This clearly demonstrates the scalability and reliability of our strategy.

**Combinatorial drug-response screening.** Motivated by the urgent need to screen therapy options directly on patient material, and by the idea that the small reaction volumes of ~0.5 μL per plug make our technology compatible with the screening of patient biopsies, we applied the above-described technology to screen cellular responses to drug combinations. Indeed, the amount of starting material (and thus viable cells) is the most limiting factor in the application of standard screening technologies directly on patient samples, and miniaturization allows screening one to two orders of magnitude more conditions using the same starting material, but simply 10x–100x smaller assay volumes. Accordingly, only ~100 cells were encapsulated in each plug together with media, one or two compounds and a rhodamine 110 (green-fluorescent dye) conjugated substrate of Caspase-3, which is an early marker of apoptosis. The cell suspension contained Alexa Fluor 594 (orange-fluorescent dye) to verify its addition to all plugs. In turn, this also allowed us to monitor the correct operation of all valves since the malfunctioning of one valve would result in an unbalanced mixture of the components, thus affecting the orange signal of the corresponding plug. This is also true for the single compound conditions for which we added only media instead of the second compound.

For initial screens, we included ten well-characterized drugs and biologicals (listed in Table 1) that were tested alone and in pairwise combinations. Specifically, we included two drugs that are currently used in clinical chemotherapy as first line treatment for pancreatic cancer (Gemcitabine and Oxaliplatin), seven drugs that have specific kinase targets which play key roles in different pathways (i.e., IKK, MEK, JAK, PI3K, EGFR, AKT and PDPK1 inhibitors) and one cytokine (TNFa) that activates the extrinsic apoptosis pathway. Compounds were stored in ten syringes which were connected to the corresponding inlets in the chip (Fig. 1a).

Additionally, we included two syringes with media to be able to generate the single-compound conditions (cells + Caspase-3 substrate + compound X + media) and the control condition (cells + Caspase-3 substrate + media + media). Plugs for different experimental conditions were generated sequentially and each condition was preceded by barcoding plugs as described in the previous paragraph. We generated 12 consecutive plugs (replicates) for each of the 55 perturbed conditions and 20 for each control condition (Fig. 2a). In order to monitor the stability of the system over time, the control condition was repeated at the beginning and at the end of the sequence, as well as after every 10 conditions. This resulted in a total of 62 sequential conditions corresponding to 800 assay plugs and 340 barcoding plugs (1140 in total). In order to rigorously assess the robustness of the results, the whole sequence of conditions (which we call run) was repeated multiple times (at least two consecutive runs for patient material and six runs, also on different days, for cell lines available in big quantities). Plugs were stored in gas permeable tubing with a total length of 5 m (having a capacity for about 3500 plugs in total) and incubated for 16 h (see details in Online Methods).

**Data extraction and quality assessment.** In the fluorescence data, each plug corresponds to a peak in one or more channels (i.e., green, orange and blue), as shown in Fig. 2b. When processing the data, we first identified the peaks in the blue channel, representing the barcodes, and we used them to separate and identify the peaks corresponding to different conditions (Online Methods). For each experimental condition we obtained multiple peaks (typically 12 replicates per run, 20 for control conditions) with signals both in the green and in the orange channel. The height of each peak is proportional to the measured fluorescence intensity: the intensity in the green channel represents the activation of Caspase-3 (thus apoptosis) while the intensity in the orange channel represents the concentration of the orange-fluorescent dye, which was added to the cell suspension. Since each plug was produced by mixing four components (cells, Caspase-3 substrate and two compounds/media), the intensity in the orange channel allowed assessing the quality of this mixture and was used to discard conditions/peaks with extreme values (i.e., outliers, see Online Methods). The width of the peak represents the length of the plug: Thin or wide peaks were discarded as they correspond to split or fused plugs, respectively. Additionally, the first peak for each condition was discarded to avoid any possible effect of cross contamination between conditions, which could be due to the dead volume (causing a leftover of reagents from previous plug; see Supplementary Note 1) in the chip. Even if two to three peaks per condition are discarded at this step, >1200–1300 actual data points for each sample remain (even when only two runs are available). As illustrated in Fig. 2a, data were first processed separately for each run, obtaining a z-score and a p-value for each condition, and then combined to get overall scores (details in Online Methods).

![Fig. 1](image-url)
Combinatorial screening of pancreatic cancer cell lines. To optimize and validate our microfluidics platform, we performed combinatorial screening of drugs and biologicals on two pancreatic cancer cell lines with different genotype and phenotype\(^\text{32}\): AsPC1 and BxPC3. The sequence of 62 conditions was repeated six times and data from these 6 runs are shown in Fig. 3a (data refer to BxPC3 cells, but sequence of tested conditions was the same for both cell lines). These results (Fig. 3a for BxPC3 and Supplementary Fig. 6 for AsPC1) show that repeated control (same for both cell lines). These results (Fig. 3a for BxPC3 and Supplementary Fig. 6 for AsPC1) show that repeated control condition are preceded by binary barcode plugs to encode the corresponding identification number (high concentration of blue fluorescent dye = 1, low concentration = 0, followed by an end of barcode signal).

| Condition | Control (10 plugs) | Perturbation (17 plugs) | Perturbation (12 plugs) |
|-----------|--------------------|------------------------|------------------------|
| Run 1     |                    |                        |                        |
| Run 2     |                    |                        |                        |
| Run N     |                    |                        |                        |
| Condition X |                 |                        |                        |

**Fig. 2** General workflow of data acquisition and processing. a Different conditions are generated sequentially with multiple replicates (aqueous plugs) produced for each condition and collected in a tube. The whole experiment (run) is repeated multiple times and data for each run are processed separately and then combined. b Example of a plug sequence and corresponding readout. Each aqueous plug is generated by mixing the following components: cells (with orange-fluorescent dye to verify the proper mixing of all components), caspase-3 substrate and one or two compounds. Plugs of each tested condition are preceded by binary barcode plugs to encode the corresponding identification number (high concentration of blue fluorescent dye = 1, low concentration = 0, followed by an end of barcode signal).

Cell line-specific drug combinations and validation. We then focused on the cell line-specific effects, as this allows excluding drug combination that are generally toxic (which would show strong effect in both cell lines) and illustrates the concept of personalized medicine prioritizing the therapy most suited for each patient (cell line in this case). As a general observation (Fig. 3d), we noticed that PHT-427 induced apoptosis in BxPC3 cells in combination with multiple drugs ([z-score range = [0.86, 2.36]; median z-score = 1.39]), while it showed little or no effect on AsPC1 cells (z-score range = [−0.95, 0.16]; median z-score = −0.58). For a more systematic identification of strictly cell line-specific effects, we considered the efficacious perturbations for each cell line (as described in the previous section) and assessed if the measured effect is statistically stronger than the one exerted on the other cell line (using a Wilcoxon rank-sum test). The resulting top 10 treatments which are strictly specific for each cell line (i.e., significantly stronger, with p-value <0.05) are shown in Fig. 3e.

The strongest effect for BxPC3 cells was measured in response to combinatorial treatment with PHT-427 (AKT and PDPK1 inhibitor acting on the PH domain) and MK-2206 (allosteric inhibitor of AKT). We compared the combinatorial effect on BxPC3 cells with the corresponding single drug treatment (Fig. 4a); either single treatment showed an effect that was not significantly higher than zero (p-value 0.21 and 1 for PHT-427 and MK-2206, respectively, one-tailed t-test), while the combinatorial treatment showed a strong and significant effect (effect
size = 2.24, Cohen’s d; p-value = 0.001, one-tailed t-test). On the contrary, no significant effect was shown for AsPC1 cells for PHT-427, MK-2206, or their combination (p-value = 1, 0.68 and 0.28, respectively, one-tailed t-test), suggesting that the efficacy of the drug combination on BxPC3 cells is not caused by a general toxicity of the drugs when administered in combination, but rather by a cell line specific effect. Similarly, the most promising treatment specific for AsPC1 cells is the combination of Gefitinib (EGFR inhibitor) with ACHP (IKK inhibitor), which showed a strong efficacy for AsPC1 cells (effect size = 1.34, Cohen’s d; p-value = 0.01, one-tailed t-test) but not for BxPC3 cells (p-value = 0.83, one-tailed t-test).

For both examples, results were validated in vitro in subsequent tissue culture experiments (Fig. 4b–d), confirming the behaviour observed in the microfluidic system. Encouraged by these results, we went on to investigate if our findings would also hold in an in vivo setting, using xenograft tumour models generated using AsPC1 and BxPC3 cell lines (see Online Methods). The BxPC3-specific combination of PHT-427 and MK-2206, as predicted from the microfluidics data, was significantly more effective than the standard of care Gemcitabine for the BxPC3 mice but not for AsPC1 mice (Fig. 5a; p-value 0.08, 0.03, 0.02, respectively, at day 9, 11, 14 for BxPC3; p-value always >0.1 for AsPC1). This specificity can be further assessed by

**Fig. 3** Microfluidic combinatorial drug screening in cell lines. **a** Boxplot of the sequence of conditions across multiple replicates for BxPC3 cells (z-scores of Caspase-3 activity) with control conditions in purple and conditions which are not significantly (Wilcoxon rank-sum test; p-value < 0.05) better than the control with light grey borders and dots. For each condition, replicates are shown as dots, and summary statistics are represented using a horizontal line for the median and a box for the interquartile range. The whiskers extend to the extreme data point, which is no >1.5 times the length of the box away from the box. **b** Heatmap representation of the same conditions as in a (BxPC3 cell line) using median value across 6 replicates. Colored red scale starting from z-score equal to 0 (i.e., median activation across all conditions) while negative values are represented in grey. Conditions which are not significantly better than the control are marked with an x. **c** Heatmap representation for AsPC1 cells. **d** Overall drug efficacy for each cell line, computed as median z-score across all conditions (single or combinatorial perturbations) involving the drug. **e** Top 10 cell line-specific conditions for each cell line.
Combinatorial screening of patient pancreatic tumour biopsies. After optimizing and validating our approach in tissue culture and mouse models, we aimed for demonstrating its applicability to clinical samples. Starting with biopsies from five patients, the resected solid tissue was dissociated to create a single cell suspension that was then used for the screening (Fig. 6a; protocol in Online Methods). Similar to the pipeline used to screen cell lines, a total of 62 conditions were screened, with 12 sequential plugs for each perturbed condition and 20 for the control conditions (each plug with about 100 live cells). The whole sequence of conditions was repeated two or three times (different runs) depending on the amount of viable cells that were obtained from each patient biopsy, with ~1 million viable cells being sufficient to perform two full runs. As in the case of the cell lines, unreliable peaks/conditions were discarded based on the intensity in the orange channel and on the length of the peaks. Only conditions passing the quality assessment in at least two runs and with consistent values across runs were considered for further analysis (boxplot with data for each run shown in Supplementary Fig. 8, more details in Online Methods). As previously described, control conditions were repeated regularly throughout the experiment and used to assess if cell occupancy and viability were robust over time. For one patient sample this
Looking at the patient-specific response to combinatorial drug treatments in more detail (Fig. 6c, d) we observed that the top hit for treatment with Gefitinib or combination of MK2206 and PHT-427. For both panels, p-values are computed using one-tailed t-test and error bars represent the standard error of the mean. The experiment started with 5 mice per each group, error bars are not reported for the condition Gefitinib + ACHP after the third day, where only one mouse survived.

The four successfully screened samples (Supplementary Fig. 9) include two primary tumours from patients diagnosed with pancreatic cancer (classified as stage pT3 pN1 M0 and pT3 pNx M0, respectively) as well as a intraepithelial neoplasia (pre-tumour stage, PanIN1/2) and a liver metastasis of pancreatic cancer patient (stage pT3 pN1 M1). Heterogeneous samples were included to show that the developed technology can be used to screen different tissue types and (pre-)tumour stages.

Looking at the overall efficacy of each drug across all combinations (i.e., the median z-score across all conditions involving the drug; Fig. 6b), PHT-427 showed the highest efficacy for primary tumour #1 (median z-score = 0.75), but showed very little efficacy for primary tumour #2 (median z-score = 0.04), thus reproducing the strong specificity of this drug in certain pancreatic cancer samples as also observed for the cell lines. The strongest overall efficacy for primary tumour #2 was in response to Gemcitabine (median z-score = 0.41), which is indeed the standard of care in pancreatic cancer. Interestingly, the intraepithelial neoplasia was highly responsive to Oxaliplatin (median z-score = 0.72) which is also a first line treatment in pancreatic cancer. A possible explanation for this strong response is that cancer precursor lesions (PanIN) are known to be highly proliferative and Oxaliplatin targets DNA replication. The strongest overall response for the liver metastasis was observed for treatment with Gefitinib (median z-score = 0.82) which is in line with the previously observed efficacy of EGFR inhibitors in patients with advanced pancreatic cancer.

Looking at the patient-specific response to combinatorial drug treatments in more detail (Fig. 6c, d) we observed that the top hit is different for each patient (PHT-427 and Oxaliplatin for primary tumour #1, TNFa and Oxaliplatin for primary tumour #2, GDC0941 and MK-2206 for the intraepithelial neoplasia and AZD6244 and PHT-427 for liver metastasis). It is worth noticing that none of these four top combinations was effective in all samples (suggesting that they are not just generally toxic), but they were all effective in at least two samples (Fig. 6e). This suggests that they might be promising combinations for clinical applications, especially the combination of PHT-427 and Oxaliplatin, which showed strong efficacy for both primary tumour samples (z-score = 2.78 and 0.85, respectively). Overall, the two standard care treatments, Gemcitabine and Oxaliplatin, were recurrent across the top combinations, especially for primary tumours where Oxaliplatin is among the top hits for both patients. Another interesting observation is that both AKT inhibitors (MK-2206 and PHT-427) are effective in combination with different drugs across different patients (Fig. 6e). This supports the current interest in combinatorial treatments targeting AKT as druggable target downstream of KRAS in pancreatic cancer, including pre-tumour and metastasis cases.

Discussion
We describe here a fully integrated microfluidic platform enabling the fast screening of many drug combinations in cell-based assays. The very small assay volumes (~100 cells per 0.5 μL plug) allowed us to perform comprehensive screens directly on patient biopsies. Importantly, these can be done without the need for any intermediate cultivation steps, which might introduce significant cellular changes and/or the selection of individual clones. Hence our approach opens the way for comprehensive screens that have
so far been restricted to blood tumours, for which patient-derived cancer cells are available in large quantities\(^\text{[13]}\). Furthermore, the low volume and the high level of automation allow such screens to be performed cost-efficiently and fast enough to adjust therapeutic treatments in a clinical setting. Within 48 h after tumour resection or biopsy, and with consumable costs of less than US$...
150 (Supplementary Note 2), which is considerably lower than routine procedures, such as MRT scans or surgical interventions. Therefore, we envision a potential translation of this technology into clinical application, supported by our in vivo validation of best specific therapy options on mouse xenograft models.

Functional combinatorial screening has great potential in predicting personalized therapy as revealed by the data presented in this paper. Our cell line screening recapitulates the lower sensitivity of KRAS mutants (AspPC1, vs wild-type BxPC1) to PHT-427, for (the single-drug treatment, as well as across combinatorial treatments involving PHT-427). In addition, we could suggest and validate a novel and particularly strong and specific drug combination for BxPC3 when treated with PHT-427 and MK-2206. These two compounds are both AKT inhibitors, although they act through different sites, and PHT-427 additionally inhibits PDK1. Subsequently, similar efficacious specific combinations were suggested for each patient sample. Interestingly, no combination showed strong efficacy across all cell lines/patients suggesting that the tested treatment options are not just generally toxic but rather cell line/patient specific. This inter-patient heterogeneity in response to treatment highlights the importance of our approach to personalized medicine and is in agreement with previous findings, where sensitivity profiles were shown to be particularly patient specific for pancreatic cancer.

A network-based perspective can be informative when investigating the best combinatorial therapies. For example, mapping the effect of the kinase specific inhibitors on the signalling pathways can help to prevent the mechanisms of drug resistance by suggesting combinatorial therapies that act on parallel pathways. An interesting example is the combination of PHT-427 (AKT inhibitor) and AZD6244 (MEK inhibitor), which was highly effective (z-score >0.35) in three out of the four tested patient samples. These drugs in fact block two important parallel pathways which are the MEK-ERK and the PI3K-AKT pathways. Although, as far as we know, synergistic combinations targeting these pathways have been previously studied for pancreatic cancer, especially in the context of KRAS mutants (>90% of pancreatic cancer patients are KRAS mutants), this is the first evidence of potential synergistic effect between PHT-427 and AZD6244. The efficacy of this treatment also in BxPC3 cells (KRAS wild type) might suggest a more general applicability.

In this study, we prioritized the number of tested drug combinations over the possibility of testing multiple time points or multiple drug concentrations; although, we are aware that they are both important aspects that can affect cellular drug response. The choice of maximizing the number of tested combinations, rather than testing multiple time points, is in line with other recent studies focusing on large-scale screening despite the risk of missing drug combinations showing only slow effects (false negatives). Similarly screening a single, relatively low concentration only implies the risk of false negatives, but should never result in false positive hits, especially when focusing on the effects that are patient/cell line-specific rather than generally toxic. Therefore, we believe that our setup is already informative for the clinical community as it allows patient-specific prioritization of therapeutic strategies among a set of approved (or under trial) alternative. Nonetheless, the extension to time- and dose-dependent results is an important focus for future work.

Compared to other personalized approaches our procedure has specific advantages: While organoids and xenografts are particularly well-suited for mimicking three-dimensional tumour architecture and the in vivo microenvironment, respectively, the use of individualized tumour cells as shown here facilitates rapid, massively parallelized assays at low cost. In order to ensure a very physiological sample composition, we deliberately decided to avoid cell sorting based on specific markers (e.g. by FACS) and to avoid any cultivation step ahead of the screen. A similar approach has also been used by Montero and colleagues and is supported by the fact that there are many publications showing that tumour heterogeneity plays an important role and, in particular, stroma cells have a direct impact on the drug response. The presented technology could be exploited further by implementing single-cell droplet assays, which require fewer cells and also allow to investigate intratumoral heterogeneity. In addition to deriving an averaged readout over tens to hundreds of single-cell replicates per treatment option, one could quantitatively determine the number of non-responding cells for each drug cocktail. Such data would probably be very valuable to overcome therapy resistances.

Apart from using it for personalized cancer therapies, our microfluidic platform should also be of interest for further applications, such as the screening of cocktails for targeted stem cell differentiation or combinatorial chemistry. In addition, the possibility of controlling the flow in individual channels of a microfluidic platform should facilitate the construction of cheap and versatile multi-way cell- and droplet-sorters. This should pave the way for further significant developments on the technology side.

**Methods**

**Microfluidic setup.** Microfluidic chips were fabricated using standard soft-lithography methods. In brief, molds were fabricated on 4-inch silicon wafers (Siltronix) using AZ-40XT positive photoresist (Microchemicals) according to the manufacturer’s instructions. Patterning was achieved by projecting 25400 dpi photomasks (Selba) onto the photoresist using light with a wavelength of 375 nm (Suss Microtec MB3 Mask Aligner). All channels had a height of ~50 μm and widths ranging from 50 to 400 μm (400 μm for all valve sections). PDMS chips were manufactured using elastomer and curing agent in a ratio of 1:10 (Sylgard 186 elastomer kit, Dow Corning Inc) cured overnight at 65 °C. To allow for valve actuation (compression of the valve sections by the Braille pins) the PDMS chips were not bonded to glass, but rather to a thin elastic PDMS membrane. This membrane was prepared using elastomer and curing agent in a 1:20 ratio, poured on a transparency sheet and spin coated at 700 rpm (Laurell WS 650) before overnight curing. Bonding was performed in a Diener Femto Plasma Oven.

Connections to inlets and outlets were punched using 0.75 mm Harris Unicore biopsy punches. Before use, chips were flushed with Aquapel (PPG industries) from the outlet up to the T junction to render the channel surface hydrophobic. For screening, the Braille valve chip (Fig. 1a) was mounted onto an SC-9 Braille display (KGs Corporation, Japan) using an in-house holder as shown in Fig. 1b. This holder includes a Plexiglas bar with built-in screws to push the PDMS chip onto the Braille pins. The design of the chip ensures that all fluid connections (inlets and outlets) are outside the area covered by the Plexiglas bar. Movement of the Braille pins was controlled by an in-house LabVIEW program (available for free academic download at www.merten.embl.de/downloads.html), enabling to actuate all individual pins according to a pre-defined sequence (corresponding to well/assays/stock/stock). Barcodes were generated using binary combinations of Cascade Blue (16 μM and 48 μM for low and high, respectively), as detailed in Supplementary Note 3.

For all experiments the aqueous fluids were injected at a flow rate of 500 μl/h using Harvard Apparatus Syringe pumps. Based on the continuous injection mode the ab/adsorption of small molecules in PDMS is negligible as further detailed in Supplementary Note 4. AFC-40 containing 0.5% perfluoro-octonol (PFO, ABCR) and mineral oil were injected at rates of 200 μl/h and 175 μl/h. Resulting aqueous plugs were 1.5–2 mm in length and 424–560 nL in volume. The sequential generation of the 1140 plugs required ~1 h (3 per plug). Plugs were incubated for 16 h at 37 °C in a 5% CO2 atmosphere. The incubation time was chosen based on observations from kinetic experiments (in plate reader format), showing that robust results with good signal to noise ratios can be obtained for an overnight workflow.

Subsequent to incubation, the fluorescence of all plugs was determined inside PIPPI (Fig. 1d, ef) with an inner diameter of 600 μm using the optical setup shown in Supplementary Fig. 4. The flow rate used for the sequential measurement of the plugs was adjusted to match the time required for plug generation. In this way we could guarantee the same incubation time for each plug. The resulting data were analyzed using custom R-scripts (see paragraph “data processing” for details).

**Setup of the fluidic system and choice of additives and oils.** We used fluorinated oil without stabilizing surfactant as a carrier phase (only 0.5% of the anti-corrosion agent fluoro-octonol PFO was added), which turned out to have two major advantages: upon reaching the outlet, small droplets generated at the T-junction fused and formed larger plugs that completely filled the collection tubing, thus allowing to...
incubate all conditions in a sequential order. Furthermore the lack of surfactant prevented the formation of micelles, which can cause the exchange of reagents between the droplets. 

**Integration of an autosampler.** To allow for upscaling of the screens, we integrated an autosampler into our microfluidic platform\(^2\). One of the inlets of the microfluidics chip was connected to a Dionex 3000SL Autosampler, aspirating samples from 96-well plates and injecting them sequentially into a target tubing connected to an external Harvard Apparatus Syringe pump. While the resulting throughput for loading compounds from different wells is rather low (~90 s per reagent), each of them can be mixed with all of the drugs injected directly into the Braille valve chip. Therefore, the maximal throughput in terms of pairwise combinations is much higher (e.g., 9 s per combination when mixing with 10 further reagents directly connected to the Braille display chip). However, one effect had to be taken into account: the concentration of compounds coming from the autosampler varied due to dispersion of the samples in the miscible carrier phase (the buffer used in the robotic system)\(^3\). To overcome this effect, we implemented a feedback loop between the autosampler and the Braille valves: the relay signal of the autosampler was sent to the beginning of each sample from the microtiter plates to the waste, using the two Braille valves controlling the relevant inlet on the microfluidic chip. This process allowed to overcome sample dispersion and is illustrated in Supplementary Fig. 2. This way only the centre part of each sample segment, showing constant concentration, was mixed with the drugs injected into the Braille display chip. To verify this process, we mixed fluorophores stored in a 96-well plate with fluorophores injected directly into the autosampler and measured the resulting fluorescence signal of the droplets (Supplementary Fig. 3). All combinations showed the expected signal intensities, confirming the feasibility of the approach if a sufficient amount of cells is available for screening.

**Single cell suspension from pancreatic primary tumours.** Primary tumour samples were obtained from routine resections from patients who signed an informed consent approved by the EMBL Bioethics Internal Advisory Committee. Informed consent was obtained by the patients for research use of the samples. A viable single cell suspension was obtained from the patients for research use of the samples. A viable single cell suspension was maintained at low temperature using ice and constantly stirred.

**Preparation of cells for microfluidic experiments.** AsPC1 and BxPC3 cell lines were obtained from ATCC for this study and were tested negative for mycoplasma contamination using the MycoAlertTM PLUS Mycoplasma Detection Kit from Lonza. Both cell lines were cultured using the recommended media RPMI-1640 (Gibco, Life Technologies) and diluted according to the standard protocol. To a final concentration of 8 × 10^5 viable cells/ml (counted with trypan blue exclusion method using a BioRad cell counter). Quantity of 15 μg/ml of the fluorescent dye Alexa Fluor 594 (ThermoFisher, #A33082) were added and the solution was centrifuged at 250 g for 5 min. Supernatant was then removed and 2 ml of 0.05% trypsin-EDTA (Gibco, Life Technologies) were added and the solution was incubated for 5 more minutes at 37 °C. Subsequently 15 ml of DMEM supplemented with 10% fetal bovine serum were added and the solution was centrifuged again for 5 min at 250 g. After removing the supernatant, the pellet was resuspended in FreeStyle medium (ThermoFisher).

For validation experiments in 96 well plates, compounds were instead prepared in 5 consecutive fivefold dilutions (25, 5, 1, 0.2, 0.004 μM). The compound dispensed to the 384-well plate (Z-DEVD)-RII10 was purchased from Biomol (#ABD-13430). Volume of 3 ml of substrate working solution were prepared by adding: 2400 μl 5X Reaction buffer (20 ml of 50 mM PIPES, pH 7.4, 10 mM EDTA, 0.5% CHAPS), 60 μl DTT (1 M), 540 μl dH₂O and 44 μl Z-DEVD-RII10 substrate (5 μM). Caspase-3 activity is a marker of apoptosis and is a good early marker of cell viability (Supplementary Fig. 1f).

**Data processing.** Data were acquired using an in-house LabVIEW program (available for free academic download at www.merten.embl.de/downloads.html) allowing fluorescence detection in three separate channels (i.e. blue = fluorescence barcodes, green = Caspase-3 activity and orange marker dye to monitor mixing of reagents) as exemplified in Fig. 2b. Peaks in each of the three channels were identified by defining an empirical threshold both on the intensity and the duration of the measured signal in order to distinguish real peaks from background noise. Peaks in the blue channel (corresponding to the barcode plugs) were detected and used to separate the different conditions consisting of sequences of peaks (replicates) in the green channel. Conditions with multiple peaks having either very low or very high orange signals (e.g., due to occasional cell clogging) were manually inspected. P-values were computed separately for each run, FDR-corrected for multiple hypotheses testing and combined across different runs using Fisher’s method. Significance and z-score for all data shown in the manuscript is illustrated in Supplementary Fig. 11.

**Xenograft mouse models.** Xenograft mouse experiments were performed by an external company (EPO Berlin). All animal experiments were carried out in accordance to the German Animal Welfare Act as well as the UKCCCR (United Kingdom Coordinating Committee on Cancer Research). The respective pancreatic carcinoma cell suspensions of the human AsPC1 or BxPC3 cells were injected subcutaneously into the flank of anaesthetized female Rj:NMRI-Foxn1 nu/nu mice from Janvier. The age of the animals was 6–8 weeks. Tumours were allowed to establish a palpable size (about 0.1 cm²), before the treatment was started. A total of 40 mice were grouped in the animals in four groups for each cell line (five animals per group). Mice in the four groups were treated respectively with: 1. Gemcitabine (100 mg/kg i.p. once a week in PBS); 2. combination of Gefitinib (75 mg/kg p.o. sequence days 0, 2, 4, 6, 8, 10, 12 in 0.5% Polysorbate) and ACHP (8 mg/kg i.v. q4d—day 0, 4, 8, 12 in 0.9% NaCl); 3. combination of MK2206 (120 mg/kg p.o. sequence days 0, 2, 4, 6, 8, 10) and PHT-427 (200 mg/kg p.o. twice a day in 40–50 mg/ml Sesamol); 4. vehicle p.o. alone. During the study tumour volumes were measured in two dimensions with a caliper. Tumour volumes (TV) were calculated by the formula: TV = (length × width)^2 × 0.5. During the study mice were maintained under sterile and controlled conditions (22 ± 5°C, 50% relative humidity, 12 h light–dark cycle, autoclaved food and bedding, acidified drinking water) and monitored for body weight and health condition.

Treatment with MK2206 + PHT-427 was interrupted for both AsPC1 and BxPC (at day 5 and 8, respectively) due to massive body weight loss for AsPC1 mice, and then continued until day 13. A total of 40 mice were used for each group of the animals in four groups for each cell line (five animals per group). Mice in the four groups were treated respectively with: 1. Gemcitabine (100 mg/kg i.p. once a week in PBS); 2. combination of Gefitinib (75 mg/kg p.o. sequence days 0, 2, 4, 6, 8, 10, 12 in 0.5% Polysorbate) and ACHP (8 mg/kg i.v. q4d—day 0, 4, 8, 12 in 0.9% NaCl); 3. combination of MK2206 (120 mg/kg p.o. sequence days 0, 2, 4, 6, 8, 10) and PHT-427 (200 mg/kg p.o. twice a day in 40–50 mg/ml Sesamol); 4. vehicle p.o. alone. During the study tumour volumes were measured in two dimensions with a caliper. Tumour volumes (TV) were calculated by the formula: TV = (length × width)^2 × 0.5. During the study mice were maintained under sterile and controlled conditions (22 ± 5°C, 50% relative humidity, 12 h light–dark cycle, autoclaved food and bedding, acidified drinking water) and monitored for body weight and health condition.

**Preparation of drugs and Caspase-3 substrate.** Cyt387 (#52219), PHT-472 (#51536), MK-2206 (#51078), DCD9341 (#51065), Gefitinib (#51025), Oxaliplatin (#51220), D6246 (#51228) and Gemcitabine (#51440) were purchased from Selleckchem. ACHP (#4547) was purchased from Tocris. All compounds were diluted in DMSO to a 20 mM stock solution. When preparing the syringes for the microfluidic experiment, compounds were further diluted in FreeStyle medium to a final concentration of 5 μM in the plugs. Tumour Necrosis Factor-α (TNF) (#PH33015) was purchased from Life Technologies and diluted in FreeStyle medium according to the manufacturer’s instruction to a 10 μg/ml stock solution. It was further diluted in FreeStyle medium for microfluidic experiments to a final concentration of 5 μg/ml.

**Data availability.** The microfluidics-based drug screening data referenced during the study are available in Zenodo https://doi.org/10.5281/zenodo.1248886. The standard protocol to analyze the data by students is published as Supplementary Protocol 1. All other data supporting the findings of this study are available within the article and its Supplementary Information Files and from the corresponding author upon reasonable request.
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18. Ma, W.-Y. et al. A novel 96-well-formatted micro-gap plate enabling drug screening on cells, Jan Korbel and Denes Turei for critical reading of the manuscript. F.E. designed and performed experiments, performed the computational analysis under the supervision of J.S.-R. and shaped the overall study design. R.U. designed and performed experiments, Jan Korbel and Denes Turei for critical reading of the manuscript. F.E. thanks the European Molecular Biology Laboratory Interdisciplinary Post-Docs (EMBL EIPOD) and Marie Curie Actions (COFUND) for funding.

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Author contributions

F.E. designed and performed experiments, performed the computational analysis under the supervision of J.S.-R. and shaped the overall study design. R.U. designed and performed experiments and developed the microfluidic platform under the supervision of C. A.M. D.M. performed experiments. U.P.N. supervised the surgical specimen collection and provided clinical patient data. T.C. provided clinical samples and contributed to the design of the study. T.L. provided patho-histological patient data. J.S.-R. and C.A.M. conceived the project, designed experiments and supervised the study. F.E., J.S.-R. and C.A.M. interpreted the results and contributed to manuscript development. All authors approved the final manuscript.

Additional information

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Competing interests: C.A.M. and R.U. are inventors on patent applications covering parts of the technology described here. All the remaining authors declare no competing interests.
