

Ex vivo Drug Sensitivity Imaging-based Platform for Primary Acute Lymphoblastic Leukemia Cells

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Abstract

Resistance of acute lymphoblastic leukemia (ALL) cells to chemotherapy, whether present at diagnosis or acquired during treatment, is a major cause of treatment failure. Primary ALL cells are accessible for drug sensitivity testing at the time of new diagnosis or at relapse, but there are major limitations with current methods for determining drug sensitivity *ex vivo*. Here, we describe a functional precision medicine method using a fluorescence imaging platform to test drug sensitivity profiles of primary ALL cells. Leukemia cells are co-cultured with mesenchymal stromal cells and tested with a panel of 40 anti-leukemia drugs to determine individual patterns of drug resistance and sensitivity ("pharmacotype"). This imaging-based pharmacotyping assay addresses the limitations of prior *ex vivo* drug sensitivity methods by automating data analysis to produce high-throughput data while requiring fewer cells and significantly decreasing the labor-intensive time required to conduct the assay. The integration of drug sensitivity data with genomic profiling provides a basis for rational genomics-guided precision medicine.

Key features

- Analysis of primary acute lymphoblastic leukemia (ALL) blasts obtained at diagnosis from bone marrow aspirate or peripheral blood.
- Experiments are performed *ex vivo* with mesenchymal stromal cell co-culture and require four days to complete.
- This fluorescence imaging-based protocol enhances previous *ex vivo* drug sensitivity assays and improves efficiency by requiring fewer primary cells while increasing the number of drugs tested to 40.
- It takes approximately 2–3 h for sample preparation and processing and a 1.5-hour imaging time.

Keywords: Ex vivo drug sensitivity, Functional precision medicine, Acute lymphoblastic leukemia, Pharmacogenomics, Pharmacotyping, Fluorescence imaging

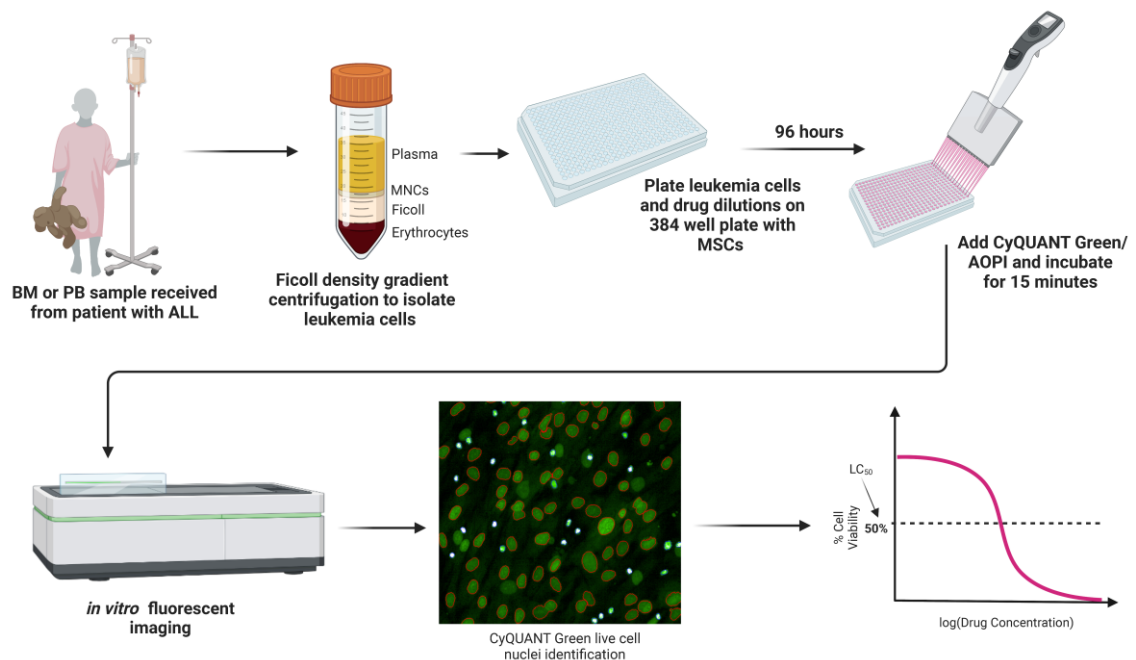
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Graphical overview



BM: bone marrow; PB: peripheral blood; ALL: acute lymphoblastic leukemia; MNCs: mononuclear cells, which include leukemia cells when present; MSCs: mesenchymal stromal cells; LC₅₀: drug concentration that kills 50% of the leukemia cells

Background

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease that can be grouped into multiple B- and T-lineage genomic subtypes (Inaba and Mullighan, 2020; Inaba and Pui, 2021; Brady et al., 2022). While five-year survival rates for many pediatric B-cell ALL (B-ALL) subtypes can exceed 90%, survival rates are lower for adults who have more treatment-resistant B-cell ALL, particularly those with Ph-like ALL (Foà et al., 2011). Similarly, survival for certain subtypes of T-cell ALL (T-ALL) remains below 80% (Karrman and Johansson, 2017) and outcomes following relapse for both pediatric and adult patients with ALL remain dismal (Pocock et al., 2021; Laukkanen et al., 2022; Wudhikarn et al., 2022). Because of its high incidence, ALL remains the most common cause of death from childhood cancer. Treatment efficacy in ALL varies by molecular subtype and secondary genomic alterations (Pui et al., 2012; Brady et al., 2022). Progress in understanding the heterogeneity of the genomics of ALL has provided insight into actionable drug targets (Meyer et al., 2013; Irving et al., 2014; Khaw et al., 2016; Gocho et al., 2021; Brady et al., 2022). Molecularly targeted agents have shown efficacy in some subtypes of high-risk B-ALL disease. There is a need for further development of molecularly targeted therapy optimized based on characteristics of an individual patient's cancer cells (Carroll et al., 2003; Brady et al., 2022; Lee et al., 2023).

Functional precision medicine approaches combine direct drug sensitivity profiling (i.e., pharmacotyping) with genomic testing to elucidate the biological basis of variability in treatment response (Frismantas et al., 2017; Kornauth et al., 2022; Lee et al., 2023). Defining inter-patient variability in leukemia cell drug sensitivity is a starting point for observing patterns of sensitivity to antileukemia agents. Drug sensitivity profile groups have been associated with treatment outcome, including event-free survival and cumulative risk of relapse (Holleman et al., 2004; Pieters et al., 2007; Lee et al., 2023). ALL drug sensitivity phenotype has likewise been associated with clinical characteristics and somatic genomic features (Pieters et al., 1993; Ramakers-van Woerden et al., 2002; Holleman et al., 2004; Ramakers-van Woerden et al., 2004; Lugthart et al., 2005; Pui et al., 2019). Pharmacotyping

profiles have the potential to drive research to uncover new subtype-specific therapeutic opportunities for ALL. Both flow cytometry-based and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT)-based drug-resistance assays have served as reliable methods in our laboratory for quantification of B- and T-ALL leukemia cell viability upon exposure to antineoplastic agents (Holleman et al., 2004; Paugh et al., 2015; Autry et al., 2020; Gocho et al., 2021; Lee et al., 2023). We have used flow cytometry-based methods and MTT-based methods for sensitivity testing of leukemia cell lines, patient-derived xenografts (PDX), and primary patient samples; however, in our experience, these assays are markedly limited by requirements for high cell count at plating, prolonged time requirements to complete the assay and data analyses, and lack of high-throughput potential for screening of antineoplastic drugs against samples (Gocho et al., 2021). Ex vivo models have used human bone marrow mesenchymal stromal cells (MSC) to support leukemia cells in culture for functional experiments (Boutter et al., 2014). Other groups have published functional precision medicine methods using automated microscopic imaging to test drug sensitivity of PDX cells from clinically relevant ALL subgroups (Frismantas et al., 2017). Here, our group has established an ex vivo pharmacotyping platform to directly profile leukemia cells from individual patients for drug sensitivity using high-content imaging. Coupling data derived from this platform with patient genomic profiles has the potential to catapult the development of new ALL therapy using actionable target and biomarker discovery.

Here, we present our methodology for a high-throughput imaging-based assay for leukemia cell viability to identify effective cytotoxic and targeted anti-leukemia agents. Using a biologically realistic leukemia cell and MSC co-culture as described by other groups (Frismantas et al., 2017), our imaging system has produced high-throughput viability data with potential for further image analyses. This method is used to assess inter-patient differences in drug sensitivity related to patient age, sex, molecular subtypes of leukemia, and genomics, and to discover mechanisms of drug resistance in patients with ALL (Autry et al., 2020; Gocho et al., 2021; Lee et al., 2023). This knowledge may in turn be used to build more robust precision medicine approaches to improve cure rates, and to create future avenues to facilitate the rational use of molecularly targeted therapy. This protocol includes methods for this imaging-based functional precision medicine assay.

Materials and reagents

Biological materials

1. hTERT immortalized human bone marrow mesenchymal stromal cells (MSCs) (Applied Biological Materials, Inc., catalog number: T0523)

Reagents

1. AIM-V medium, 1,000 mL (Gibco, catalog number: 12055-083)
2. Hydrocortisone-water soluble (Sigma, catalog number: H0396)
3. RPMI 1640 medium, 500 mL (Gibco, catalog number: 11875-093)
4. Fetal bovine serum, certified, heat inactivated (Gibco, catalog number: 10082-147)
5. DMSO (Sigma, catalog number: D2660-100ML)
6. PBS, pH 7.4, 500 mL (Gibco, catalog number: 10010-023)
7. CyQUANT™ Direct Cell Proliferation Assay Green (Invitrogen, catalog number: C35012)
8. ViaStain AOPI staining solution (Nexcelom, catalog number: CS2-0106-25mL)
9. Trypsin-EDTA 0.25% (Gibco, catalog number: 25200-056)
10. Antibiotic antimycotic 100× (Gibco, catalog number: 15240-062)
11. RPMI 1640 without phenol red & L-glutamine (Lonza, catalog number: 12-918F)
12. 0.5 M EDTA pH 8.0 (Gibco, catalog number: 15575-038)
13. 0.9% sodium chloride (Baxter, catalog number: 2F7123)
14. Insulin-transferrin-selenium 100× (Gibco, catalog number: 41400045)
15. L-Glutamine 200 mM (Gibco, catalog number: A2916801)

16. 0.1 N sodium hydroxide (Fisher Scientific, catalog number: AC124190010)
17. Ambion™ nuclease-free water (Invitrogen, catalog number: AM9937)
18. Ultrapure (Type 1) water
19. Drug stock solutions (Table 1)

Table 1. Drug stock solutions

#	Name	Vendor	Catalog number	Stock concentration	Storage	Diluent	Concentration range	Dilution factor
1	Trametinib	MedChemExpress	HY-10999	10 mM	-80 °C	DMSO	0.01–1,000 nM	10
2	Gilteritinib	MedChemExpress	HY-12432	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
3	Venetoclax	MedChemExpress	HY-15531	10 mM	-80 °C	DMSO	0.001–100 nM	10
4	Inotuzumab	Pfizer	NA	0.25 mg/mL	-80 °C	Nuclease-free water	0.0025–250 ng/mL	10
5	Palbociclib	MedChemExpress	HY-50767A	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
6	CHZ868	MedChemExpress	HY-18960	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
7	Dasatinib	Selleckchem	S1021	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
8	Ibrutinib	MedChemExpress	HY-10997	50 mM	-80 °C	DMSO	1.5625–50 mM	2
9	6-mercaptopurine	Acros Organics	226520050	5 mg/mL	-20 °C	0.1 N NaOH	91.8–2,938 mM	2
10	Prednisolone	Pfizer	00009004722	12.5 mg/mL	-20 °C	0.9% NaCl	0.015–503.5 mM	8
11	L-asparaginase	BioVendor	RP1792872500	500 IU/mL	-80 °C	Type 1 water	0.0032–10 IU/mL	5
12	Vincristine	Hospira	61703030906	0.25 mg/mL	-20 °C	Type 1 water	0.0017–54.169 mM	8
13	Nelarabine	Sigma-Aldrich	SML1736	200 mM	-20 °C	DMSO	1.03–250 mM	3
14	Bortezomib	St. Jude Compound Management	SJ000518968	10 mM	-80 °C	DMSO	0.98–1,000 nM	4
15	Vorinostat	Selleckchem	S1047	100 mM	-80 °C	DMSO	102.88–25,000 nM	3
16	Daunorubicin	Teva	00703523313	0.1 mg/mL	-20 °C	Type 1 water	0.004–3.55 mM	4
17	Luminespib	MedChemExpress	HY-10215	10 mM	-80 °C	DMSO	0.01–1,000 nM	10
18	Clofarabine	MedChemExpress	HY-A0005	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
19	Olaparib	MedChemExpress	HY-10162	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
20	Selinexor	Selleckchem	S7252	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
21	Ponatinib	MedChemExpress	HY-12047	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
22	Saracatinib	MedChemExpress	HY-10234	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
23	Acalabrutinib	MedChemExpress	HY-17600	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
24	Momelotinib	MedChemExpress	HY-10961	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
25	Midostaurin	MedChemExpress	HY-10230	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
26	Fostamatinib	MedChemExpress	HY-13038A	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
27	Larotrectinib	MedChemExpress	HY-12866	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
28	Repotrectinib	MedChemExpress	HY-103022	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
29	Sorafenib	MedChemExpress	HY-10201	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
30	Everolimus	MedChemExpress	HY-10218	10 mM	-80 °C	DMSO	0.1–10,000 nM	10

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31	Sotorasib (AMG-510)	MedChemExpress	HY-114277	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
32	Birinapant	MedChemExpress	HY-16591	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
33	SNDX-5613	MedChemExpress	HY-136175	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
34	Iadademstat	MedChemExpress	HY-12782T	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
35	EPZ4777 (EPZ004777)	MedChemExpress	HY-15227	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
36	JQ1	MedChemExpress	HY-13030	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
37	Eprentapopt (APR-246)	MedChemExpress	HY-19980	10 mM	-80 °C	DMSO	0.1–10,000 nM	10
38	S63845	MedChemExpress	HY-100741	10 mM	-80 °C	DMSO	0.001–100 nM	10
39	Navitoclax	MedChemExpress	HY-10087	10 mM	-80 °C	DMSO	0.01–1,000 nM	10
40	Carfilzomib	MedChemExpress	HY-10455	10 mM	-80 °C	DMSO	0.0001–10 nM	10

Solutions

- 1 mM hydrocortisone stock solution (see Recipes)
- Cell medium (see Recipes)
- MSC medium (see Recipes)

Recipes

1. 1 mM hydrocortisone stock solution

Reagent	Final concentration	Amount
Hydrocortisone-water soluble	1 mM	100 mg
Type 1 water	n/a	23.45 mL
Total	n/a	23.45 mL

2. Cell medium

Reagent	Final concentration	Amount
RPMI w/o phenol red & L-glutamine	n/a	500 mL
Antibiotic antimycotic	1×	6.25 mL
L-Glutamine	2 mM	6.25 mL
Insulin-transferrin-selenium	1×	6.25 mL
Heat-inactivated fetal bovine serum	20%	125 mL
Total	n/a	643.75 mL

3. MSC medium

Reagent	Final concentration	Amount
1 mM hydrocortisone-water soluble	1 μ M	0.625 mL
RPMI-1640	n/a	500 mL
Heat-inactivated fetal bovine serum	20%	125 mL
Total	n/a	625.625 mL

Laboratory supplies

- 384-well plates (PerkinElmer, Phenoplate, catalog number: 6057302)
- 10 mL pipettes (Corning, catalog number: 4101)
- 25 mL pipettes (Corning, catalog number: 4237)
- 15 mL conical tubes (Falcon, catalog number: 352097)
- 50 mL conical tubes (Falcon, catalog number: 352098)
- Microtubes 1.5 mL (Axygen, catalog number: MCT-150-L-C)
- Microtubes 0.6 mL (Axygen, catalog number: MCT-060-L-C)
- Stericup quick release Millipore express 0.22 μ M PES 500 mL (Millipore, catalog number: 6346)
- 10 cm dishes (Corning, catalog number: 353003)
- 20 μ L filter tips (Rainin, catalog number: 30389225)
- 200 μ L filter tips (Rainin, catalog number: 30389239)
- 1,000 μ L filter tips (Rainin, catalog number: 30389212)
- 125 μ L tips XYZ rack of 384 griptips (Integra, catalog number: 6465)
- 125 μ L tips ECO rack of 384 griptips (Integra, catalog number: 3425)
- 150 mL reagent reservoirs (Integra, catalog number: 6318)

Equipment

1. Operetta CLS™ high-content analysis system (PerkinElmer, HH1600000)
2. STR4 plus series centrifuge (Thermo Scientific, catalog number: 75016037)
3. BX41 microscope (Olympus, catalog number: BX41-PH-B)
4. Tuberoller (Benchmark, catalog number: R3010)
5. Nikon Eclipse TS100 microscope (Nikon, catalog number: NI-TS100)
6. IsoTemp digital controlled water bath (Fisher Scientific, model: 2320)
7. TC10 automated cell counter (Bio-Rad, catalog number: 145-0010)
8. Sorvall Legend X1R centrifuge (Thermo Scientific, catalog number: 75004260)
9. Heracell 150i CO₂ incubator (Thermo Scientific, catalog number: 51032719)
10. VIAFLO 384 automated pipette (Integra, catalog number: 6031)
11. VIAFLO 384-well 5–125 µL pipette head (Integra, catalog number: 6132)
12. VOYAGER 12-channel 5–125 µL pipette (Integra, catalog number: 4732)
13. 1300 Series Class II A2 biological safety cabinet (Thermo Scientific, catalog number: 1323TS)

Software

1. Harmony software (4.9, 2019)
2. Prism v9.3 (GraphPad, 11/15/2021)
3. Microsoft office suite

Procedure

Procedure overview

Note: AOPI staining in the drug treatment wells as well as in the quality control wells would allow direct determination of cell death; however, the method described herein uses the more cost-efficient CyQUANT stain in the drug treatment wells to keep the assay costs down.

Drug response of primary human leukemia cells is evaluated using a co-culture system and a fluorescence imaging-based assay, building on a previously published assay (Boutter et al., 2014) with adaptations as described herein. hTERT immortalized MSCs are first seeded in a 384-well plate. After 24 h, leukemia cells are added to the stromal cell layer in AIM-V medium at a ratio of 1:10 MSCs to leukemia cells, along with a drug solution prepared in the same medium at six drug concentrations in duplicate. After a 96-hour incubation, images are acquired with a 20× objective, and the total number of live leukemia cells is evaluated. The leukemia cells and MSCs are analyzed in the same fluorescence channel based on the size and intensity of the nuclei. Drug-induced inhibition at each concentration is estimated by comparing to leukemia cells treated with the vehicle alone. The drug concentration that kills 50% of the leukemia cells (LC50) is determined using a dose-response model and is used as a measurement of drug sensitivity. Quality control is performed to remove cases with low viability (defined as less than 500 viable blast cells in each well in the absence of drugs on day 4).

Study approval

This study was approved by the respective institutional review boards at St. Jude Children's Research Hospital, MD Anderson Cancer Center, and University of Chicago, and written informed consent was obtained from parents, guardians, and/or patients, as appropriate, before enrollment, in accordance with the Declaration of Helsinki.

Items to prepare in advance

To help with the following procedure, it is advised to prepare MSC medium and cell medium before starting. It is also recommended to prepare drug stocks to the concentrations stated in Table 1 or other concentrations desired.

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MSC cell culture procedure

1. Grow and expand the MSC cells. MSC cells are cultured in MSC medium as described in Recipes.
2. Store MSC cells in incubator at 37 °C.
3. Twenty-four hours before receipt of a fresh primary sample, prepare MSC plate as below.
4. Remove a 10 cm dish of MSC from the incubator. Aspirate old medium.
5. Add 5 mL of PBS to the MSC dish. Rinse the monolayer by gently rocking the dish.
6. Aspirate all PBS from dish.
7. Slowly add 1 mL of trypsin-EDTA to the dish. Spread evenly by gently rocking the dish.
8. Incubate dish for 3 min in incubator (37 °C) until cells release from plate.
9. Microscopically verify that cells have released from the plate. Cells should be rounded and move when the dish is gently tapped.
10. Add 9 mL of MSC medium to the dish. Resuspend cells and medium 3–5 times, then transfer the suspension to a 15 mL (or 50 mL) Falcon tube.
11. Centrifuge at 300× g for 5 min at 4 °C.
12. While cells are spinning, prepare a 384-well plate by adding 50 µL of PBS per well to outside border wells.
13. Remove cells from centrifuge and aspirate the supernatant.
14. Resuspend cells in 5–10 mL of MSC medium.
15. Aliquot 10 µL of cells and count cells using an automated cell counter.
16. Calculate volume of MSC medium needed to dilute to a 1×10^5 cells/mL cell density.
17. Add appropriate volume of MSC medium to the Falcon tube containing cells.
18. Transfer 25 µL of the MSC suspension per well ($= 2.5 \times 10^3$ cells/well) using a multichannel pipette.
19. Place plate in incubator and incubate overnight (37 °C, 5% CO₂).

Notes before starting

1. AIM-V media that will be used for MSC plate washing and sample preparation should be warmed to 37 °C prior to use.

Primary sample procedure

Note: This method is for fresh cells processed within 24 h of collection. If the sample will not be plated on day of collection, keep cells rotating at 4 °C until ready to do so.

1. Leukemia blasts are obtained from either bone marrow or peripheral blood samples after undergoing Ficoll gradient centrifugation. The readers are referred to the Methods of the following references: Gocho et al. (2021), Lee et al. (2023).
2. To proceed to testing with the pharmacotyping assay, the sample must meet the following parameters: viability above 80%, red blood cell (RBC) contamination below 30%, and a blast percentage above 85%.
3. If the sample has more than 30% RBC contamination but above 85% blasts, the RBCs are removed from the sample through RBC depletion using an institutional procedure (see Supplemental Methods).
4. If the sample has a blast percentage of less than 85%, the sample is enriched for leukemia by using magnetic cell separation (Miltenyi Biotec) with a negative selection for leukemia cells using an institutional procedure (see Supplemental Methods).
5. Keep primary sample suspended in cell medium and rotating at 4 °C until ready to proceed.

Day 0 cell culture procedure

Note: While this protocol is designed for primary patient samples, it can be adapted for experiments with cell lines.

1. Prepare drug dilutions in AIM-V medium to achieve the final concentration ranges listed in Table 1.
Note: AIM-V media does not need to be warmed to 37 °C for drug plate preparation.
2. Remove the 384-well imaging plate containing MSCs from the incubator.
3. Aspirate the 25 µL of MSC medium from each well using the VIAFLO 384 automated pipette with a 384-channel head on a speed setting of 4 or lower to prevent MSC detachment.

Note: Automated pipettes are not required for aspiration steps described in this procedure; however, if done manually, make sure to remove media slowly and without touching the bottom of the well to prevent MSC removal.

4. Wash each well by adding 40 μ L of AIM-V medium.
5. Centrifuge primary sample at 300 \times g for 5 min at 4 $^{\circ}$ C.
6. Aspirate cell medium of primary cells and flick pellet to resuspend cells.
7. Resuspend primary patient samples at 6.25×10^5 cells/mL in AIM-V medium for plating in the 384-well MSC plate.
8. Aspirate the AIM-V medium from each well using the VIAFLO 384 automated pipette on a speed setting of 4 or lower.
9. Add 40 μ L of AIM-V medium to the MSC-only control wells.
10. Add 40 μ L of the cell suspension to every well except the MSC-only control wells.
11. Add 10 μ L of the drug dilutions to the designated drug wells (Figure 1).

A1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
B	Blast QC	Control	Drug 1	Drug 1	Drug 2	Drug 2	Drug 3	Drug 3	Drug 4	Drug 4	Drug 5	Drug 5	Drug 6	Drug 6	Drug 7	Drug 7	Drug 8	Drug 8	Drug 9	Drug 9	Drug 10	Drug 10	
C	Blast QC	Control	Drug 1	Drug 1	Drug 2	Drug 2	Drug 3	Drug 3	Drug 4	Drug 4	Drug 5	Drug 5	Drug 6	Drug 6	Drug 7	Drug 7	Drug 8	Drug 8	Drug 9	Drug 9	Drug 10	Drug 10	
D	Blast QC	Control	Drug 1	Drug 1	Drug 2	Drug 2	Drug 3	Drug 3	Drug 4	Drug 4	Drug 5	Drug 5	Drug 6	Drug 6	Drug 7	Drug 7	Drug 8	Drug 8	Drug 9	Drug 9	Drug 10	Drug 10	
E	Blast QC	Control	Drug 1	Drug 1	Drug 2	Drug 2	Drug 3	Drug 3	Drug 4	Drug 4	Drug 5	Drug 5	Drug 6	Drug 6	Drug 7	Drug 7	Drug 8	Drug 8	Drug 9	Drug 9	Drug 10	Drug 10	
F	Blast QC	AOPI	Drug 1	Drug 1	Drug 2	Drug 2	Drug 3	Drug 3	Drug 4	Drug 4	Drug 5	Drug 5	Drug 6	Drug 6	Drug 7	Drug 7	Drug 8	Drug 8	Drug 9	Drug 9	Drug 10	Drug 10	
G	Blast QC	MSC	Drug 1	Drug 1	Drug 2	Drug 2	Drug 3	Drug 3	Drug 4	Drug 4	Drug 5	Drug 5	Drug 6	Drug 6	Drug 7	Drug 7	Drug 8	Drug 8	Drug 9	Drug 9	Drug 10	Drug 10	
H																							
I																							
J	Blast QC	Control	Drug 11	Drug 11	Drug 12	Drug 12	Drug 13	Drug 13	Drug 14	Drug 14	Drug 15	Drug 15	Drug 16	Drug 16	Drug 17	Drug 17	Drug 18	Drug 18	Drug 19	Drug 19	Drug 20	Drug 20	
K	Blast QC	Control	Drug 11	Drug 11	Drug 12	Drug 12	Drug 13	Drug 13	Drug 14	Drug 14	Drug 15	Drug 15	Drug 16	Drug 16	Drug 17	Drug 17	Drug 18	Drug 18	Drug 19	Drug 19	Drug 20	Drug 20	
L	Blast QC	Control	Drug 11	Drug 11	Drug 12	Drug 12	Drug 13	Drug 13	Drug 14	Drug 14	Drug 15	Drug 15	Drug 16	Drug 16	Drug 17	Drug 17	Drug 18	Drug 18	Drug 19	Drug 19	Drug 20	Drug 20	
M	Blast QC	Control	Drug 11	Drug 11	Drug 12	Drug 12	Drug 13	Drug 13	Drug 14	Drug 14	Drug 15	Drug 15	Drug 16	Drug 16	Drug 17	Drug 17	Drug 18	Drug 18	Drug 19	Drug 19	Drug 20	Drug 20	
N	Blast QC	AOPI	Drug 11	Drug 11	Drug 12	Drug 12	Drug 13	Drug 13	Drug 14	Drug 14	Drug 15	Drug 15	Drug 16	Drug 16	Drug 17	Drug 17	Drug 18	Drug 18	Drug 19	Drug 19	Drug 20	Drug 20	
O	Blast QC	MSC	Drug 11	Drug 11	Drug 12	Drug 12	Drug 13	Drug 13	Drug 14	Drug 14	Drug 15	Drug 15	Drug 16	Drug 16	Drug 17	Drug 17	Drug 18	Drug 18	Drug 19	Drug 19	Drug 20	Drug 20	
P																							

Figure 1. Example plate layout

12. Add 10 μ L of AIM-V medium to wells in Columns 2 and 3 in Figure 1.
13. Incubate the plate at 37 $^{\circ}$ C (5% CO₂) for 96 h for primary patient samples.

Day 4 procedure

Note: Stain each plate individually so that incubation times are ~15 min, to reduce the chance of MSCs detaching from the plate; longer (> 1 h) incubation times are associated with detachment of MSCs, which may cause an experiment to fail.

1. Dilute CyQUANT Direct nucleic acid stain 1:26 and the CyQUANT Direct background suppressor 1:5.2 in PBS.
Note: Add the suppressor to the PBS first, because the nucleic acid stain will separate out of solution if added to PBS without the suppressor.
2. Add 5 μ L of the CyQUANT staining solution to each well except AOPI control well.

Note: Verify visually that CyQuant was added to every well before moving to the next step. This can be done by checking the color of the well.

3. Add 50 μ L of AOPI staining solution to the AOPI control well for viability analysis.
 4. If desired, measure blast percentage of samples in Blast QC wells using Cytospin or institutional procedure.
 5. Incubate the plate at 37 °C (5% CO₂) for 15 min.
- Critical step: Prolonged incubation with CyQuant can lead to cell apoptosis, which can influence the results of the assay.*

The CyQUANT imaging procedure

Note: The imaging procedure described here applies specifically to the Operetta CLS; however, imaging can be performed with any imager that can acquire images in the green channel from a 384-well plate.

1. Remove imaging plate from incubator.
2. Spray a Kimwipe with 70% alcohol and wipe the bottom of the plate, as dust particles on the bottom of the plate may cause autofocusing errors during imaging.
3. Click the Eject button in Harmony software.
4. Properly position the plate in the plate holder of the imager.
5. Click the Load Plate button in the Harmony software.
6. Click the Setup tab in the Harmony software.
7. Click New in Experiment window to create a new experiment.
8. Use the following settings for the experiment:
 - a. Plate Type: 384 PerkinElmer CellCarrier Ultra
 - b. Autofocus: Two Peak (Default)
 - c. Objective: 20 \times Air, NA 0.4
 - d. Opt. Mode: Confocal
 - e. Binning: 2
9. In the Channel Selection window, add the Brightfield and CyQuant Green channels with the following settings:

Note: CyQuant Green may not be a pre-provided channel. To create the CyQuant Green channel, use wavelengths 460–490 for excitation and 500–550 for emission. Green Fluorescent Protein (eGFP) has similar excitation and emission wavelengths.

 - a. Brightfield:
 - i. Time = 20 ms
 - ii. Power = 60%
 - iii. Height = 8.0 μ M
 - b. CyQuant Green:
 - i. Time = 60 ms
 - ii. Power = 100%
 - iii. Height = 8.0 μ M
10. In the Navigation window, under the define layout tab, highlight all the wells that will be imaged and click Select. Within the Well window, highlight the nine inner squares, which correspond to the number and position of images being taken per well, and click Select. If the well/field of view has only been highlighted, it will be orange, but if it has been selected, it will be grey.
11. To take a snapshot: highlight one of the control wells and a single field of view. Then, within the Channel Selection window, click Snapshot in any of the channels to take one image to check if the leukemia cells are in focus. If cells are not in focus, change the heights for CyQuant Green and Brightfield channels until the cells are in focus (Figure 2).

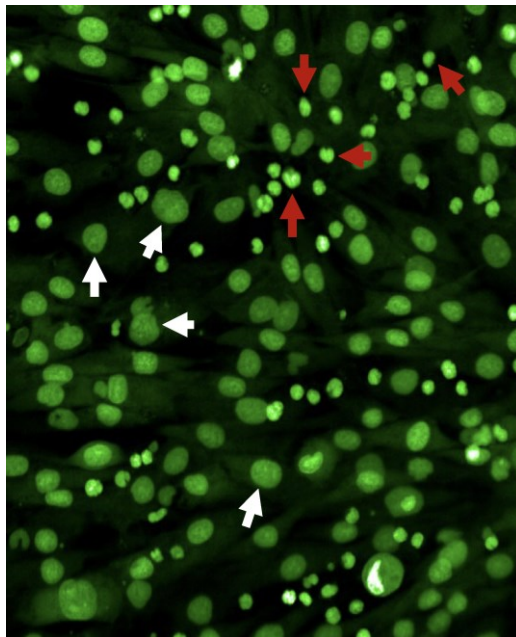


Figure 2. Fluorescence imaging using a 20× objective of a primary patient leukemia sample overlaid with mesenchymal stromal cells (MSCs) stained with CyQUANT Green. White arrows indicate MSCs, and red arrows indicate leukemia cells.

12. Save the experiment.

Note: Once experiments have been saved, they can be reused by clicking the “...” icon to the right of the experiment name.

13. Click the Run Experiment tab in the Harmony software.
14. Fill out Plate Name with desired file name as this is what the measurement will be titled.
15. Click Start to run measurement.

AOPI imaging procedure

1. Click the Setup tab in the Harmony software.
2. Click New in experiment window to create a new experiment.
3. Use the following settings for the experiment:
 - a. Plate type: 384 PerkinElmer CellCarrier Ultra
 - b. Autofocus: Two Peak (Default)
 - c. Objective: 20× Air, NA 0.4
 - d. Opt. Mode: Confocal
 - e. Binning: 2
4. In the Channel Selection window, add the Acridine Orange, Propidium iodide, and Brightfield channels with the following settings:
 - a. AO:
 - i. Time = 40 ms
 - ii. Power = 80%
 - iii. Height = 8.0 μ M
 - b. PI:
 - i. Time = 20 ms
 - ii. Power = 60%
 - iii. Height = 8.0 μ M
 - c. Brightfield
 - i. Time = 20 ms

- ii. Power = 60%
- iii. Height = 8.0 μ M
5. In the Navigation window, under the Define Layout tab, highlight all the wells that will be imaged and click Select. Within the Well window, highlight the nine inner squares, which correspond to the number and position of images being taken per well, and click Select. If the well/field of view has only been highlighted, it will be orange, but if it has been selected, it will be grey.
6. To take a snapshot: highlight one of the control wells and a single field of view. Then, within the Channel Selection window, click Snapshot in any of the channels to take one image to check if the leukemia cells are in focus. If cells are not in focus, change the heights for AO, PI, and Brightfield channels until the cells are in focus (Figure 3).

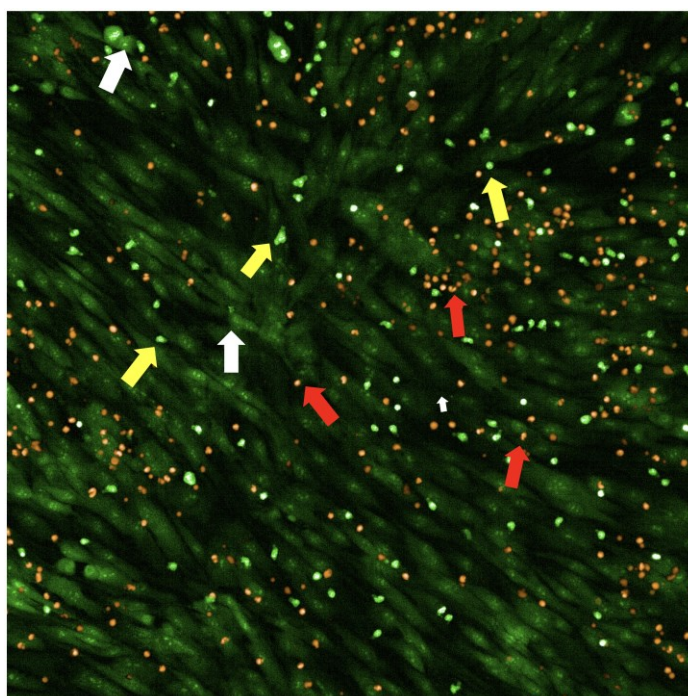


Figure 3. Overlay of primary patient leukemia cells and mesenchymal stromal cells (MSCs) stained with acridine orange/propidium iodide (AO/PI) for calculating percent cell viability. White arrows indicate MSCs, red arrows indicate dying leukemia cells, and yellow arrows indicate live leukemia cells.

7. Save the experiment.
8. Click the Run Experiment tab in the Harmony software.
9. Fill out Plate Name with desired file name as this is what the measurement will be titled.
10. Click Start to run measurement.

Data analysis

Data evaluation

The Harmony software for the PerkinElmer Operetta CLS™ high-content analysis system is used for the data analysis of the primary samples. The analysis pipeline distinguishes viable leukemia nuclei from MSC nuclei by setting nuclei area and CyQUANT fluorescence intensity cutoffs. The pipeline identifies and draws an outline of the nuclei in an image. Any nuclei that touch the border of the image are removed. Next, the CyQUANT intensity and area of the nuclei are measured. Manually select the viable leukemia nuclei with intensity values above 2,000 and

an area less than $70 \mu\text{m}^2$ (Figure 4) (Note: Intensity cutoff values and/or area cutoff values may be adjusted for individual samples). Nine fields of view per well at a $20\times$ magnification are evaluated for both the control and drug treatment wells. The data output is the number of viable leukemia cells per well. This output is copied into a Microsoft Excel analysis template, in which calculations are made for the relative viabilities of each drug treatment in comparison to the average of the untreated control wells.

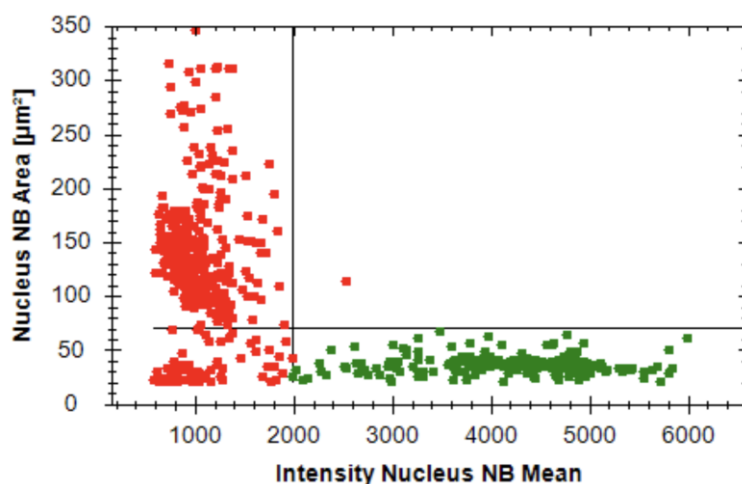


Figure 4. Representative scatter plot of an analysis pipeline. Perpendicular lines represent cutoffs for nuclei size and nuclei intensity. Green dots indicate cells gated as leukemia cells and red dots indicate cells gated as mesenchymal stromal cells (MSCs).

Data analysis procedure for CyQUANT

Note: Attach an assay layout to the measurement you are analyzing before starting data analysis.

1. Create an assay layout by clicking the Settings button in the Harmony software and then select the Assay Layout Editor.
2. Complete the Compound, Concentration, Cell Type, and Cell Count entries that match your experiment plate layout.
3. Save the assay layout.
4. Attach assay layout to desired experiment.
5. Click the Image Analysis tab in the Harmony software.
6. Click the button to the right of Analysis and load the analysis pipeline you would like to use or, if setting up a new pipeline, press the New button under Measurement.
7. For setting a new pipeline:
 - a. Press the “+” button at the bottom of the tab to add a building block to your pipeline.
 - b. Click Find Nuclei.
 - c. Under Find Nuclei, select the channel you would like to use and the method that best labels the cells in your image.
 - d. Label the output population as Nuclei.
 - e. Press the “+” button again and click Select Population.
 - f. Select the Remove Border Object box and use these settings:
 - i. Population = Nuclei
 - ii. Method = Common Filters
 - iii. Output Population = Nuclei NB
 - g. Press the “+” button again and click calculate intensity properties.
 - h. Use these settings:
 - i. Channel = CyQuant Green
 - ii. Population = Nuclei NB

- iii. Region = Nucleus
 - iv. Method = Standard
 - v. Property Prefix = Intensity Nucleus NB
- i. Press the “+” button again and click calculate morphology properties, select all boxes, and use these settings:
 - i. Population = Nuclei NB
 - ii. Region = Nucleus
 - iii. Method = Standard
 - iv. Property Prefix = Nucleus NB
- j. Press the “+” button again, click select population, and use these settings:
 - i. Population = Nuclei NB
 - ii. Method = Filter by Property
 - iii. Intensity Nucleus NB Mean > 2,000
 - iv. Nucleus NB Area [μm^2] < 70
 - v. Output Population = Nuclei NB Leukemia Viable
- k. Press the “+” button again, click select population, and use these settings:
 - i. Population = Nuclei NB
 - ii. Method = Filter by Property
 - iii. Nucleus NB ≥ 75
 - iv. Output population = Nuclei NB MSC
- l. Press the “+” button again, click define results, and use these settings:
 - i. Under Population: Nuclei NB Leukemia Viable select the number of objects box
 - ii. Apply to All = Individual selection
 - iii. Intensity Nucleus = Mean
 - iv. Intensity Area [μm^2] = Mean
 - v. Under Population: Nuclei NB MSC select the number of objects box
 - vi. Apply to all = Individual selection
 - vii. Intensity Nucleus = Mean
- m. Click the save button and name the pipeline accordingly.
8. Click the Evaluation tab in the Harmony software.
9. Check that you have the correct analysis pipeline and measurement files.
10. Select all the wells and fields of view that you want to analyze.
11. Click the Start button to analyze the plate.

Data analysis procedure for AOPI

1. Verify that the correct assay layout is attached to your plate.
2. Click the Image Analysis tab in the Harmony software.
3. Click the button to the right of Analysis and load the analysis pipeline you would like to use or, if setting up a new pipeline, press the new button under measurement.
4. For setting a new pipeline:
 - a. Press the “+” button at the bottom of the tab to add a building block to your pipeline.
 - b. Click Find Nuclei.
 - c. Under Find Nuclei, select the channel you would like to use and the method that best labels the cells in your image.
 - d. Label the output population as AO Nuclei.
 - e. Press the “+” button again and click select population.
 - f. Select the remove border object box and use these settings:
 - i. Population = AO Nuclei
 - ii. Method = Common Filters
 - iii. Output Population = AO Nuclei NB
 - g. Press the “+” button again and click calculate intensity properties.
 - h. Use these settings:

- i. Channel = Brightfield
- ii. Population = AO Nuclei NB
- iii. Region = Nucleus
- iv. Method = Standard
- v. Property Prefix = AO Intensity
- i. Press the “+” button again and click calculate morphology properties, select area box, and use these settings:
 - i. Population = AO Nuclei NB
 - ii. Region = Nucleus
 - iii. Method = Standard
 - iv. Property Prefix = AO Nucleus
- j. Press the “+” button again, click select population, and use these settings:
 - i. Population = AO Nuclei NB
 - ii. Method = Filter by Property
 - iii. AO Intensity Mean > 200
 - iv. AO Intensity Mean < 100
 - v. Output Population = AO Viable Nuclei
- k. Press the “+” button again, click find nuclei, and use these settings:
 - i. Channel = Brightfield
 - ii. ROI = None
 - iii. Method = C
 - iv. Output population – PI Nuclei
- l. Press the “+” button again, click select population, and use these settings:
 - i. Population = PI Nuclei
 - ii. Method = Common Filters
 - iii. Click the remove border object box
 - iv. Output population = PI Nuclei NB
- m. Press the “+” button again and click calculate intensity properties.
- n. Use these settings:
 - i. Channel = Propidium Iodide
 - ii. Population = PI Nuclei NB
 - iii. Region = Nucleus
 - iv. Method = Standard
 - v. Property Prefix = PI Intensity
- o. Press the “+” button again and click calculate morphology properties, select the area box, and use these settings:
 - i. Population = PI Nuclei NB
 - ii. Region = Nucleus
 - iii. Method = Standard
 - iv. Property Prefix = PI Nucleus
- p. Press the “+” button again, click select population, and use these settings:
 - i. Population = PI Nuclei NB
 - ii. Method = Filter by Property
 - iii. PI Intensity Mean > 1,000
 - iv. PI Nucleus Area [μm^2] < 100
 - v. Output Population = PI Dead Nuclei
- q. Press the “+” button again, click define results, and use these settings:
 - i. Under Population: AO Viable Nuclei select the number of objects box
 - ii. Apply to All = Individual selection
 - iii. AO Intensity = Mean
 - iv. AO Nucleus Area [μm^2] = Mean
 - v. Under Population: PI Dead Nuclei select the number of objects box
 - vi. Apply to all = Individual selection

- vii. PI Intensity = Mean
- viii. Method = Formula Output
- ix. Formula = $[a/(a+b)] \times 100$
- x. Variable a = AO Viable Nuclei – Number of Objects
- xi. Variable b = PI Dead Nuclei – Number of Objects
- xii. Output name = Viability %
- r. Click the save button and name the pipeline accordingly.
- 5. Click the Evaluation tab in the Harmony software.
- 6. Check that you have the correct analysis pipeline and measurement files.
- 7. Select all the wells and fields of view that you want to analyze.
- 8. Click the Start button to analyze the plate.

Data evaluation

- 1. Click the Evaluation tab in the Harmony software.
- 2. Load the correct evaluation using the button next to the Evaluation line.
- 3. The evaluation results will populate in the middle of the screen.
- 4. Data from the CyQuant Green wells will populate as number of viable leukemia cells per well.
- 5. Data from the AOPI well will populate as Viability % per well and can be used as an internal control for the sample.
- 6. Copy data into a Microsoft Excel analysis template to calculate the relative viabilities of each drug treatment compared to the average of the untreated control wells. Dose response curves are generated from the raw data using software of choice.

Validation of protocol

Our group has measured and reported ex vivo drug sensitivity of eight drugs used in the current method (i.e., L-asparaginase, daunorubicin, bortezomib, mercaptopurine, nelarabine, prednisolone, vincristine, and vorinostat) via an MTT-based method as previously described (Holleman et al., 2004; Lugthart et al., 2005; Autry et al., 2020). Likewise, our group has measured and reported ex vivo drug sensitivity of eight drugs used in the current method (i.e., CHZ868, dasatinib, gilteritinib, ibrutinib, inotuzumab ozogamicin, palbociclib, trametinib, and venetoclax) via a flow cytometry-based method as previously described (Gocho et al., 2021; Lee et al., 2023). To validate the current imaging-based method for these 16 drugs, a minimum of 15 patient samples (either primary or obtained from patient-derived xenograft models) were studied in parallel by the current imaging assay and either the MTT-based or flow cytometry-based method (Figure 5). Cell viability was calculated using each method. For each individual concentration data point, percent cell viability was charted and R^2 values were generated. Correlation of the cell viability results was reproducibly achieved, with all R^2 values above 0.5 (except for two drugs: 6-mercaptopurine, which has historically been tested via an MTT-based method, for which the observed R^2 value was 0.4780, and CHZ868, which has historically been tested via a flow cytometry-based method, for which the observed R^2 value was 0.4412).

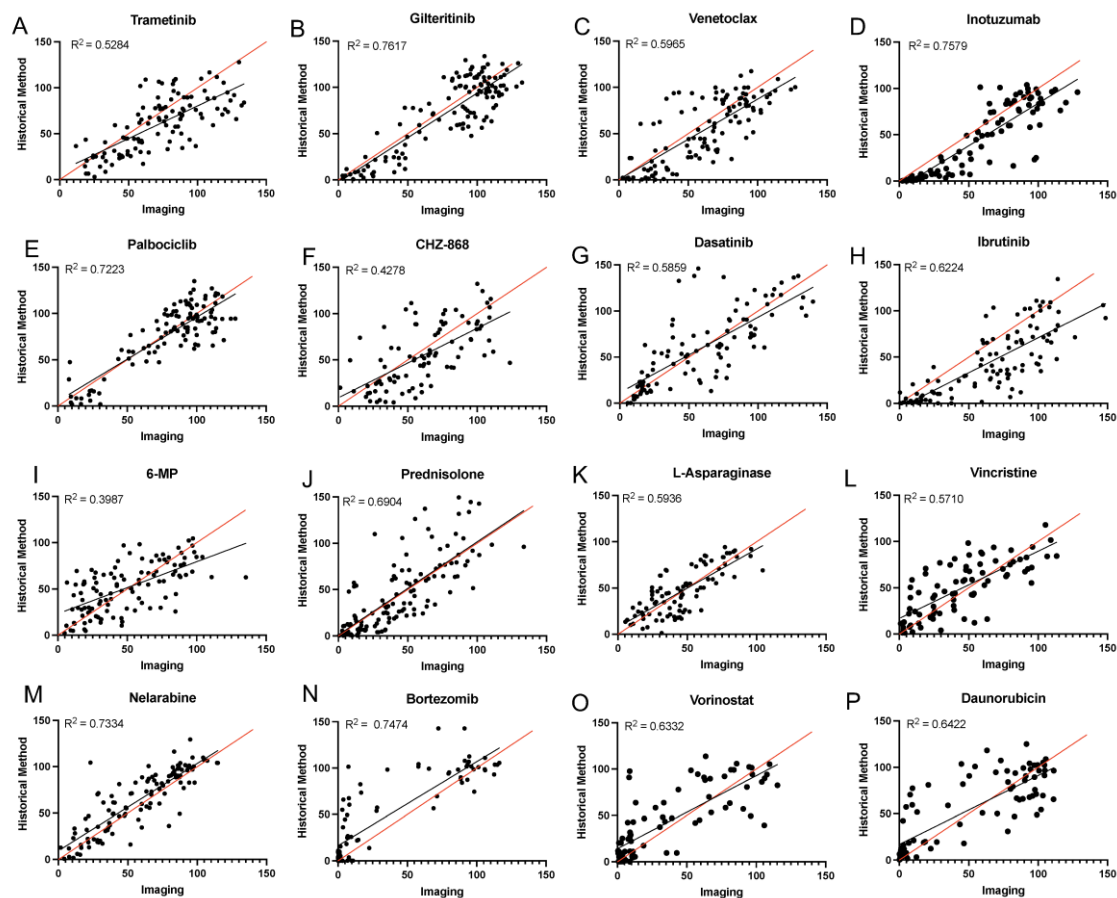


Figure 5. Comparison of imaging assay with previous methods. (A–H) Correlation plots showing the correlation between the imaging method and the gold standard flow cytometry–based mesenchymal stromal cell (MSC) co-culture method. (I–P) Correlation plots showing the correlation between the imaging method and the gold standard MTT-based metabolic activity method. Leukemia cells were treated for 96 h in the presence of increasing concentrations of each agent and cell viability was determined. For each individual plot, viability determined by the current imaging assay is shown on the x-axis, and viability determined by the gold-standard assay method is shown on the y-axis.

In addition to the 16 drugs for which we already had a validated method established, we introduced ex vivo sensitivity testing of an additional 24 drugs with the current imaging-based method. We determined drug sensitivity of MSCs alone to each single agent after a 96-hour incubation for all 40 drugs used with the proposed assay (Figure S1). Optimal concentration ranges were chosen based on clinically achievable concentrations in vivo and on the frequency distribution of LC50 values for each drug.

We measured ex vivo drug sensitivity to our drug panel of 40 antineoplastic agents with the current imaging-based method in 30 primary adult and pediatric ALL samples. Samples were assayed as received and reflect the variability of the population to be studied with this method. We observed varying patterns of drug sensitivity across patients (Figure 6). Leukemia cells with certain molecular markers had a high probability of showing a predictable sensitivity pattern (e.g., samples positive for the *ETV6::RUNX1* fusion are usually sensitive to asparaginase; samples positive for the *TCF3::PBX1* fusion are usually sensitive to dasatinib) (Frismantas et al., 2017; Lee et al., 2023). In this pilot dataset, we observed a lower median asparaginase LC50 value in cases with the *ETV6::RUNX1* fusion ($n = 4$) vs. other cases ($p = 0.02$; Figure 6B), and a lower median dasatinib LC50 value in cases with the *TCF3::PBX1* fusion ($n = 3$) vs. other cases ($p = 0.009$; Figure 6C).

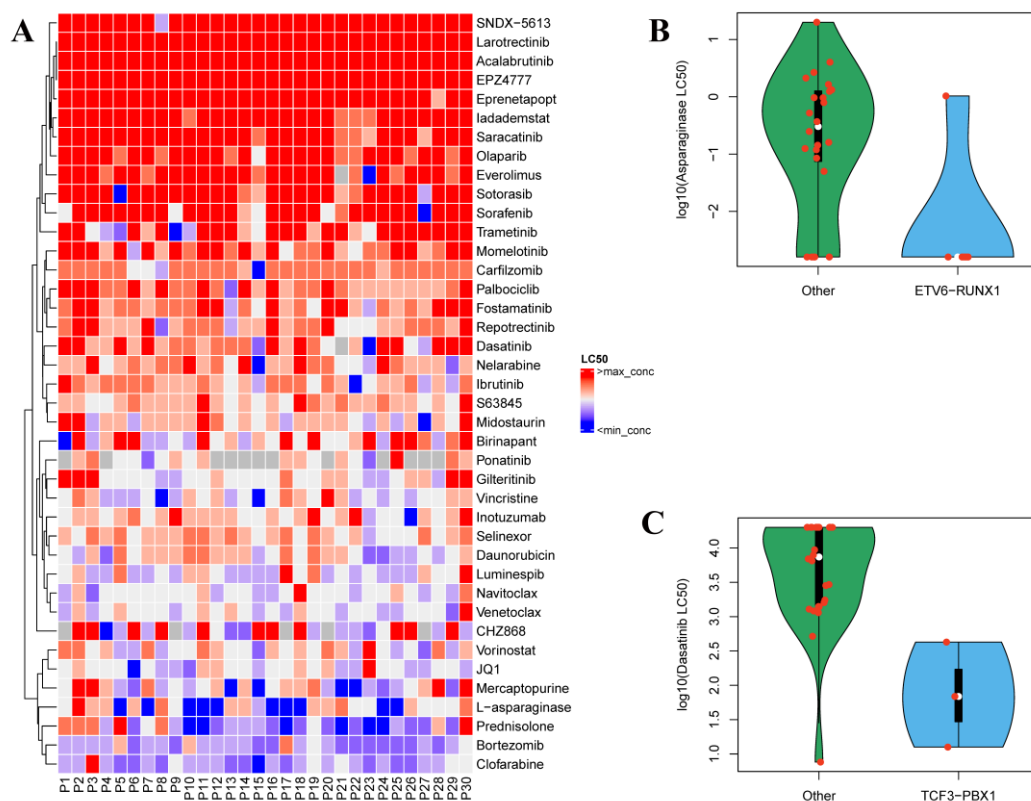


Figure 6. Validation of imaging assay with primary patient samples. (A) Heatmap indicates the sensitivity of 30 primary leukemia samples to 40 anti-leukemia agents. LC50 values for each drug were ranked, with red indicating most resistant cases and violet indicating most sensitive cases. Grey squares indicate no data. (B) LC50 values of asparaginase in 30 primary ALL cases displayed as cases with the *ETV6::RUNX1* fusion (n = 4) vs. other cases (n = 26). Median LC50 value for each group is shown as a white circle (p = 0.02 by Wilcoxon rank-sum test). (C) LC50 values of dasatinib in 30 primary ALL cases displayed as cases with the *TCF3::PBX1* fusion (n = 3) vs. other cases (n = 27). Median LC50 value for each group is shown as a white circle (p = 0.009 by Wilcoxon rank-sum test). LC50, the concentration required to kill 50% of cells.

General limitations and potential problems

This protocol has potential limitations. This method is designed for analyzing fresh primary samples, one at a time. If a laboratory adopting this protocol cannot receive fresh primary samples, adjustments would potentially need to be made to the method. The imager used in this protocol may not be easily acquired by every institute. While this protocol may be adapted for other high-throughput imaging platforms, this adaptation would need to be validated beforehand. The ability to separate two cell populations is essential and is not exclusive to the imaging platform. There are options for free open-source image analysis software that could be used to analyze images taken from other platforms, which have the ability to perform batch analysis.

Due to the heterogeneity of primary samples, occasionally, samples may contain visible *clumps* upon receipt. If this occurs, samples can be strained using a 40 μ m cell strainer to remove cell clumps before processing. Finally, due to the amount of manual pipetting, either with a single-channel or multichannel pipette, there is potential for variability among samples, and great care needs to be taken to minimize error.

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Competing interests

All authors declare no competing interests.

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Supplementary information

The following supporting information can be downloaded [here](#):

1. Supplemental Methods
2. Figure S1. MSC Drug Sensitivity Curves for the 40 Therapeutic Agents Tested