Enzymatic-based cytometry, a sensitive single-cell cytometric method to assess BCR-ABL1 activity in CML

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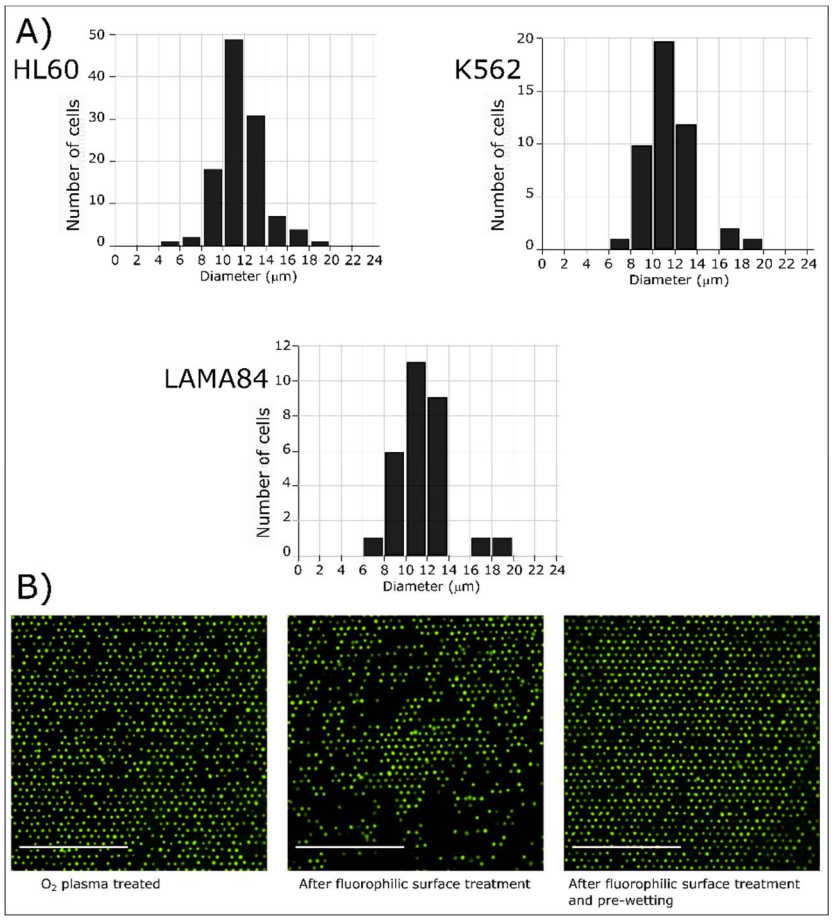
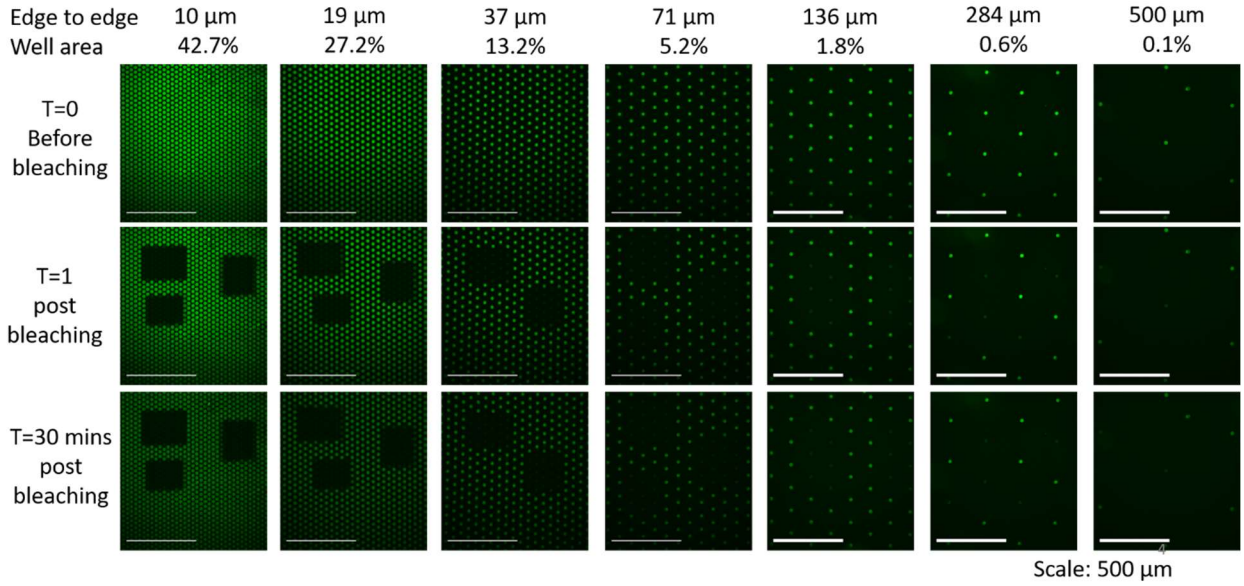
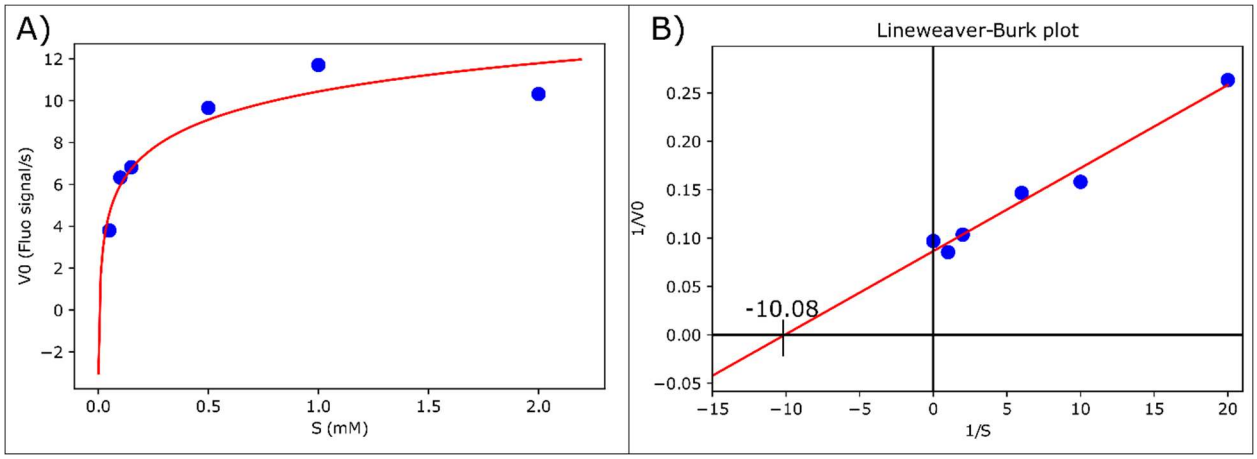
Supplemental figures



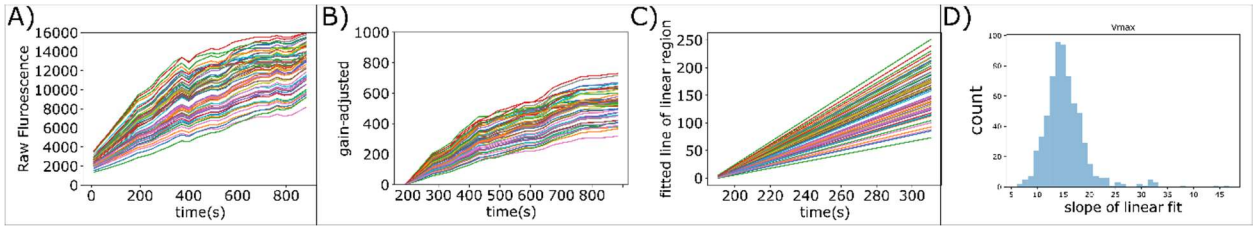
***Supp. Fig. 1**. A) Measurement of the cell diameter for the different CML cell lines. B) Cell loading in the microwells is aided by a pre-wetting step. The scale bars represent 500 μm.*



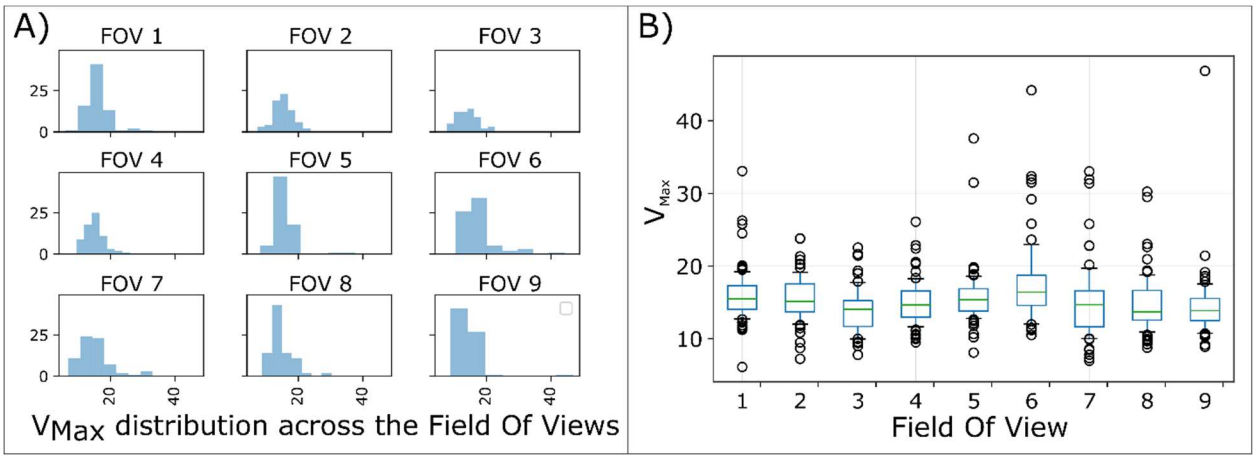
***Supp. Fig. 2**. Photobleaching experiments to validate microwell sealing. The horizontal rows represent different time points and the vertical rows represent arrays with different well densities. Microwells are loaded with a solution of 1 mM fluorescein and sealed with FC-70 oil. At T=1, selected microwells are photobleached. The absence of fluorescence recovery at T=30 mins indicates that fluorescein in adjacent microwells have not diffused into the photobleached areas.*



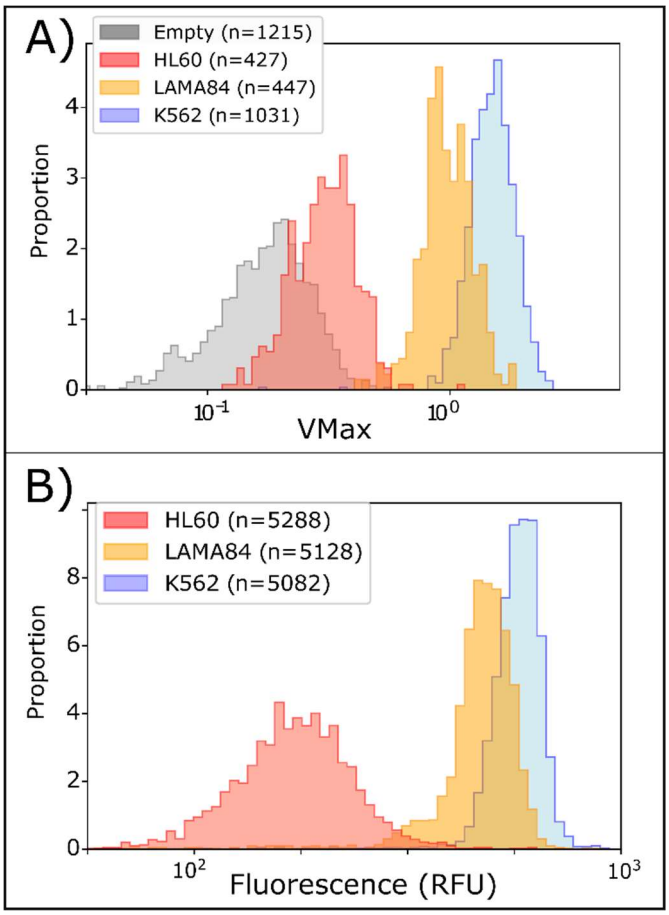
***Supp. Fig. 3**. Determination of K_M for the couple AP and 4-MUP on the array of microwells. A) The initial enzyme velocity is reported as a function of substrate concentration. B) The data are plotted using the Lineweaver-Burk transformation to determine the value of K_M obtained as the inverse of the intersect with x-axis.*



***Supp. Fig. 4**. Data analysis pipeline. Example of the analysis of data extracted from a single field of view for LAMA84 cells. A) Raw data. B) Traces are corrected for the camera gain. C) The initial phase of the gain-adjusted traces is linearized to measure V_{Max}. D) The distribution of V_{Max} across the cell population is computed.*



Supp. Fig. 5. *Quality control of the data across the field of views. A) V_{Max} distribution across the different fields of view. B) Boxplot representation of the distribution of V_{Max} for each field of view allows direct comparison and identifying possible artefacts. FOV: Field Of View.*



Supp. Fig. 6*. A) Normalized histograms of the phosphorylation level of CrkL in different CML cell lines obtained with the enzymatic-based cytometry (log scale for the x-axis). B) Normalized histograms of the phosphorylation level of CrkL in different CML cell lines obtained with flow cytometry (log scale for the x-axis).*

Materials and Methods

Microwell Design, Fabrication, and Surface treatment

Microwell fabrication was manufactured using soft lithography [1]. The design was generated with DraftSight (Dassault Systèmes, Yvelines, France) and printed onto a Fuji transparent film at 25,400 dpi (CAD/Art Services Inc., OR, USA) to create a mask. The design consists of an array of hexagonally packed 20 μm wells with an area fraction of 21% to optimize well density and sealing efficiency. The negative photoresist SU8-2025 (Microchem, MA, USA) was patterned onto a silicon wafer (SSP orientation 100, University Wafer, USA) using optical lithography. A layer of Omnicoat (Microchem) and a homogeneous 5 μm layer of SU8-2005 were used to improve the adhesion between the silicon wafer and the patterned photoresist. After completion, a vapor deposition of 1H,1H,2H,2H-Perfluorooctyltrichlorosilane (007146, Oakwood Chemical, SC, USA) was performed for 3 hrs to improve the release of cured polydimethyl-siloxane (PDMS).
The microwell arrays were manufactured by spin coating PDMS (Sylgard 184 Silicone Elastomer Kit, Dow Corning, MI, USA), at a 1:10 ratio of curing agent and polymer base, on the master at 300 rpm for 30 s. Once cured, the microwell arrays were bonded to a glass slide using oxygen activation (PDC-32G, Harrick Plasma, NY, USA) to provide mechanical support. The device was then incubated in an 80 °C oven for several hours to complete the bonding. The top of the array was rendered fluorophilic by stamping it onto a solution of (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane diluted at 1% v/v in fluorinated oil FC-70 (3M, MN, USA) spread onto a silicon wafer, for 5 min. The device was then dried at 150 °C for 5 min to evaporate traces of FC-70 and immersed in alkaline phosphatase (AP) buffer (see below) to lock in the hydrophilicity of the microwells until use.

Cell lines and cell culture

The human cell lines K562 [2] (ATCC CCL-24), and LAMA-84 [3] (ACC-168) cells derived from patients with chronic myeloid leukemia in blast crisis were purchased from the American Type Culture Collection (ATCC, MD, USA) and the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Germany). They were maintained in RPMI 1640 medium (cat. # 11875085, Thermo Fisher Scientific, MA, USA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, MO, USA) and 1% (vol/vol) penicillin-streptomycin (cat. # 15140-122,Thermo Fisher Scientific).The BCR-ABL1 negative human acute myeloid leukemia cell line HL-60 [4] (ATCC CCL-240) was maintained in RPMI 1640 medium with 20% FBS, and 1% penicillin-streptomycin.
An imatinib-resistant K562 cell line (K562R) was established by exposing the parental cell line to increasing concentrations of imatinib mesylate (IM) (sc-202180A, Santa Cruz Biotechnology, CA, USA) with the following sequence: from 0.05 μM (5 days), 0.1 μM (5 days), 0.2 μM (6 days), 0.4 μM (8 days), 0.6 μM (9 days), 0.8 μM (11 days), to 1 μM (35 days). Cells were seeded at 0.1x 10^6 cells/ mL in antibiotic-free RPMI-1640 supplemented with 10% FBS and IM. Stock dilutions of IM were prepared in dimethyl sulfoxide (DMSO) and stored as a 10 mM solution at -20 °C. Fresh dilutions of IM were prepared in phosphate-buffered saline (PBS) (Corning, NY, USA). After approximately 3 months, cells developed resistance to 1 μM IM and monoclonal cell populations were isolated by limiting dilution. The K562R cell line was propagated in RPMI 1640 medium supplemented with 10% FBS and 1 μM IM.
All cell lines were passaged every 2-3 days, seeded at 0.5 million cells/mL and maintained for up to 3 months in a humidified atmosphere of 5% CO_2 at 37 ℃. Experiments were performed using

logarithmically growing cells ($1x10^6$ cells/mL). Cell lines were routinely screened for *mycoplasma* contamination (e-Myco™ Mycoplasma PCR Detection kit version 2.0, iNtRON biotechnology, Corea) and authenticated by STR (short tandem repeat) DNA profiling analysis (ATCC, VA, USA).

Cell fixation and permeabilization

Cells were harvested, clarified *via* centrifugation at 200 x g for 4 min, washed with warm PBS and fixed with freshly prepared 4% formaldehyde (BDH0500-1LP, VWR, PA, USA) for 15 min at room temperature (RT). Cells were then permeabilized with ice cold 90% (v/v) methanol (BDH1135-1LP, VWR) and immediately stored at -20°C in the permeabilization solution until antibody labeling. All cells used in flow cytometry and microwell experiments were fixed within one month of use.

Immunofluorescence and cell enzyme labeling

Fixed and permeabilized cells were washed (3x 15 min) in blocking buffer (0.5% (w/v) fatty acid-free Bovine Serum Albumin (BSA) in PBS, cat. # 126609, Sigma-Aldrich). Subsequently, cells were incubated with a rabbit anti-phospho-CrkL (Tyr207) polyclonal antibody (product #3181S, 1µg/ 10^6 cells, Cell Signaling Technology, MA, USA) overnight at 4 °C. In parallel, an equal amount of anti-rabbit (DA1E) mAb IgG XP isotype control antibody (product #3900S, CST) was used as negative control. The following day, samples were washed (3 x 5 min) in blocking buffer. For flow cytometry experiments, samples were incubated with an Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (cat. #, A-11008, dilution: 1/500, Thermo Fisher Scientific) for 1 hr in the dark. Labeled cells were characterized on a FACSCalibur system (Becton Dickinson, NJ, USA) and analyzed using Cell Quest software (Becton Dickinson, NJ, USA). Dead cells were excluded by propidium iodide (Sigma) staining. Cells were gated on forward and side scatter to exclude dead cells, cell aggregates and debris from the analysis.

For microwell experiments, samples were incubated with a biotin-conjugated goat anti-rabbit IgG polyclonal antibody (65-6140, 10 ug/ 10^6 cells, Thermo Fisher Scientific) for 1 hr. Cells were then washed for (3 x 5 min) in AP buffer (100 mM Tris-HCl buffer with 1 % BSA, 10 mM $MgCl_2$, 0.05% Tween 20, pH 9) [5]. Cells were further incubated with streptavidin-conjugated alkaline phosphatase (SA-5100, 40 ug/10^6 cells, Vector Laboratories, CA, USA) for 1hr. Labeled cells were then washed (3x5 min) in AP buffer and each cell line was stained with a fluorescent nucleic acid stain with unique spectral properties. SYTOX green and orange dyes (S7020, S11368, Thermo Fisher Scientific) were diluted in AP buffer to working concentrations of 60 nM, and to 800 nM for SYTO 62 (S11344, Thermo Fisher Scientific). Each cell line was incubated in their specific dye solution for 30 min before being combined and immediately loaded on microwell arrays in AP buffer.

In all experiments, antibodies were diluted in blocking buffer and incubations and washes were carried out at RT under gentle agitation, unless notified otherwise.

Alkaline phosphatase fluorometric assay

AP hydrolyzes 4-Methylumbelliferyl phosphate (4-MUP) to 4-Methylumbelliferone (4-MU) following the Michaelis-Menten kinetics. The enzymatic product 4-MU is fluorescent with an excitation peak at 372 nm and an emission peak at 445 nm (DAPI channel).

Microwell loading and image Acquisition

Labeled cell lines were mixed and immediately deposited on the microwell arrays at a density of 2 cells/well. The cell suspension was let to settle in the microwells for 10 min at 4°C before removing the excess of cells with AP buffer. The microwell array was washed with 8mM 4-MUP (M6491, Thermo Fischer Scientific) diluted in AP buffer. The excess of substrate solution was swept away with FC-70 oil to assure isolation of the microwells into individual reactors [6]. Microarray wells were immediately transferred to an automated microscope stage for imaging.

Images were taken on an automated stage (Prior Scientific, MA, USA) with a DS-Qi2 camera (Nikon, Japan) mounted on a Nikon AZ100 monozoom microscope equipped with an AZ-Plan Fluor 5x lens (NA=0.5), an Aura-2 LED light engine (Lumencor, OR, USA), and a polychroic quad bandpass filter-set (89401, Chroma Systems Solutions, CA, USA). The enzymatic product, 4-MU, was illuminated at 10% of the maximum intensity of the UV LED. Time-lapse imaging was acquired at a frame rate of 1.42 fps. Multi-field imaging was set up with a serpentine path. Cell line identification was performed by collecting multispectral images at the end of each experiment.

To control for the unevenness of the illumination over the field of view, we generated an intensity map with a blue auto-fluorescent plastic slide (92001, Chroma, VT, USA). We used the intensity map with the function "Shading correction" of the NIS elements microscope imaging software (Nikon, JP) for spatially correcting the intensity. We did not observe any positional bias in the data.

Data extraction and analysis

Multichannel images were pre-processed with Nikon's NIS Elements microscope imaging software and data was extracted with an in-house custom software written in Python (Python Software Foundation, https://www.python.org/). A binary mask was generated after segmenting the microwells. The size of all microwells was fixed to control for the total number of pixels used to integrate intensity values. Microwells located at the image borders were discarded. The binary mask was used to identify the cell line by extracting the intensity values from the SYTO and SYTOX fluorescence channels. Each microwell was plotted onto a three-dimensional space so that a gate could be set to select the different cell lines and create the corresponding sub-masks. Microwells located at the image borders or with multiple cells were excluded from the analysis. The sub-masks were then used to extract the evolution of the fluorescence intensity of the 4-MU channels of specific cell line. The slope of the initial velocity was extracted by computing a linear fit using 7 points on each trace. The data was presented as histograms and as probability density functions using the kernel estimation function from the Python Seaborn package. The figures were generated with the Python Matplotlib package.

Analysis of the Michaelis-Menten kinetics

A Michaelis-Menten kinetics characterizes enzymatic reactions where free enzyme (E) and substrate (S) exist and where the enzyme-substrate complex (ES) irreversibly produces the product (P) and regenerates free enzyme [7]:

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \quad \textbf{(Eq. 1)}$$

The Michaelis-Menten model provides a relationship between the rate of product formation and the substrate concentration. The relationship is simplified when the amount of enzyme is much lower than the amount of substrate:

$$\upsilon = \frac{d[P]}{dt} = V_{max}\ \frac{[S]}{K_M+[S]} = \ k_{cat}.[E]_0\ \ \frac{[S]}{K_M+[S]}$$ **(Eq. 2)**

where [P], [S], $[E]_0$ represent the concentrations of the product, substrate and initial enzyme concentration, respectively. V_{max} and K_M are the maximum rate of product generation and the substrate concentration at which the rate is half the value of V_{max}, respectively. Interestingly, equation 2 links the rate of product formation, which can be inferred by the progression curve, to the initial amount of enzyme that is assumed to reflect the amount of pCrkL in our experiments. Equation 2 also indicates that the rate of product is proportional to the concentration of substrate when the substrate concentration is much lower than KM. This situation occurs at the end of the reaction when the substrate is depleted.

Nucleic acid isolation and cDNA synthesis

To minimize the impact of batch effects between experiments, a proper experimental design was ensured. For each experiment, all cell lines were harvested from cell culture at the same time, washed once with cold PBS and cell pellets (5.10^6 cells) were immediately stored at -80°C for 24 hrs prior to nucleic acid extraction. Genomic DNA and total RNA were isolated from the cell lines using the ZR-Duet™ DNA/RNA miniprep kit according to the manufacturer's instructions (Zymo research, Irvine, CA, USA). The integrity and concentration of the purified nucleic acids was subsequently assessed using the Agilent2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the Qubit 3.0 fluorometer (Thermo Fisher Scientific, Wilmington, DE, USA), respectively. cDNA synthesis was performed for all cell lines at the same time using the same reverse transcription (RT) master mix. First-strand cDNA synthesis was carried out with 1 ug of total RNA using the superscript IV VILO master mix with ezDNase kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The resulting cDNA was stored at -20°C. Positive (plasmid) and negative controls (water and HL60) were included alongside each experiment.

PCR and sequencing reactions

The complete ABL1 tyrosine kinase domain of the *BCR-ABL1* fusion gene was amplified using primers from a previously published hemi-nested PCR assay [8]. First-round PCR was carried out with 2 uL cDNA template (100 ng RNA equivalent input) and 1 uL of first-round PCR product was directly used as template in second-round PCR. PCR reactions were performed in 25 uL reaction volumes using the Invitrogen Platinum™ hot start PCR 2X master mix supplemented with platinum GC enhancer, according to the manufacturer's recommendations. PCR reactions were run on a SimpliAmp™ thermal cycler (Applied Biosystems, CA, USA) with the following thermal cycling conditions: 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C and 2 min at 72°C. The purified PCR product was sequenced in both directions using the BigDye Terminator V3.1 cycle sequencing kit (Life Technologies) and a 3700 DNA Sequencer (Applied Biosystems) at the Stony Brook University DNA sequencing core facility.

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) analysis of *BCR-ABL1* Mbcr (p210) was performed with previously published assays by the EAC (Europe against cancer) initiative [9]. The beta-glucuronidase (*GUSB*) was used as a reference gene for qPCR data normalization. Reactions were

carried out with 2 uL of cDNA template (100 ng equivalent RNA input) added to a final volume of 20 µL containing 1x TaqMan® fast advanced Master Mix (Applied Biosystems) with 900 nM of each primer pair (Eurofins Genomics USA, Louiseville, KY, USA) and 250 nM of probe (Eurofins Genomics US). qPCR reactions were run on a QuantStudio 3™ thermal cycler (Applied Biosystems) with the following thermal cycling conditions: 50°C for 2 min; 95°C for 20 sec min; 35 cycles of 95°C for 1 sec, 60°C for 20 sec.
DNA standards for *BCR-ABL1* Mbcr(p120) (GenBank accession number AJ131466.1, (nt1-662)) and *GUSB*, (GenBank accession number M15182.1, (nt 1739-1879)) were synthesized in a single pCR2.1 TOPO vector at Eurofins Genomics USA. Normalized levels of *BCR-ABL1* were calculated as the ratios between *BCR-ABL1* and *GUSB* transcripts. All qPCR reactions were conducted in triplicates on a QuantStudio™ 3 real time PCR system (*Applied Biosystems*). Water instead of cDNA was included in each experiment to control for PCR contamination.

Fluorescence *in-situ* Hybridization (FISH)
The genomic copy number of *BCR-ABL1* in a cell was characterized using the Vysis LSI BCR/ABL dual color, dual fusion translocation probe (probes: 05J82-001 and 05J82-010, Abbott Laboratories, IL, USA). The kit comprises a FISH probe for *BCR* and *ABL1* labeled with a green and an orange dye, respectively. The *BCR-ABL1* fusion gene is identified by the presence of an overlap or the proximity of the two colors when metaphasic cells are observed under microscope at high magnification [10, 11]. The slides were prepared following the manufacturer's recommendations under the guidance of a technician in the pathology department of Stony Brook Hospital. Fluorescence images were obtained with either a 60x oil immersion objective or with a Nikon N-SIM Super Resolution Microscope System (Microscopy facility, Stony Brook University, USA). The number of fusion genes was recorded for each cell line and statistical differences were assessed with a T-test (http://www.quantitativeskills.com/sisa/index.htm).

Supplemental Material

> consensus sequence-K562R tyrosine kinase domain

CTCGCATGAGTTCATAGACCTTCTCTGGGCAGCCTTCTGGGCGCTCCATGCGGTAGT
CCTTCTCTAGCAGCTCATACACCTGGGACAGGTCAATTCCCGGGTAAGGGGACATGC
CATAGGTAGCAATTTCCCAAAGCAATACTCCAAATGCCCAGACGTCGGACTTGATGG
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CAGCATGGGCTGTGTAGGTGTCCCCTGTCATCAACCTGCTCAGGCCAAAATCAGCTA
CCTTCACCAAGTGGTTCTCCCCTACCAGGCAGTTTCGGGCAGCAAGATCTCTGTGGA
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> consensus sequence-K562 tyrosine kinase domain

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> consensus sequence-LAMA 84-Tyrosine kinase domain

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