Abstract
The inhibition of tyrosine kinases is a successful approach for the treatment of cancers and the discovery of kinase inhibitor drugs is the focus of numerous academic and pharmaceutical laboratories. With this goal in mind, several strategies have been developed to measure kinase activity and to screen novel tyrosine kinase inhibitors. Nevertheless, a general non-radioactive and inexpensive approach, easy to implement and adapt to a range of applications, is still missing. Herein, using Bcr-Abl tyrosine kinase, an oncogenic target and a model protein for cancer studies, we describe a novel cost-effective high-throughput screening kinase assay. In this approach, named the BacKin assay, substrates displayed on a bacterial cell surface are incubated with kinase and their phosphorylation is examined and quantified by flow cytometry. This approach has several advantages over existing approaches, as using bacteria (i.e. *Escherichia coli*) to display peptide substrates provides a self-renewing solid support that does not require laborious chemical strategies. Here we show that the BacKin approach can be used for kinetic and mechanistic studies, as well as a platform to characterize and identify small-molecule or peptide-based kinase inhibitors with potential applications in drug development.

Introduction
Tyrosine kinases (TKs) are implicated in the development of many cancers [1,2] and are the focus of numerous drug discovery projects [3–5]. These critical enzymes are involved in the activation and amplification of signaling pathways within cells [2], and when deregulated can promote unregulated cell growth and cancer progression [1]. Chronic myeloid leukemia (CML) is an example in which a deregulated TK, Bcr-Abl kinase (Figure 1), gives rise to cancer [6]. As the sole molecular abnormality in the early stage of this cancer [7], Bcr-Abl kinase is an ideal oncogenic target [8] and was a landmark case in validating the clinical application of TKs as drug targets to treat cancer [9–11].

Imatinib was the first TK inhibitor (TKI) approved against Bcr-Abl kinase and gave rise to the so-called molecular revolution in cancer therapy [12]. This designed small molecule targets the ATP binding site in the Abl TK domain [8,13] (Figure 1) and it is very efficacious in the treatment of CML [14]. Although remarkably effective at inhibiting cancer progression, mutations in the ATP binding site render imatinib ineffective and resistance occurs in many patients after a few years of imatinib treatment [11,14]. As a consequence, much recent effort has been directed at identifying more potent TKIs against Bcr-Abl kinase and other oncogenic TKs [1–3,5,12,15].

So far the discovery and development of TKIs has been based on the screening of small molecules that bind in the ATP cleft. Although a proven approach, the activity of small molecules is sensitive to kinase mutations. All human kinases have similar ATP binding pockets and therefore designing molecules targeting sites with both high specificity and a lack of susceptibility to the development of resistance is very challenging [16]. Peptide-based drugs that disrupt the interactions between TKs and their substrates [16,17] have been proposed as alternative TKIs. The substrate-binding domain in TKs is less conserved than the ATP cleft, and peptides typically display larger contact with their targets than small molecules [2]; therefore, peptides can potentially yield higher specificity and have been a focus in our laboratory.

Assays to measure kinase activity and test and screen kinase inhibitors are essential tools for drug discovery and in the last few years several strategies have been established [10,19]. The activity of TKs is classically measured by radiometry with radioactive phosphate transferred from *[^32]P*ATP to a kinase substrate. Although the quantification of radiolabelled ATP is the ‘gold standard’ in kinase activity assays, high levels of radioactivity are required when thousands of compounds are to be screened [19]. With safety and environmental issues in mind, novel non-radioactive methods have emerged in recent years, including colorimetric methods quantifying ATP consumption [19], mass spectrometry quantification of phosphorylation by measurement of mass change upon phosphate incorporation into a peptide substrate [20,21], an assay based on magnetic beads and matrix-assisted laser desorption/ionization [22], peptide microarray with surface plasmon resonance imaging [23] or methods using...
The peptides were quantified by absorbance at 280 nm (\(e_{280} = 1490 \text{ M}^{-1} \text{ cm}^{-1}\)) and had a purity \(\geq 95\%\).

In the absence of an inexpensive high-throughput screening (HTS) kinase assay that could be used to identify both substrates and peptide inhibitors, measure kinase activity and compare potential lead compounds, we developed a novel kinase assay that is cost effective. In this method, substrates displayed on a bacterial cell surface are incubated with kinase and their phosphorylation is examined and quantified by flow cytometry (FC). Bcr-Abl TK, a recognized oncogenic target and a model for cancer studies, and abltide, its preferred substrate, were used to validate our method. This novel approach can be used to measure TK activity and to characterize TK inhibitors, but can also be used to identify new binders using a peptide library displayed at the bacterial cell surface and by sorting using fluorescence-activated cell sorting (FACS). It has the potential to help facilitate the rapid development of TKI drugs against Bcr-Abl kinase and can be applied for other TKs of oncogenic interest. To the best of our knowledge, this is the first kinase assay approach using peptide-displaying bacteria.

**Materials and Methods**

**Peptide synthesis and purification**

Based on a previously identified recognition Abl TK substrate sequence (Ile/Val-Tyr-Xaa-Xaa-Pro, where Xaa is any amino acid) [25], the peptide EAIYAAPFKKK, named abltide, was used as an optimal substrate. Soluble abltide and the analogue [Y4F,F8Y] (EAIYAAPFYKKK), in which the Tyr4 and Phe8 residues are swapped, were synthesized using Fmoc chemistry on a Liberty Automated Microwave Peptide Synthesizer (CEM corporation, USA). The mass and the purity of the synthetic peptides were confirmed by ESI-MS and analytical reverse phase-HPLC. The peptides were quantified by absorbance at 280 nm (\(e_{280} = 1490 \text{ M}^{-1} \text{ cm}^{-1}\)) and had a purity \(\geq 95\%\).
Bacterial surface display construct

Abltide was displayed on the surface of *E. coli* by fusing the C-terminus of the sequence KKGEEAYAAPFA to the N-terminus of the enhanced circularly permuted outer protein OmpX (eCPX) display scaffold [26]. The spacer sequence GGQSGQ [26] was included to efficiently display abltide at the cell surface (Figure 2). The plasmid pBAD33-eCPX, which contains an arabinose promoter (pBAD) and resistance to chloramphenicol (Cmr) [26], was used to prepare the abltide construct (Figure 2A). *E. coli* strain MC1061 was transformed to display the abltide fused to eCPX. Assembly PCR was performed using KOD Hot Start DNA polymerase (Novagen) with overlapping primers including the abltide sequence, the linker, SfiI site and an eCPX link (GTAGCTGGCCAGCTTGCCAGCGAAGGCG-GATTATGCGGCGCCGTTTGCGGGAGGGCAGTCTGGG-CAGTC) (forward primer outside), AGGGCAAGCGGTAT-TATGCGGCGCGGTTFGGCGAGGCGATCTGGG-CAGTC (forward primer inside). The primer GGTCGAAATCTTCTCTCTC was used as reverse primer and pBAD33-eCPX as the template. The product of PCR reaction and the vector pBAD33-eCPX were digested with SfiI (New England Biolabs) and ligated. The ligation product (pBAD33-eCPX-abltide) was desalted and electroporated into electrocompetent *E. coli* MC1061 with 2 mm electroporation cuvette and pulse at 25 kV, 50 μF, and 100 Ω. Transformed cells were grown in super optimal broth supplemented with glucose and isolated clones were plated and the sequence confirmed by plasmid DNA sequencing.

Bacterial Growth and induction conditions

An isolated clone of *E. coli* transformed with pBAD33-eCPX-abltide was grown overnight in 3 mL LB broth supplemented with 34 μg/mL chloramphenicol (LB-CM) at 37°C. Sixty μL of overnight culture were subcultured into 3 mL of fresh LB-CM and incubated at 37°C with shaking at 225 rpm for 2 h (optical density at 600 nm is ~0.6). To induce protein expression, 60 μL of L-arabinose 2% (w/v) (final concentration 0.04% (w/v)) and 60 μL of 100 mM EDTA (final concentration 2 mM) were added to cell culture and incubated for an additional 45 min at 37°C with shaking at 225 rpm. Arabinose induces protein synthesis and promotes abltide cell surface expression, whereas EDTA facilitates the peptide display. After induction, cells were kept at 4°C and used within 30 h. The same procedure was followed with *E. coli* expressing the eCPX scaffold, which was used as a control in the kinase assay.

Kinase activity, incubation time, ATP and kinase concentration optimization

Thirty five μL of induced cells were used in each kinase reaction. Before starting the reaction, the cells were spun down at 3000 g for 5 min at 4°C and washed with cold phosphate buffer solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). Cells were pelleted, the supernatant was removed and stock solutions were added to have a final kinase reaction volume of 35 μL in all the conditions tested. All the reagents in the kinase reaction were solubilized in kinase buffer (NEBuffer) and the kinase reaction was started by addition of ATP from stock solutions with 10-fold concentration. The conditions and final concentrations of each reagent are as detailed below.

To evaluate the time-course for the phosphorylation of abltide by Abl TK, cells expressing abltide were resuspended with Abl TK 0.5 U/mL, and the kinase was activated by adding 500 μM ATP and incubated at 37°C for different times (0, 10, 20, 30, 40, 50, 60, 90 and 120 min) with gentle shaking. The effect of ATP concentration on kinase activity was tested by incubating the induced *E. coli* cells with 0.5 U/mL Abl TK at 37°C for 30 min with final ATP concentrations ranging from 500 μM to 0.5 μM. To choose the best kinase concentration, *E. coli* cells were incubated with kinase concentrations ranging from 2 μU/mL to 0.015 μU/mL and incubated for 30 min with 500 μM ATP. To stop the reaction, cells were immediately centrifuged at 4°C and washed with cold PBS.

After the reaction was stopped, phosphorylated abltide was detected by incubating the cells with 40 μL of biotinylated antibody anti-phosphorytrosine 4G10 (Merck Millipore, 1 μg/mL in PBS) at 4°C for 45 min with gentle shaking. Cells were pelleted by centrifugation and incubated for further 45 min at 4°C with 40 μL streptavadin-R-phycocerythrin (SAPE; Invitrogen, 5 μg/mL in PBS). Cells were centrifuged and resuspended with 500 μL of ice-cold PBS (~107 cells/mL) and analyzed by flow cytometry (FC). Each experiment was done in duplicate and repeated three times.

Inhibition and competition assay

Inhibition of Abl TK activity was evaluated by incubating induced cells with several concentrations of imatinib (ranging from 32 to 0.008 μM). Competition with soluble abltide was evaluated with peptide concentrations ranging from 64 to 0.25 μM. The abltide analogue [Y4F,B8V] was used as a control. Inhibitor, or peptide, were solubilized in kinase buffer and co-incubated with abltide-expressing cells, 0.5 U/mL, kinase and 500 μM, or 50 μM ATP for 30 min. The reaction was started with the addition of ATP and incubation at 37°C and stopped with centrifugation as described above. Samples were fluorescently-labelled and treated as described above. Each experiment was done in duplicate and repeated three times.

Quantification by flow cytometry

FC measurements were performed with a BD FACSCanto II flow cytometer instrument (BD Biosciences). The population of cells was selected based on forward scatter and side scatter measurements. Cells with phosphorylated abltide were identified with fluorescence emission signal at 585/42 nm (excitation with 488 nm laser and detection at 585 nm with 42 nm bandpass); to gate out dark events and select the fluorescent cells, the background fluorescence emission of control cells (eCPX-displaying cells) was screened and a gate drawn to include only 0.2% or less of fluorescent cells. The mean fluorescence of the cell population and the percentage of fluorescent cells was determined by screening 100,000 cells per sample.

Flow cytometry data analysis

The background fluorescence from unspecific binding was very low and was neglected. The mean fluorescence was normalized to the maximum response in each assay. The *K*~H~ (kinase or ATP concentration required to phosphorylate half of the abltide molecules in the sample) was calculated by fitting the plot of the normalized mean fluorescence versus Abl TK or ATP concentration to the sigmoidal dose-response with variable slope

\[ F = \frac{[X]^H}{K_H^* + [X]^*} \]

in which *F* is the mean fluorescence, [X] is kinase or ATP concentration and *H* is the Hill slope.

The half-time (t½, time required to phosphorylate half of the abltide molecules displayed on the bacteria surface) was calculated assuming pseudo-first order association kinetics. The plot of the
The normalized mean fluorescence versus incubation time was fitted to the equation $F = F_{\text{max}} \left[1 - \exp(-kt)\right]$, in which $k$ is the rate constant and $t$ is the time. The $t_1/2$ was calculated from:

$$t_{1/2} = \frac{\ln(2)}{k}$$

To calculate the percentage of inhibition, the mean fluorescence response obtained without inhibitor was considered as 100% of abltide phosphorylation and 0% of inhibition. The inhibitor concentration required to inhibit the phosphorylation of half of the abltide molecules (IC$_{50}$) was calculated by fitting the inhibition percentage fluorescence as a function of inhibitor concentration to the sigmoidal dose-response with variable slope

$\%\text{inhibition} = \frac{100\% \times [I]^H}{IC_{50} + [I]^H}$

in which $[I]$ is the inhibitor concentration and $H$ is the Hill slope.

**Results and Discussion**

The BacKin assay is a novel kinase assay based on substrates displayed on a bacterial surface and FC detection. As a proof-of-concept, abltide, the optimal substrate of Abl kinase, was displayed on the surface of $E. coli$ by fusing the C-terminus to the N-terminus of the eCPX display scaffold (see Figure 2A). After incubation with Abl kinase, phosphorylated abltide was fluorescently-labeled with biotinylated-anti-phosphotyrosine antibody (biotin-anti-PY) followed by incubation with streptavidin-phycoerythrin (SAPE). Mean fluorescence of kinase-treated cells is quantified by flow cytometry and compared with mean fluorescence of kinase untreated cells.

**Validation of BacKin assay**

To validate the BacKin assay, the fluorescence emission of abltide-expressing cells was compared with that for cells expressing eCPX scaffold (Figure 3). After 30 min incubation with Abl kinase, ~96% of abltide-displaying cells were fluorescent, whereas eCPX-displaying cells were not fluorescent even after 2 h of incubation.
The observed fluorescence profile reveals that: i) abltide is efficiently displayed at the cell surface; ii) surface exposed abltide can be efficiently phosphorylated by Abl kinase and iii) phosphorylated abltide can be fluorescently labeled by anti-phosphotyrosine and SAPE.

**Characterization of Abl kinase activity using BacKin assay**

To evaluate whether the BacKin assay could be used to characterize Abl TK activity, phosphorylation of abltide was studied as a function of kinase and ATP concentration and reaction time. The results in Figure 4A show an increase in abltide phosphorylation with the kinase concentration, followed by a steady state. The concentration required to phosphorylate half of the abltide molecules displayed on the surface of E. coli cells was found to be 0.26 ± 0.02 μM/L. At a concentration equal to, or higher than 0.5 μM/L Abl TK, almost all the abltide molecules are phosphorylated; therefore, 0.5 μM/L Abl TK was the kinase concentration chosen to conduct the following kinase studies.

To examine the effect of ATP on the kinase reaction, concentrations ranging from 0.5 to 500 μM of ATP were tested in a 30 min reaction (Figure 4B). As expected, the phosphorylation of abltide catalyzed by Abl TK is ATP-dependent and no reaction occurs in the absence of ATP. The ATP concentration required to phosphorylate half of the abltide molecules was found to be 18.2 ± 0.7 μM. To guarantee that ATP is not a limiting reagent 500 μM ATP was chosen to use in the following studies.

The kinetic of the reaction was followed for two hours and fitted with a pseudo-first order kinetics (Figure 4C). The rate constant of abltide phosphorylation catalyzed by 0.5 U/mL Abl TK was found to be (4.4 ± 0.3) × 10^−2 min^−1^ and the half-time of the reaction was 15.7 ± 2.3 min. Altogether the results in Figure 4 confirm that the BacKin assay can be used to characterize and evaluate Abl kinase activity under various conditions.

The reaction time-course was also followed using a cell-free assay and quantified by LC-MS. The reaction was conducted with 60 μM of soluble abltide, 500 μM ATP and 0.5 U/mL Abl TK (see Figure 4C). These conditions yielded a reaction rate of (3.8 ± 0.1) × 10^−2 min^−1^ and a half-time of 18.5 ± 1.3 min. The kinetic curves obtained with the BacKin assay and the LC-MS assay are identical, suggesting that under the conditions tested for the BacKin and cell-free assays the concentration of abltide is high enough to induce the maximum reaction velocity of Abl TK. These results confirm that Abl kinase activity can be efficiently measured using BacKin assay and results can be compared with other methods.

**Inhibition of Abl tyrosine kinase and substrate competition followed with BacKin assay**

To evaluate whether the BacKin assay can be used to characterize kinase inhibitors quantitatively, the kinase inhibition induced by the clinically used drug imatinib (Figure 5A) was studied. Imatinib dose-response was studied with 0.5 U/mL Abl TK and 500 μM, or 50 μM, ATP for 30 min. Ablide phosphorylation decreased with increasing concentrations of imatinib. Comparison of inhibition curves obtained with 50 μM vs. 500 μM ATP confirmed that inhibition of Abl kinase by imatinib is ATP-dependent with a 10-fold difference in IC50, but with identical Hill slope (Table 1). These results are consistent with imatinib and ATP competing for the same binding site and IC50 values obtained are comparable with previously reported values [20,27,28].

The BacKin assay can also be used in substrate competition studies as shown here with soluble abltide and with the analogue [Y4F,F8Y] as a negative control (Figure 5B). Incubation of abltide-expressing cells with increasing concentrations of soluble abltide induced a decrease in the fluorescent signal, suggesting that the soluble peptide and the cell-immobilized peptide compete for the enzymatic catalytic center. The analogue [Y4F,F8Y], although

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**Figure 3. Validation of BacKin assay examined with flow cytometry.** (A) Dotplot of side scatter and fluorescence emission intensity at 585/42 nm for eCPX-displaying cells and abltide-displaying cells. After treatment, the percentage of fluorescent cells (shown in green) is ~0.1% in eCPX-displaying cells and ~96% in abltide-displaying cells. The incubation time with kinase is given in brackets. All samples were fluorescently labeled by incubation with biotin-anti-PY and SAPE. F is the mean fluorescence signal obtained for 100,000 cells analyzed.

![Figure 3](image-url)

**Figure 4. BacKin assay kinetic studies.** B is the mean fluorescence signal obtained for 100,000 cells analyzed. (A) Characterization of Abl TK activity using BacKin assay.

![Figure 4A](image-url)

![Figure 4B](image-url)
having a very similar sequence to abltide and with a Tyr residue, is not phosphorylated by Abl kinase and did not inhibit kinase activity in the concentration range tested.

As expected, the effect of ATP concentration on the inhibition of Abl kinase by the substrate abltide is distinct from the effect induced by the ATP-competitor imatinib (see Figure 5 and Table 1). Whereas the dose-response curve for imatinib has the same Hill slope but a 10-fold difference in IC50, the inhibition curve profile induced by abltide differs approximately 2-fold in the Hill slope and IC50 values when 500 μM ATP is compared with 50 μM ATP. The ATP concentration affects enzymatic kinetics (see Figure 4B), which explains the differences in the abltide IC50 and Hill slope with ATP concentration.

Considerations for optimization of BacKin assay

However good was the reproducibility obtained with the BacKin assays, pitfalls might arise from using live organisms to display the peptide substrate. In particular, variable density of expressed peptide on the bacterial membrane might occur. As the kinetics of enzymatic reactions are dependent on the substrate concentration, the amount of abltide molecules expressed on the bacteria influences the reaction. Nevertheless, a good reproducibility was obtained when growth and induction conditions were kept constant. Indeed larger variability was found when different batches of kinase or if different kinase buffers were used (e.g. commercially available vs. prepared in house).

Although in the current study the assay was conducted using a tube-based approach, in principle it can be optimised into an automated HTS using a 96-well plate configuration compatible with many FC instruments. With such a configuration the volume and kinase concentration per sample could be further decreased.

Conclusion

Using Bcr-Abl TK, a recognized oncogenic target, we developed a new cost-effective kinase assay. This non-radioactive BacKin assay, based on detection of phosphorylation of peptide substrates displayed on the bacteria surface and quantification by FC, can be used to: i) characterize and measure kinase activity; ii) screen small-molecule or peptide-based inhibitors or substrates; iii) compare and identify kinase substrates and iv) obtain mechanistic information on inhibition modes of action.

Figure 4. Characterization of Abl kinase using the BacKin assay. Mean fluorescence was converted into fraction of phosphorylation, assuming maximum response as 100% of abltide phosphorylation. Each data point is the average of three independent experiments ± SD (A) Abtide phosphorylation catalyzed by increasing concentrations of Abl kinase in the presence of 500 μM ATP and incubated for 30 min at 37°C. The kinase concentration required to achieve half of the maximum response, K0±SD, was calculated by fitting a nonlinear sigmoidal curve. (B) ATP concentration effect on abtide phosphorylation catalyzed by 0.5 U/mL Abl Kinase for 30 min at 37°C. The ATP concentration required to achieve half of the maximum response, K0±SD, was calculated by fitting a nonlinear sigmoidal curve. (C) Time-course of abtide phosphorylation catalyzed by 0.5 U/mL Abl kinase in the presence of 500 μM ATP at 37°C obtained with BacKin assay (circles) or with LC-MS assay (squares). The time required to phosphorylate half of the abtide molecules, t1/2±SD, was calculated by fitting a pseudo-first order association kinetics.

doi:10.1371/journal.pone.0080474.g004

Figure 5. Kinase inhibition study using the BacKin assay. Inhibition of surface-displayed abtide phosphorylation by (A) imatinib or (B) soluble abtide. Reaction was catalyzed with 0.5 U/mL Abl kinase in the presence of 50 (squares) or 500 μM ATP (circles) and incubated for 30 min at 37°C. Mean fluorescence was converted into percentage of inhibition. Each data point representing the average of three independent experiments ± SD and data were fit with a sigmoidal curve. The abtide analogue [Y4F,F8Y] is shown in black circles and was tested with 500 μM ATP, average of two replicates are shown.

doi:10.1371/journal.pone.0080474.g005
Importantly, peptide libraries displayed on the surface of bacteria [29] can be used in this approach to identify kinase peptide substrate/inhibitors. Bacterial peptide libraries are readily amplified by growth and are self-renewing; therefore, BacKin assay can be used to screen such libraries in a cost effective way to identify potential peptide-based leads. Clones that express positive hits can be selected and isolated, individually tested and sequenced and developed as potential drug leads for cancer treatment. This is a major advantage compared with a LC-MS approach, which even though applicable to screen and identify peptide substrates, would require all peptides to be screened and sequenced individually and therefore would not be appropriate to screen large libraries.

Although the BacKin assay was developed with abtlide-displaying cells and Abl kinase, it could be similarly applied to other kinases and their respective substrates. The development of efficient screening techniques for measuring kinase activity is of major importance for drug research, as malfunction of protein TK activity is a hallmark of numerous diseases, including cancers, diabetes and immune diseases.

Acknowledgments

Dr. Quentin Kaas (UQ, IMB) is acknowledged for help with Pymol and the Abl kinase model.

Author Contributions

Conceived and designed the experiments: STH LT YHH JAG. Performed the experiments: STH LT YHH JAG. Analyzed the data: STH LT YHH. Contributed reagents/materials/analysis tools: PSD DJC. Wrote the paper: STH DJC.

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Table 1. Kinase inhibition parameters[a]

|          | IC50 (μM)          | H      |
|----------|--------------------|--------|
| Abltide  | 50 μM ATP          | 0.040±0.004 | 0.71±0.05 |
|          | 500 μM ATP         | 0.409±0.040 | 0.71±0.05 |
| Imatinib | 50 μM ATP          | 21.51±2.64 | 0.89±0.10 |
|          | 500 μM ATP         | 37.47±3.95 | 1.80±0.31 |

[a]Inhibitory concentration (IC50) and Hill slope (H) determined by fitting dose-response plots with a sigmoidal curve (see Figure 5).