MOLECULAR SENSING

APPLICATION NOTE

# Label-free, Free-Solution Binding Characterization of Bcr-Abl Kinase Inhibitors by Back-Scattering Interferometry


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## ABSTRACT

This application note describes the use of Back-Scattering Interferometry (BSI) to characterize the binding affinity of Bcr-Abl kinase inhibitors (dasatinib, imatinib, and nilotinib) with wild type and four mutant Bcr-Abl kinases (H396P, M351T, Q252H, and T315I).

BSI successfully demonstrated facile determination of equilibrium dissociation constants ($K_d$) for all systems, with a high degree of concordance with competition assay derived $IC_{50}$ results. Using crude cell lysates (27% purity) and positive control compounds, binding assays were optimized within a couple of days of experimentation, and for the entire study, less than twenty micrograms of total enzyme was consumed.

These results indicate that BSI binding studies for both class I and class II kinase inhibitors can easily be performed, allowing for confirmation of target engagement as well as direct binding assessment of type 2 kinase inhibitors against inactive Bcr-Abl kinase.

In this fashion, BSI analysis has demonstrated superiority when compared to radioligand and other typically employed activity assays, uniquely positioning the technology to advance research in the search for new second and third generation kinase inhibitors.

Taken collectively, the unique strengths of BSI make it an attractive biophysical technique for the study of second and third generation kinase inhibitors to address the challenges of kinase inhibitor drug resistance.


## INTRODUCTION

In addition to playing an essential role in cellular energetics, kinases and their associated signaling pathways are principally responsible for the regulation of intracellular processes. When abnormally expressed or controlled, kinase activity can cause cellular dysregulation and contribute to the onset of several diseases, including cancer.

Based on the fundamental understanding of kinase malfunction in cancer biology, the discovery of small organic molecules to alter kinase function has culminated in the development of targeted cancer therapy[1].

As of now, a dozen kinase inhibitors are on the market and several hundreds more are in clinical trials. However, limited selectivity and the emergence of drug resistance remain fundamental challenges for current modern medicinal chemistry research for the development of kinase inhibitors that are effective in long-term treatments[2].

Chronic myelogeneous leukemia (CML) is caused by the Bcr-Abl oncogene, which encodes a chimeric Bcr-Abl (breakpoint cluster region, abeleson) protein that has constituently activated AB1 tyrosine kinase activity[3-5]. In 2000, O'Dwyer demonstrated the potential treatment of CML using the kinase inhibitor STI571, later known as imatinib or Gleevec®[6].

Most known kinase inhibitors are Type I inhibitors, ATP-competitive compounds such as staurosporine, erlotinib (Tarceva®) and dasatinib (Sprycel®), that bind to the ATP binding site and hydrogen bond with the hinge region of the kinase.

Type II inhibitors are compounds which bind partially in the ATP binding site and extend past the gatekeeper and into an adjacent allosteric site that is present only in the inactive kinase conformation. Compared to Type I inhibitors, Type II inhibitors have been shown to possess advantageous pharmacological properties, including improved target specificity[2]. As such, many Type II inhibitors currently on the market, such as imatinib (Gleevec®), are very effective anti-cancer drugs.

Mutations resistant to classical ATP-competitive (Type I/II) inhibitors are emerging at a rapid pace and often limit the success of newly available targeted cancer therapies. Point mutations at the gatekeeper position, in which a relatively small amino acid side chain (classically Thr) is mutated into a larger hydrophobic residue (Ile or Met), are common among protein kinase targets in cancer such as Bcr-Abl (T315I)[7]. Such mutations often result in a steric hindrance that obstructs inhibitor binding to the hinge region of the ATP pocket.

For example, imatinib affinity to T315I is less than that for wild type Bcr-Abl[8]. Three other Bcr-Abl mutants, M315T and Q252H[9] as well as H396P[10], have been isolated and associated with clinical relapse following initial response to imatinib. At present, there are more than 50 mutation sites and more than 70 individual mutations conferring different levels of imatinib resistance found within CML patients [9, 11, 12]. As such, the need to identify and develop reversible inhibitors that are resistant to such mutations and bind with a high affinity is the focus of many academic and industrial research projects.

Compounds binding exclusively to less-conserved allosteric sites outside the ATP pocket (Type III inhibitors) bind beyond the gatekeeper residue and are expected to have superior selectivity profiles and represent new antineoplastics. This allosteric site is formed when the activation loop, a crucial structural component of the substrate binding cleft participating in the recognition of substrates and influencing the arrangement of the catalytic residues, adopts the DFG-out conformation characteristic of inactive kinases[2, 13]. Hence, the identification of allosteric inhibitors that bind to and stabilize inactive kinase conformations is expected to provide valuable insights into the development of new chemical principles to address the challenges of limited selectivity and drug resistance[14].

Because classical kinase inhibition assays rely upon the arrest of kinase activity, such assays must, by definition, be applied to activated kinases in the DFG-in conformation[15, 16]. As such, kinase inhibition assays do not lend themselves well to the discovery of Type II or Type III kinase inhibitors.

Back-Scattering Interferometry (BSI) is a label-free, free-solution molecular interaction technology that has demonstrated ability to characterize small molecule – large target interactions[17]. BSI functions by optically detecting changes in molecular conformation and hydration state. Class I kinase inhibitors, such as staurosporine, have been have been linked to significant induced-fit rearrangement of the enzyme, resulting in demonstrable target conformational change by x-ray crystallography[18]. Recently, BSI has been used to characterize allostery in the M4 muscarinic receptor as expressed in a commercial CHO cell line[19].

The authors propose that the inherent strengths of BSI to detect target conformational change in a free-solution/label free manner, could manifest as a valuable new biophysical means to advance kinase inhibitor research.

In this study, we have applied BSI to study the interaction of Type I (dasatinib and nilotinib) and Type II (imatinib) kinase inhibitors to wild type Bcr-Abl kinase

as well as to Bcr-Abl H396P, M351T, Q252H, and T314I mutants. Overall assay design and performance was straight forward, making for facile determination of direct binding affinity. The demonstrated strengths of BSI to measure small molecule inhibitor binding to both active and non-activated Bcr-Abl combined with its ability to measure direct binding in allosteric systems make BSI an amenable tool for the discovery of type III kinase inhibitors.

## MATERIALS AND METHODS

### Back-Scattering Interferometry

The Back-Scattering device is a micro-scale interferometer with a simplified optical bench (see figure 1). The BSI device consists of a light source (HeNe laser), an object (the microchip), and a detector (CCD Array). Central to the system is the fluidic microchip. The microchip receives coherent light from a HeNe laser. Light impinges upon the sample containing channel. As light passes into the channel, combinations of reflection and refraction at the air-chip interface (front and back) as well as fluid-chip interface creates constructive and destructive interference fringe patterns in the back-scattered direction. These fringes are collected by a mirror and directed to strike a video camera (CCD or CMOS array) that images the fringes as shown in Figure 1.

When molecules combine, the resultant complex causes a constitutive change in molecular mean polarizability that is measured in the detection zone as a phase shift in many of the frequencies that comprise the interference pattern. Monitoring the change in phase as a function of ligand concentration allows equilibrium dissociation constant, $K_d$, measurements to be performed.

The BSI chip is essentially a flow cell, in which previous samples are displaced with subsequent injections. Unlike many other bio-sensing techniques, with BSI there is no need for surface regeneration, as each measurement is truly performed in free solution.

### *Setting up the BSI Assay*

Free solution BSI $K_d$ determinations are performed in end-point fashion, with target and ligand pre-incubated to establish equilibrium. Target and ligand concentrations are chosen to initiate pseudo-first order binding conditions, for which the target is typically held at sparing concentration and the ligand in excess to insure against depletion during the binding process.

The concentration of target can range from pM to µM depending on the $K_d$ of the ligand – target equilibrium. A series of range-finding experiments are launched to determine the optimal concentration of the target, which is ideally established to be about one-tenth of the system's $K_d$. Usually, the analyte concentration ranges from one-fifth to 10 times the concentration of the system $K_d$.

Figure 2 illustrates the RI change for a constant concentration of target A (light blue trace, constant RI), the increase in RI as ligand B is increased (red trace) as a control and finally, the binding isotherm RI curve for the AB complex after mixing and equilibration of A and B (dark blue trace). In practice, control B is run simultaneously in a reference channel with complex AB probed in the analytical channel. The difference is plotted as AB-B against an offset of A. A single site binding isotherm model is fitted to the AB curve to determine the binding maximum or $B_{max}$. $K_d$ is then established as ½ $B_{max}$.

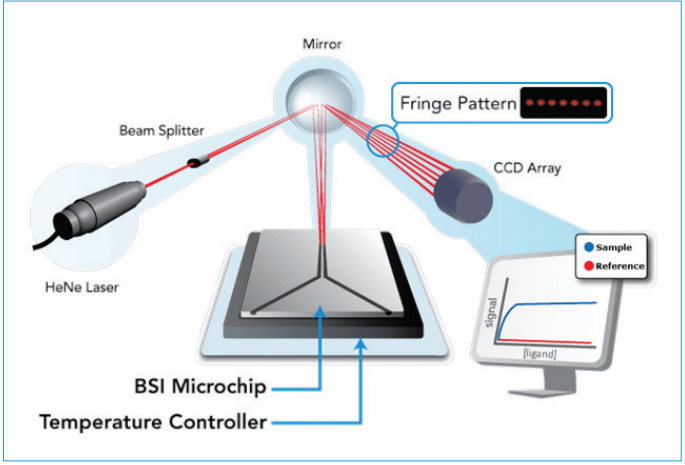


**Figure 1:** *Back-Scattering Interferometer schematic. Light from a HeNe laser is split into two beams and then directed to strike a microfluidic chip that serves as an interferometric chamber. The resultant interference fringe pattern is picked up by a mirror and directed to strike a camera. See text for further details.*

### *Preparation of Bcr-Abl Kinase Target Solutions*

Wild-type Bcr-Abl Kinase as well as H396P, M351T, Q252H, and T315I mutants were sourced from Millipore (EMD Millipore, Darmstadt, Germany). All Bcr-Abl were expressed via baculovirus in Sf21 insect cells and provided in aliquots of 10 ug of enzyme (27% purity) in 100 uL of 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 270 mM sucrose, 1 mM benzamide, 0.2 mm PMSF, 0.1 mm EGTA, 0.1% 2-mercaptoethanol, 0.03% Brij 35 and kept frozen at -70° C until used. Assay and diluent buffer was 8 mM MOPS, pH 7, 10 mM Mg Acetate, 0.2 mM EDTA, and 1% DMSO. Kinase working solutions were created by diluting the above noted stock solutions to concentrations that approximated 1/50 of the target $K_d$ for each system (concentration range: 0.5 nM – 10 nM).

### *Preparation of Kinase Inhibitors*

Imatinib, dasatinib, and nilotinib were purchased from LC Laboratories (Woburn, MA, USA) and were first brought up as 50 mM working stocks in 100% DMSO. Dose response series were created by diluting each working stock with the a fore mentioned MOPS buffer to establish the appropriate target concentration range for each binding system using a 12-point doubling dilution approach (range: 50 pM – 125 µM).

### *Assay Sample Preparation and BSI Analysis*

BSI binding affinity determinations are performed under equilibrium based conditions. As such, ligands and targets are mixed together and incubated to allow equilibrium to establish and the mixtures are then read in end-point, steady-state fashion. Ligand – target stoichiometry is arranged in order for pseudo-first order kinetics to dominate, for which typically the target is presented in sparing amounts and the ligand in excess. For these Bcr-Abl kinase – kinase inhibitor assays, enzyme concentration range was varied from 0.5 nM to 10 nM, while kinase concentration ranged from 50 pM – 125 µM, dependent upon binding system anticipated $K_d$.

Two incubation series were created: assay and control. The assay concentration series consisted of equal volume mixtures of inhibitor and kinase for each point in the dose response series, while the control series comprised the dose response series alone adjusted to match the assay series' final inhibitor concentration by diluting with assay buffer. Samples were prepared using 250uL Eppendorf tubes, and allowed to incubate for four hours at room temperature (typically 22° C).

BSI measurements were performed using a dual-channel BSI prototype system (Molecular Sensing, Inc., Nashville, TN, USA). The assay series were analyzed in channel one while the control series were concom-

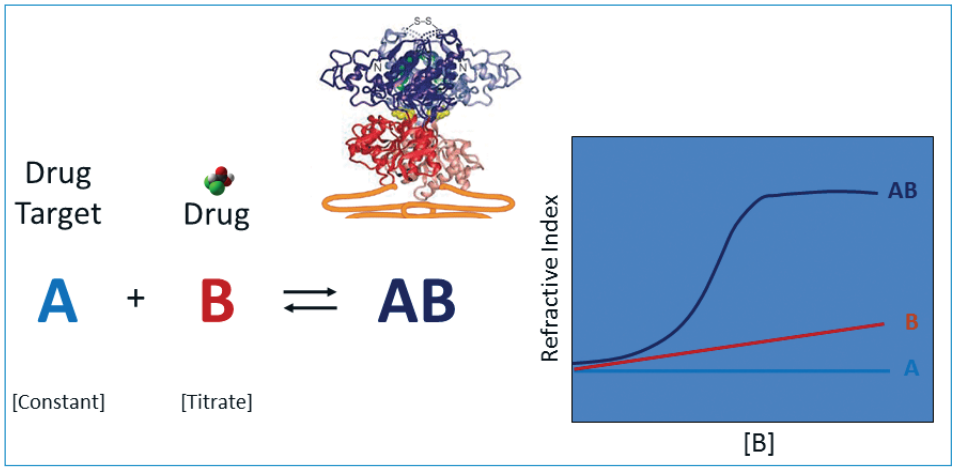


**Figure 2:** *BSI sample preparation. Drug target and lead compound are mixed together in stoichiometric proportion to insure the creation of pseudo-first order kinetics, for which the target is maintained at sparing concentrations. Mixtures are pre-incubated to establish equilibrium prior to analysis in the BSI device. See text for further details.*

itantly analyzed in channel two. Each sample was measured in triplicate.

BSI signals were generated using a proprietary algorithm that analyzes phase change for the principle spatial frequencies that comprise the fringe pattern. Difference plots of assay minus control for BSI response at each inhibitor concentration were constructed using GraphPad Prism® (San Diego, CA, USA). Single-site binding model fits were performed to determine the binding maximum ($B_{max}$) and $K_d$ determined as ½ $B_{max}$.

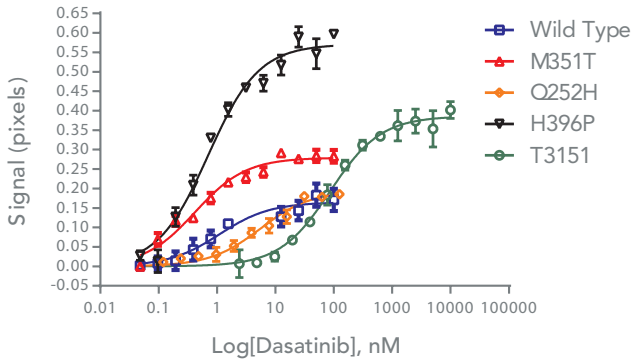


**Figure 3:** *BSI analysis of Dasatinib binding against Bcr-Abl kinase Wt and H396P, M351T, Q252H, and T315I mutants. All binding systems achieved saturation and appropriate $K_d$ determination easily ensued. See text for further details.*

# RESULTS

## Kinase Inhibitor Binding Curve Analysis

The results for measurements of dasatinib, nilotinib, and imatinib binding affinity to wild-type and mutant Bcr-Abl Kinase are respectively illustrated in Figured 3, 4, and 5. The overall binding affinity for these systems is summarized in Table 1. For each of these systems, resultant assays produced a high degree of concordance between replicate measurements (avg $K_d$ % RSD < 25 %). Table 1 also lists the final concentration of Bcr-Abl Kinase used in each assay. For most of these assay systems, total experimentation consumed on the order of 6.25 mL of enzyme solution, making the total protein consumption quite low [range 72 picomole ( 9 µg) – 0.156 picomole (0.02 µg)].

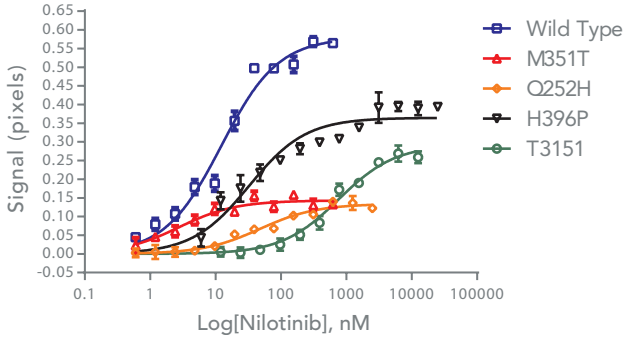


**Figure 4:** *BSI analysis of Nilotinib binding against Bcr-Abl kinase Wt and H396P, M351T, Q252H, and T315I mutants. See text for further details.*

# DISCUSSION

## Comparison with known $IC_{50}$ Data

Table 2 compares the obtained binding equilibrium constants for each system with previously reported $IC_{50}$ data as compiled by O'Hare et. al[20]. Figure 6 depicts the correlation between BSI obtained $K_d$ and $IC_{50}$ values for the studied systems (linear fit R2 = 0.9744). As is clearly demonstrated, BSI binding results highly correlated with previously compiled kinase activity inhibition assays. Moreover, determined $K_d$ values for each system are in high agreement with various modeling and x-ray crystallographic studies[21-24].

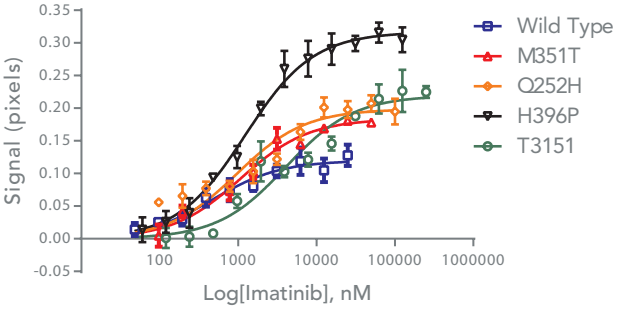


**Figure 5:** *BSI analysis of Imatinib binding against Bcr-Abl kinase Wt and H396P, M351T, Q252H, and T315I mutants. See text for further details.*

## Kinase inhibitor affinity to Bcr-Abl Kinase wild type and mutants

Specific mutations in various domains of Bcr-Abl kinase have been identified that ostensibly alter the overall effectiveness of kinase inhibitors.For this specific study, mutants were selected that represented four different mutations in distinct domains within wild type Bcr-Abl kinase: Q252H (P-Loop domain); T315I (ATP binding region); M351T (SH-2 contact); and H396R (A-Loop domain)[25].

Table 3 lists the overall fold differences for each of these mutants in terms of changes in observed $IC_{50}$ as reported by Raedelli et. al as well as mutation dependent changes in measured binding affinity by BSI. As can be seen, observed BSI 3derived binding affinities substantially agree with $IC_{50}$ results, indicating that BSI could be used as a convenient means to predict potency.

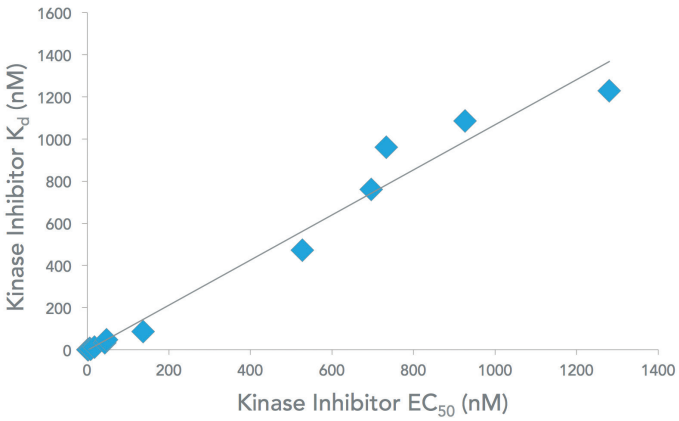


**Figure 6:** *Correlation of BSI determined $K_d$ and $IC_{50}$ for the studied kinase inhibitor systems. Determined $K_d$ values for each system are in high agreement with various modeling and x-ray crystallographic studies. See text for further details.*

## FINAL CONCLUSIONS

This study has illustrated the utility of BSI in the analysis of type I and type II inhibitors of wild-type Bcr-Abl kinase as well as four different imatinib resistant mutants.

The overall assay development process was quite facile, requiring only a couple of days and consuming minimal amounts of precious target. Assay fidelity was quite high, with substantial agreement of results for three independent replicate analyses for each system. Obtained BSI kinase inhibitor affinities agreed well with previously reported $IC_{50}$ values and are consistent with theoretical and x-ray crystallographic studies of the same systems, lending credence to the overall approach as a viable means to study potency for kinase inhibitors.

Because BSI evaluates direct target engagement independent of downstream activity, BSI is aptly suited to advance efforts in the discovery of new type II and allostery driven type III inhibitors to Bcr-Abl Kinase as well as other valuable kinase targets of medical import.

| Assay System | $K_d$ (nM) | [Bcr-Abl Kinase] (nM) |
|---|---|---|
| Dasatinib-WT | 1.10 +/- 0.24 | .025 |
| Dasatinib-H396P | 0.70 +/- 0.09 | 0.025 |
| Dasatinib-M351T | 0.42 +/- 0.065 | .025 |
| Dasatinib-Q252H | 5.53 +/- 0.81 | 0.5 |
| Dasatinib-T315I | 86.5 +/- 6.7 | 3.9 |
| Nilotinib-WT | 12.3 +/- 1.8 | 0.5 |
| Nilotinib- H396P | 32.6 +/- 6.5 | 0.5 |
| Nilotinib- M351T | 2.7 +/- 0.5 | 0.25 |
| Nilotinib- Q252H | 47.6 +/- 8.57 | 0.25 |
| Nilotinib- T315I | 761 +/- 100 | 10 |
| Imatinib-WT | 472 +/- 83 | 4.86 |
| Imatinib- H396P | 1228 +/- 92 | 13 |
| Imatinib- M351T | 1068 +/- 176 | 11.53 |
| Imatinib- Q252H | 961 +/- 272 | 9.72 |
| Imatinib- T315I | 4050 +/- 1001 | 11.53 |

**Table 1:** *BSI determined binding $K_d$ of desatinib, nilotinib, and imatinib against wild-type and mutant Bcr-Abl kinase. Final Bcr-Abl kinase enzyme concentrations for each assay system are also indicated. See text for further details.*

| | Dasatinib (nM) | | Imatinib (nM) | | Nilotinib (nM) | |
|---|---|---|---|---|---|---|
| Bcr-Abl Kinase | $IC_{50}$ | $K_D$ | $IC_{50}$ | $K_D$ | $IC_{50}$ | $K_D$ |
| Wild type | 1.83 | 1.08 | 527 | 472 | 17.69 | 13.3 |
| M351T | 1.61 | 0.42 | 926 | 1086 | 7.8 | 2.7 |
| Q252H | 5.6 | 5.49 | 733 | 961 | 46.7 | 47.6 |
| T315I | 137 | 86.5 | 9221 | 4050 | 696 | 761 |
| H3696P | 1.95 | 0.7 | 1280 | 1228 | 42.6 | 32.6 |

**Table 2:** *Comparison of BSI determined binding affinity for dasatinib, nilotinib, and imatinib for wild-type and mutant Bcr-Abl kinase as compared to determined $IC_{50}$ values obtained by radio-labeled abl-tide assays.*

| | Dasatinib | | Imatinib | | Nilotinib | |
|---|---|---|---|---|---|---|
| | Redaelli | BSI | Redaelli | BSI | Redaelli | BSI |
| Abl wt | 1 | 1 | 1 | 1 | 1 | 1 |
| M351T | 0.88 | 1.42 | 1.76 | 2.31 | 0.44 | 0.20 |
| Q252H | 3.05 | 5.13 | 1.39 | 2.03 | 2.64 | 3.60 |
| T315I | 75.03 | 80.11 | 17.50 | 8.63 | 39.41 | 57.22 |
| H396P | 1.63 | 0.65 | 3.91 | 2.60 | 3.12 | 2.45 |

**Table 3:** *Comparison of BSI determined fold in binding affinty for dasatinib, nilotinib, and imatinib for wild-type and mutant Bcr-Abl Kinase as compared to determined $IC_{50}$ fold increases by Redaelli et. al. See text for further details.*

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Company

Molecular Sensing, Inc. (MSI), is a commercial stage drug discovery tools and contract research services company with headquarters and drug discovery services laboratories in Nashville, Tennessee and an R&D center in Los Gatos, California, along with a European operations center near Frankfurt, Germany.

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