

# Integrated Drug Discovery in Continuous Flow

Gabrielle S. Fleming and Aaron B. Beeler\*

Department of Chemistry, Boston University, 590 Commonwealth Avenue, Boston, Massachusetts 02215

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There are great opportunities for innovation in the drug discovery process, particularly in the lead development phase. The traditional “design–synthesize–screen” cycle has seen little innovation as a whole despite major advances at each stage, including automated purification and synthesis as well as high throughput biological screening. It could be argued that the hit-to-lead and lead optimization processes remain slow and modular with inefficient flow of information, resulting in a loss of time and money. New flow technologies may provide a promising foundation for developing a continuous integrated small molecule optimization platform that would greatly enhance hit-to-lead and lead optimization programs. Herein, we discuss major developments in integrating synthesis, purification, screening, and machine learning into a single continuous-flow platform and provide some insight into future directions of this field.

**Keywords:** Drug discovery, medicinal chemistry, integrated drug discovery

## Introduction

Drug discovery and optimization technologies must continue to evolve to overcome high attrition rates at every stage of the development process. Arguably, the hit-to-lead and lead optimization phases remain a critical and rate-limiting step in the development process and present great potential for new enabling technologies and innovations. In this regard, there have been exciting advancements in the medicinal chemistry optimization toolbox. For example, development and application of new and innovative chemical reactions provide tools to access previously underexplored areas of chemical space, a deeper understanding of biological targets has led to more effective and relevant assays, and significant technological advances have increased the throughput of biological assays. However, there remains an opportunity to transform the way in which we approach the “design–synthesis–screen” cycle as a whole, which lies at the heart of hit-to-lead and lead optimization.

Key factors that dictate the efficiency of the traditional and iterative design–synthesize–screen cycle include time, success rate, and cost. Although medicinal chemists can access larger swaths of chemical space and generate more robust structure activity relationship (SAR) models, the major bottleneck in the cycle is at the interface of biological evaluation and chemical synthesis. Often, when an analog library is sent for biological analysis, the time required to obtain and apply the data for rational design of subsequent analogs is a major source of time and monetary loss. To push the current limits of lead development, programs will require integrated cycles that are shorter and more efficient, and provide rationally designed libraries.

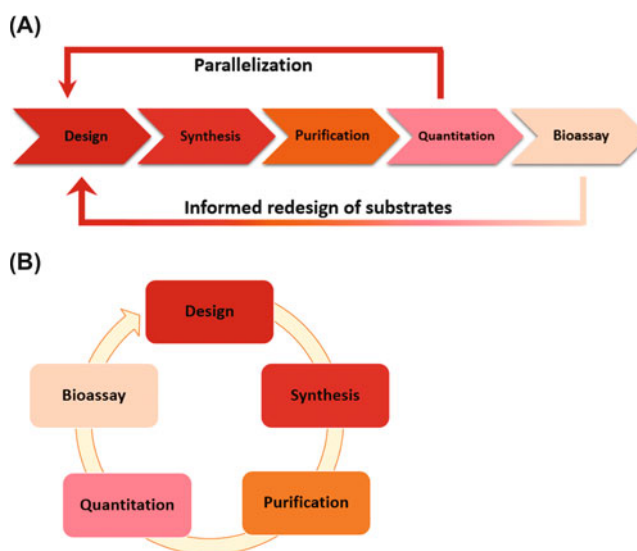
The solutions most often proposed to streamline drug lead development are parallelization and miniaturization. Parallelization is the most common approach for minimizing time between biological screening and design of substrates but can result in synthesis of irrelevant chemical analogs, which is an unwanted loss of time and money. Miniaturization has long been utilized as a potential solution for streamlining drug discovery by reducing time and costs associated with the process [1–3]. Such efforts have often focused on using microreactors and microfluidic devices for chemical library synthesis and biological screening. Specific aspects of microreactors in synthesis [4–6] and biological analysis [7, 8] have been reviewed extensively and will not be in the focus of this perspective. Instead, we will discuss the potential for implementing these technologies for integrated and automated

flow systems for lead optimization which could provide streamlined processes and more efficient flow of information.

Continuous flow could be an ideal platform for complete integration of the early stages of drug discovery. There have been significant advances in both chemical synthesis in flow and biological screening in flow and the advantages of continuous flow have pushed the boundaries of scope and efficiency in both fields. Furthermore, the continuous nature of the individual technologies makes them uniquely suited for integration into a single system which could significantly improve the time, success, and cost of the cycle, leading to a new paradigm in drug discovery (Figure 1).

## Library Synthesis, Purification, and Analysis in Flow

Continuous-flow technologies have enabled chemists to access new and more efficient processes and to push the boundaries of traditional chemical transformations [9]. The ability to run reactions safely under traditionally challenging conditions at high temperatures, pressures, and concentrations on a large scale has resulted in

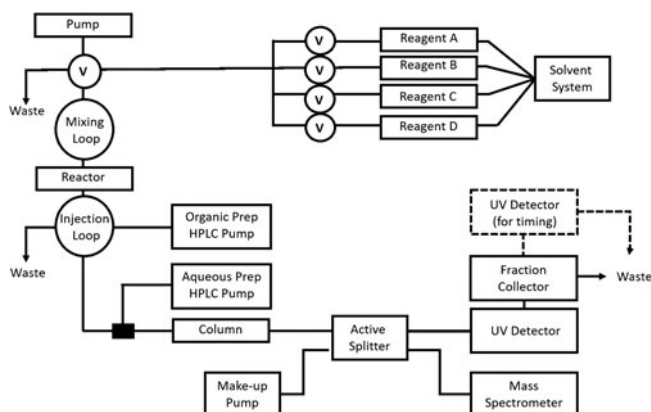


**Figure 1.** (A) The current state of the design–synthesize–screen cycle of lead development. The feedback loop between bioassay results and design of new substrates breaks the cycle and results in a time lag. A potential solution is parallelization, but uninformed design of substrates can result in synthesis of irrelevant chemical examples. (B) Integration of continuous flow to close the design–synthesize–screen cycle

\* Author for correspondence: beelera@bu.edu

adoption of continuous-flow chemistry in nearly all aspects of chemical synthesis [10–12]. Adaptation of syntheses of many active pharmaceutical ingredients (APIs) and drugs to continuous flow have also been reviewed extensively and will not be covered in this perspective [13, 14]. In addition to being an enabling technology for new chemical reactions, one of the most enticing aspects is that it affords the possibility of automation, telescoping reactions, inline purification, and even downstream inline biological assays. Nonetheless, there remain significant challenges when considering chemical library synthesis compared to single compound synthesis in flow, many of which are still unsolved. However, seminal efforts have demonstrated and highlighted the potential.

In 2011, Djuric and coworkers reported the development of a fully integrated synthesis and inline purification flow system, synthesis with integrated-flow technology (SWIFT) [15]. The platform was suitable for synthesis of 48-member libraries which could be made available for biological evaluation within 5 days, as compared to a typical two-week synthesis time using batch synthesis and purification. They achieved this by integrating high-performance liquid chromatography (HPLC) pumps for continuous flow and adapting software for validated structure analysis. In 2014, they improved upon their system by adding complete automation using more advanced custom software to reduce instrument face time for researchers, and ultimately reducing library synthesis time to 3 days (Figure 2) [16]. After completion of the reactions, crude mixtures (directed by automation) can be purified by organic and/or aqueous prep HPLC. An active splitter can then send compound for analysis, as well as to a



**Figure 2.** Schematic to illustrate the automated flow synthesis and purification using SWIFT, developed by Djuric and coworkers. Forty-eight-member libraries are made available for biological evaluation within 3 days

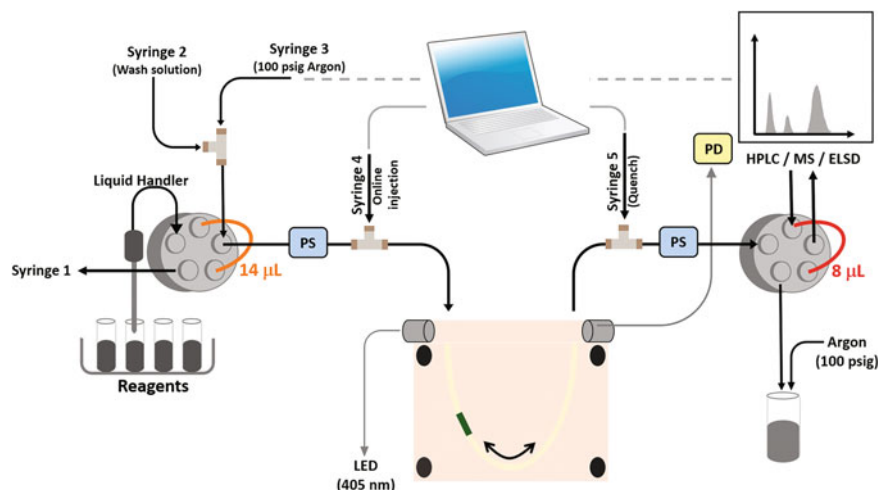
fraction collector directed by ultraviolet (UV) detection. This automated system demonstrated the ability for expedited single flow synthesis and significantly reduced the time required for library synthesis.

These examples provided innovative application of continuous flow for library synthesis. More recently, Djuric and coworkers reported a fully integrated and automated drug discovery platform, validated by testing two libraries of binders of polycomb protein EED [17]. However, challenges of throughput, purification, and integration into biological assays resulted in implementing batch-supported library synthesis. The library turnaround time is reduced to just 24 to 36 hours, which can be greatly attributed to the parallel synthesis of library members.

As was evident in the efforts by Djuric and coworkers, the serial nature of flow library synthesis can significantly minimize throughput. This is a problem that could be overcome by using multiple flow reactors in parallel to expedite compound synthesis. However, a drawback of running reactions in parallel, whether in flow or batch, can be that there is little room or incentive to optimize or to fine tune individual reactions. In flow, optimization can be particularly challenging because reactor volume, flow rate, and residence time are intimately linked and as such, optimization requires running individual reactions or even designing reactors for each tested condition. Jensen and coworkers recently disclosed their design of a horse-shoe shaped oscillatory flow reactor (OFR), which effectively decouples residence time and flow rate and highlights the advantages of using flow to run micro-scale reactions, thereby reducing waste and costs (Figure 3) [18].

The OFR is designed for the generation of small, targeted libraries and runs single microliter scale reactions individually, using inert gas as a carrier fluid. After the desired residence time, the reaction is quenched and subsequently transitioned to a linked HPLC–mass spectrometry (MS)–evaporative light scattering detector (ELSD) for analysis and UV or MS triggered collection. The short and adjustable residence time allows for efficient reaction optimization and a single reactor size and volume for a range of reaction times. As a proof of concept, they synthesized a 36-member compound library from a series of N-X bond forming reactions, as well as an inline multistep synthesis of diclofenac, wherein each reaction was able to be optimized, with little waste of time or materials given the microscale of the reactions [18]. This type of innovation in flow chemistry platforms can transform the way that we approach automated chemical library synthesis and could provide an excellent opportunity for parallel library synthesis in flow.

These examples illustrate the emerging technologies and have laid the groundwork for automated library synthesis in



**Figure 3.** Segmented flow synthesis platform in an oscillatory flow reactor (OFR) developed by Jensen and coworkers

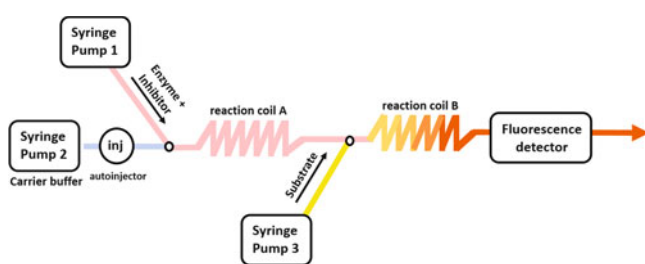
flow. When coupled with the ability to access new chemical space, these technologies are poised to enable more refined complex drug structures with significant time, material, and cost savings. However, there remain some critical challenges to be addressed. The ability to carry out flow reactions in a medium to high throughput parallel manner has not been fully realized. Moreover, in all of the abovementioned examples, purification was carried out offline using single injection HPLC, which is generally the case in library synthesis, and parallelization of purification systems can be cost prohibitive. Thus, there is a clear opportunity for innovative integrated methods for purification which would greatly advance the potential impact.

### Integrated Library Screening

The transfer of newly synthesized analogs for biological evaluation can be affected by spatiotemporal boundaries, thereby creating longer feedback times and delaying downstream chemical design and synthesis. Telescoping biological screening using flow provides an opportunity to remove this bottleneck. Since the early 2000s, there has been significant progress in adapting microfluidic devices for chemical biology applications, including biological screening of compounds [7, 19, 20, 21]. Flow can benefit screening applications by requiring only picoliter volumes of compound solution resulting in reduced waste, improved reproducibility, and shortened analysis time. Furthermore, the nature of mixing in the microfluidic channels can provide precise control over concentration profiles which can result in more accurate and consistent measurements [20].

In one of the earliest examples of biological screening in flow, Hirata and coworkers reported the development of a continuous-flow protease assay that was based on fluorescence resonance energy transfer (FRET), a commonly used screening technique (Figure 4) [22]. This flow assay was created to measure hydrolysis of an appropriately tagged human immunodeficiency virus (HIV) protease substrate in the presence of different inhibitors. In this flow system, enzyme and inhibitor are introduced into the flow system in reaction coil A, carried by carrier buffer from syringe pump 2. Once the protease substrate is introduced, the solution is allowed to reach equilibrium in reaction coil B before fluorescence is measured to evaluate the inhibition of the enzyme. Carrying out the assay with a range of concentrations generated a dose-response profile. Conceptually, this work laid the groundwork for innovative approaches toward developing biological assays in flow.

Following the initial work, Hirata and coworkers used their flow assay technology coupled to a liquid chromatography (LC) system, as a platform for screening complex mixtures for protein inhibitors [23]. However, as Kool and coworkers noted, one drawback of this approach can be relatively high consumption of compound if the system is not miniaturized [21], an issue they were able to overcome with a nano-LC-fluorescent assay hybrid system to ultimately enable identification of the bioactive member of a mixture using only 10 nL. Ultimately, these types of microfluidic biological assays coupled to methods of flow



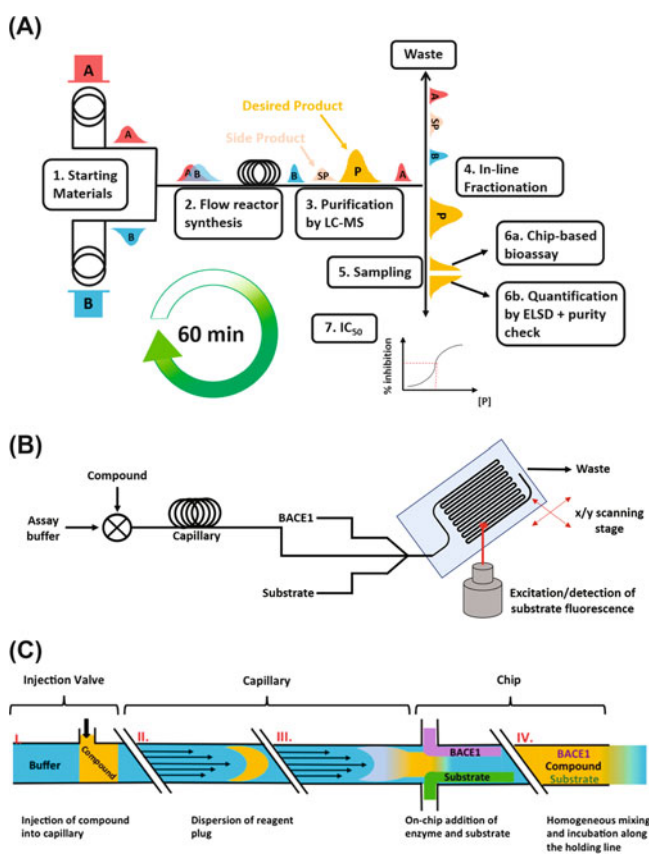
**Figure 4.** Illustration of the flow FRET bioassay developed by Hirata and coworkers

separation could be a very effective approach to screening compound mixtures.

Introducing automated flow chemistry at the front end of a biological assay could provide significant opportunity to streamline the drug discovery process by closing the design–synthesize–screen cycle. However, linking chemical synthesis and purification to biological screening into a single flow platform is a significant challenge that would require complex sampling and quantitation for accurate and meaningful biological results.

To overcome some of these issues, Werner and coworkers disclosed their efforts towards integrating flow-compatible dose-response screening into the synthesis–screening cycle using a  $\beta$ -secretase (BACE1) inhibition assay and known inhibitors to generate SAR [24]. They considered that generation of SAR is at the core of early drug development, and compartmentalized library synthesis and biological evaluation occur in specialized facilities, resulting in feedback that is rarely available prior to 1 week after submission for analysis. By integrating a flow-compatible biological assay, directly attached to inline compound synthesis and purification, they were able to gain valuable SAR information from the generation of a half-maximal inhibitory concentration ( $IC_{50}$ ) within 60 min of reagent injection (Figure 5).

To circumvent some of the automation and sampling challenges, they hypothesized that a concentration gradient could be used for a dose–response formatted assay, which would minimize the need for precise sampling across a range of concentrations. They first developed a flow process that generates a compound concentration gradient of six orders of magnitude in a



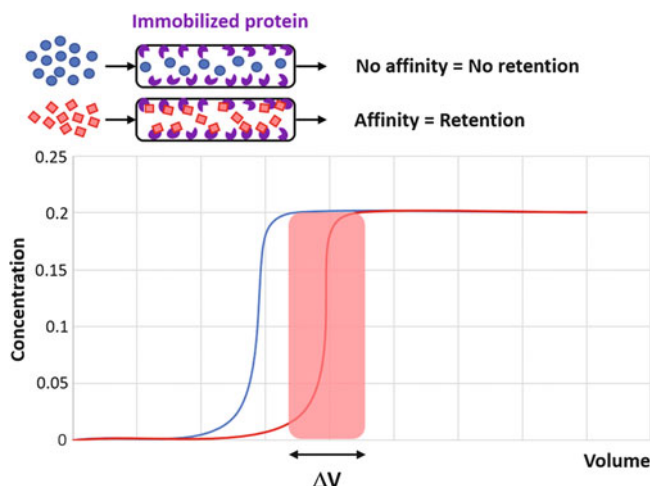
**Figure 5.** Inline gradient assay developed by Werner and coworkers. (A) A depiction of the whole system, including compound synthesis, purification, inline UV-detected fractionation, and fractionation for bioassay and quantitation, all achievable in 60 min. (B) A depiction of the flow bioassay, wherein the concentration gradient is achieved on a glass microchip which allows for detection of fluorescence. (C) A depiction of the concentration gradient achieved, followed by injection of the enzyme and substrate

glass capillary through controlled diffusion. The baseline concentration gradient is established using fluorescein, and fluorescence values are converted into concentrations using an established standard curve. A gradient plug is then generated for the molecule of interest and combined with assay reagents on a glass chip followed by fluorescence measurements across the gradient. They validated the assay using known BACE1 inhibitors, determining  $IC_{50}$  values using the flow bioassay and traditional well plate-based assays, demonstrating very good accordance.

Patel and coworkers have recently reported a microfluidic continuous-flow injection titration assay (CFITA) for monitoring inhibition of thrombin peptidase activity [25]. While this system was not coupled to continuous-flow synthesis, they did make an important observation in regard to the potential of running bioassays in flow. In general, they found that CFITA was an effective method for ranking compound potency, when compared to using standard microtiter plates (MTP). However, there was a major difference when comparing  $IC_{50}$  values of compounds with fast versus slow kinetics. Potencies of slow-acting compounds were significantly lower across the board when comparing CFITA to MTP. They found that decreasing flow rate or increasing capillary length resulted in problems including elongated equilibrium, increased tailing and dispersion, and decreased accuracy. They concluded that continuous-flow assays will likely need to include binding measurements for meaningful biological evaluation, given that the kinetics will not be known for novel targets and compounds.

Examples of translating common existing bioassays to continuous flow provide excellent proof-of-concept and can be applied to some hit-to-lead programs, but may not always provide the greatest opportunity for integration into future discovery platforms. Thus, there is a need to develop approaches to screening for bioactivity in continuous flow that would enable new assays and targets to be explored. In an effort to provide a single compatible platform to integrate synthesis and screening, Ley and coworkers developed an assay to identify  $GABA_A$  ligands using inline frontal affinity chromatography (FAC) [26]. This is a biophysical method that was originally used to study the interaction between carbohydrates and lectins but has since been used to study molecular interactions in chemical biology. The molecule of interest is flowed continuously through a column of immobilized biological target, and the retention of the molecule is proportional to affinity (Figure 6).

Ley and coworkers first developed a three-step flow platform, capable of synthesizing, purifying, and analyzing 22 imidazo[1,2- $\alpha$ ]pyridine analogs in 4 days. The synthesis platform could then be integrated inline into a flow FAC screening platform. For proof of concept, they chose to immobilize human serum albumin (HSA), a protein in human blood plasma for which there is



**Figure 6.** A depiction of frontal affinity chromatography

well characterized binding data for most drugs and many biologically relevant molecules. Most impressively, they were able to show that, if the sample is sufficiently dilute, FAC can be done without precise concentrations. They observed that the  $IC_{50}$  values determined in their system were in good accordance with literature values, and this single injection method can be used to quickly rank compounds according to their affinity. Ley and coworkers also reported the use of this FAC system to evaluate novel inhibitors targeting bromodomains, by studying measurements of the histone reader BRD9 protein [27]. In this method, FAC was combined with mass spectrometry and the results were validated by comparison to a thermal shift assay.

These examples demonstrate that synthesis and biological screening can be integrated into a single continuous platform, and such technologies could be transformative for the lead development cycle by providing inline analysis of synthesized compounds or even complex library or natural product mixtures. However, each of the examples demonstrates flow assays carried out in a serial manner and, for the technology to be truly transformative, some multiplexing or parallelization would need to be realized. There is also potential for innovative integration of flow technologies such as surface plasmon resonance (SPR) which could be used to determine binding kinetics or applied to screening crude or multiplexed reaction mixtures [28].

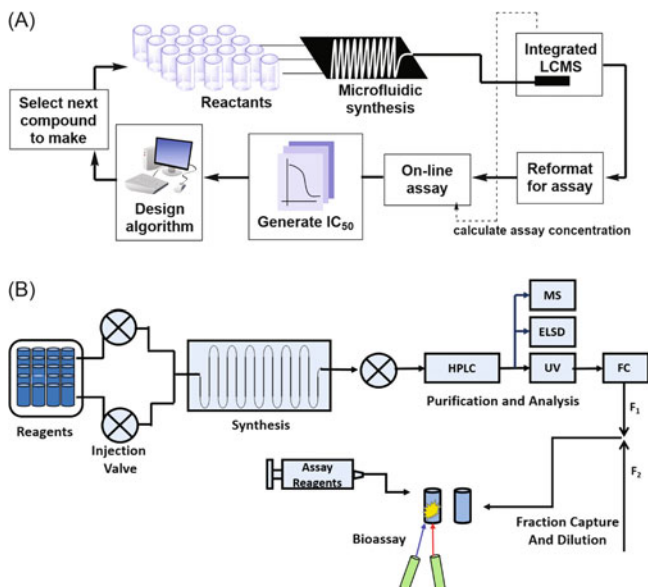
### Library Design and Machine Learning

One of the critical factors in drug discovery and optimization is the time required to close the loop of the design–synthesize–screen cycle between biological analysis and library design. As optimization of a hit or lead molecule progresses, the most effective design decisions are made using feedback from previously screened analogs to develop detailed SAR models. Even with innovative approaches integrating synthesis and biological assays into a continuous platform, there remains a gap with regard to information flow and design.

Cyclofluidic is an integrated drug discovery company that leverages flow chemistry systems in an attempt to streamline hit and lead optimization [29, 30]. Their proprietary flow platform, CycloOps™, is a closed loop structure activity platform that connects synthesis, purification and analysis, screening, and compound design. There are three major issues associated with traditional screening that this platform attempts to address: low program velocity, designing “at risk,” and assuming SAR is additive [26]. If SAR is not additive, the matrix of possible compounds will be poorly understood, which is why informed matrix development and synthesis can be critical.

Using their platform, initial hits are generated through traditional pathways using high throughput screening (HTS) or prior art reviews [29]. Once a hit is generated, known SAR is entered into the design algorithm and chemical synthesis can begin (Figure 7). Only one compound is synthesized at a time, as the objective is to use as few screening loops to map the SAR within the virtual chemical space created by the possible combinations. This provides another way to address issues of parallelization and high throughput using continuous-flow synthesis.

As a proof of concept, Wright and coworkers studied a novel series of Abl kinase inhibitors rationally designed from an X-ray structure of Ponatinib bound to the Abl active site [29]. Two portions were identified, which could be coupled via Sonogashira cross-coupling, and libraries of 10 and 27 fragments were synthesized for each portion. Once a heat map matrix is generated based on the activity of known compounds, there are two algorithms to inform which compound should be synthesized next, “most active under sampled” and “chase potency.” Each screening loop, in which 65 compounds are synthesized, takes approximately 90 min and had 71%, on average, success rate.



**Figure 7.** Cyclofluidic CycloOps™ platform, depicting (A) the overall workflow of the system and (B) the continuous-flow platform

Ultimately, the most active compounds were resynthesized and  $IC_{50}$  values were compared across batch, flow, and algorithm predicted values. In general, the data was in good agreement, thereby validating the platform.

The same team reported the application of the platform to develop xanthine based DPP4 inhibitors [30]. These examples provide compelling evidence that an automated and self-contained medicinal chemistry platform is within reach. However, there are still limitations such as implementing a wide range of chemical reactions and complex synthetic routes and a relatively limited choice of biological assays. These challenges also provide opportunity for innovation that would lead to more broadly applicable platforms.

### Future Outlook

There have been very exciting and innovative developments in flow technologies for library synthesis and biological screening. They have laid the groundwork toward developing a truly integrated platform for drug development that would have been unimaginable only a decade ago. Even with the amazing advances described above, there remain numerous challenges to fully realize the potential power of integrated flow platforms. Particularly, synthetic throughput/purification is limited and the scope of biological assays is very low when compared to traditional techniques. As academic and industrial scientists are able to address these challenges, it is likely that integrated flow platforms will disrupt the current paradigms and change the way in which we approach hit-to-lead and lead optimization. From a business perspective, they could minimize time, cost, and attrition in the discovery pipeline, but more importantly from a scientific perspective, they could enable programs to tackle increasingly

challenging problems in human disease. It could be argued that continuous processes have changed the way most industries operate, and it seems likely that in the coming years the same will be true for pharmaceuticals from discovery to manufacturing.

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