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Kit-On-A-Lid-Assays for accessible self-contained cell assays.

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Abstract

Microfluidics have demonstrated the ability to improve the control of the biomechanical and biochemical properties of cell-based assays. However, microscale methods typically rely on macroscopic reagent handling and fluidic loading protocols that are technically challenging and do not scale favorably. Here, we demonstrate a microfluidic platform technology called “Kit-On-A-Lid-Assay” (KOALA), that enables the creation of self-contained microfluidic cell-based assays, integrating all the steps required to perform cell-based assays. The operation of the KOALA platform is user-friendly and consists of bringing together a lid containing the microchannels, and a base containing the pre-packaged reagents, thereby causing fluidic exchange in all the channels simultaneously. We demonstrate that the KOALA cell-based assays can be operated from start to finish without any external laboratory equipment. Finally, we demonstrate KOALA-bases with advanced function allowing the freezing, thawing, and storing of cell-suspensions.

Introduction

In-vitro cell based assays, have become essential tools for elucidating the mechanisms of human diseases and powerful platforms for drug discovery and testing.¹ A plethora of in-vitro assays have been developed to measure cellular phenotypical features of interest, such as proliferation,² migration,^{3,4} adhesion,⁵ soluble factor secretion,⁶ co-culture,⁷ or gene expression.^{8,9} However, cell-based assays are not standardized, require a multitude of different reagents, pieces of equipment, and processing steps, and often require significant re-development. Furthermore, there is therefore a need for developing platforms that allow a more efficient use of cells and reduce logistical barriers to sharing and collaborating between clinicians and biomedical researchers.

Kits containing all the reagents required for a particular assay, such as PCR or nucleofaction, have facilitated the operation certain assays. However, few exist that integrate the whole cell-based assay including pre-packaged reagents, plastic-ware, and cryopreserved cell-suspensions. Similarly, several microfluidic platforms that allow pre-packaging of the reagents have been demonstrated and belong to two main categories. The first is point-of-care diagnostics, and comprises platforms allowing the measure of a particular bio-chemical marker in a human sample.^{10,11} The second are high-density combinatorial platforms, such as the SlipChip,¹² that allow performing a multitude of mixing combinations with a given

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sample, but does not integrate fluid handling and cell cryopreservation. Here, we demonstrate a microfluidic kit-on-a-lid-assay (KOALA) platform that integrates every aspect of cell-based assays, including reagent pre-packaging, cell cryopreservation, cell culture, and fluid handling. The KOALA platform enables the creation of self-contained cell-based assays that reduce the need for laboratory equipment and significantly reduce the time and reagent requirements for cell-based assays (Fig. 1). To our knowledge, this represents the first microfluidic platform that allows the freezing, cryopreservation, and thawing of cell-suspensions.

Microfluidic methods have been shown to improve on many traditional in-vitro assays for the studying of soluble factor interactions in multiculture systems,⁷ the interaction between cells and their substrate,^{13,14} and properties of cell migration.³ Furthermore, microfluidic methods improve various aspects of fluid handling including the control of fluid paths,¹⁵ washing efficiency,¹⁶ and reagent use.¹⁷ While the highest degree of precision is achievable with syringe-based microfluidics,¹⁸ the equipment and skill required represent a barrier to adoption in biology laboratories. An increasing body of work has identified this issue and developed more accessible open microfluidic platforms. For instance, passive pumping systems,^{19,20} capillary systems,²¹ and systems integrated into well plates,²² are operable with a traditional pipette and have been successfully applied to biological and clinical investigations. These methods, however, still require that all the reagents be prepared in a traditional way (e.g. using eppendorf tubes, pipettes, centrifuges, etc.). Furthermore, every channel is usually filled manually and sequentially, limiting the claims of efficiency and user-friendliness as device and reagent preparation can represent a large part of the experimental process.

The KOALA platform alleviates these issues by enabling the pre-packaging of all the reagents required in a cell-based assay and integrating novel fluid handling ability. The KOALA platform is composed of two parts, one is a lid containing microchannels and the other is a base containing microwells filled with the reagents to flow into the channels (Fig. 1). When the two parts are placed in contact, the fluid in every channel is replaced simultaneously by the fluid contained in the wells. This process can be repeated with a second base, in order to flow a second reagent in the microchannels contained in the lid. Furthermore, the microfluidic base containing the reagents can be sealed, frozen, and stored for extended periods of time (Supplemental movie of the KOALA process). This methodology is amenable to a multitude of assays as most fluids (e.g. cell culture media, washing buffers, cell suspensions, fixing/staining solutions, un-polymerized gels, etc...) can be handled.

We demonstrate that the KOALA platform can be used to perform simple and efficient fluid handling with pre-packaged reagents. Secondly, we show that the KOALA platform allows the culture of cells for extended periods of time. Further, we demonstrate functionalized KOALA-bases, which allow the thawing and preparation of cell suspensions for culture without requiring centrifuges or traditional laboratory equipment. Finally, we demonstrate the broad applicability of the KOALA platform by performing a cell-assay with multiple types of readouts, including live-cell dyes, molecular stains, and immunochemistry.

Materials and Methods

Fabrication of the KOALA device

The KOALA platform was fabricated using plastic micromachining methods. In brief, the lids of the KOALA device containing the channels were fabricated out of two 1.2 mm thick polystyrene (PS) sheets (640-597-67, Good fellow, UK) (Fig. 1A, Lid). The pieces were modeled in Solidworks (Dassault Systemes, USA), imported into SprutCam (Sprutcam

Technology Inc, Russia), and exported into a TAP file for milling on a CNC milling machine (PCNC 770 mill, Tormach, USA). The top piece consists of a series of posts with a horseshoe-shaped cross-section. The posts on the input side of the channel were 1 mm tall, and those on the output 0.6 mm. The post is a cylinder of 1.5 mm diameter in which was placed a 0.8 mm wide slot extending from the edge to the centre of the post. A through hole of 0.8 mm was milled at the centre of the post to connect with channels milled in a second lid part. The channels have a width of 0.8 mm and span 8 mm from the centers of the two posts. The depth of the channels varied such that the channel was 250 μ m deep near the inlet and outlet, and tapered to 1 mm deep in the center. The two pieces were bonded together using a solvent bonding approach consisting of heating one piece up to 65 degrees Celsius, applying Acetonitrile (2710004, Sigma Aldrich, USA) to the other piece, and placing them in contact on a hot plate while applying gentle pressure.²³ After 20 seconds the pressure was released, and the remaining traces of solvent were left to evaporate for 2 hours. Finally, the channel was treated with oxygen plasma (FEMTO, Diener Electronics GmbH, Germany) for 50 seconds at 50W with a 20 sccm oxygen flow. A method developed previously to induce hydrophobic recovery of plastic surfaces was used on the top surface around the posts to revert the contact angle back to that of untreated PS.²⁴ Similarly, the KOALA standard bases (Fig 1A, Base) were fabricated in two pieces of PS; one of 1.2 mm thickness in which the microwells were milled as well as a groove designed to hold a strip of absorbent pad. (CFSP223000, Millipore, USA) The piece was then solvent bonded to a 250 μ m thick PS sheet. All additional and specialized bases (Fig 1) were fabricated in a similar fashion to the KOALA-lid and standard base.

Fabrication of the KOALA cryopreservation base

The KOALA cryopreservation base was fabricated using the same micro-milling technology as described in the previous section. Four parts were fabricated: the bottom part milled in a 1.2 mm sheet, comprising of a large well for holding the dialysis media, and three parts milled in a 250 μ m sheet comprising of elliptical holes of small radius 2 mm and large radius 4 mm. A micro-porous polyester membrane with a pore size of 3 μ m (T30CP14220, GE Water & Process Technologies, USA) was bonded between upper parts, with two on one side of the membrane and one on the other, using the same weak solvent bonding approach. Finally, the bottom part was bonded to the structure containing the porous membranes.

Cell preparation and culture

HUVECs, BEAS-2B, HEK-293, and 3T3 cells suspensions were prepared from cell cultures grown to confluency in a T25 flask. After a 4 min trypsinization process, the cell suspension was diluted in culture media (following the recommendation from ATCC for each cell line), centrifuged for 3 min at 600 RCF, and re-suspended to 2 million cells per mL. Cells prepared for freezing were re-suspended in 10% dimethyl sulfoxide (DMSO), 20% fetal bovine serum, and 70% culture media. For immediate loading into the KOALA device, 8 μ L of cell suspension were loaded into each well of a KOALA-base. For freezing, 10 μ L were added to each of the wells in a KOALA cryopreservation base, and scotch tape was used to seal the device, which was subsequently placed in a -80 degrees Celsius freezer until the time of use.

Cell-based KOALA

When using the KOALA cryopreservation base, thawing was performed by removing the base from the freezer, and adding 1 mL of warm media in the dialysis chamber. The dialysis process was accelerated by gently rocking the platform by hand. When using a freshly prepared cell suspension, 8 μ L of the cell suspension at 2 million cells per mL were added to each well of a standard KOALA-base. In both cases, the cells were loaded into a KOALA channel by placing the KOALA-lid on top of the base containing the cells, thereby causing

the cell suspension to fill the channels. After loading the cells, the KOALA-lid containing the channels and cells was placed on a standard KOALA-base, which was devoid of the absorbent pad and for which the wells were filled with cell culture media to mitigate evaporation during the incubation. The lid and its accompanying base were then placed in an Omnitray (242811, NUNC, USA) containing sacrificial PBS, to further reduce evaporation. The Omnitray was then incubated in a CO₂-regulated incubator. After 24 h of culture, a series of pre-filled KOALA-bases were applied to the KOALA-lid to perform the cell-based assay. A first lid filled with 4% paraformaldhyde was applied and the channels were incubated for 30 min. A second lid filled with 0.1% triton was applied and incubated for 10 min. Then, a lid containing DAPI stain, phalloidin stain, and tubulin antibodies was applied and let incubate for 30 min. Finally a lid filled with PBS was applied to wash the microchannels. The KOALA platform was finally imaged using an Eclipse Ti fluorescent microscope (Nikon, Japan).

Results and Discussion

We demonstrate a novel class of microfluidic cell-based assays, called Kit-On-A-Lid-Assay (KOALA), allowing the creation of self-contained kits that integrate all the reagents, fluid handling methods, and cell cryopreservation methods required. The approach uses two microfluidic parts: a lid containing microchannels and a series of bases containing pre-packaged reagents, cryopreserved cell-suspensions (Fig. 1). First, we developed a method to reliably transfer fluids from the bases to the lid. As the lid is placed in contact with a base, posts located on the input and output of each microchannel in the lid come in contact with a microwell and an absorbent pad, respectively, in the base. The combination of post design and contact with the fluid and absorbent pad drive fluid from the base into the microchannel. Using a series of bases containing different reagents, a range of fluids can be sequentially flowed into the microchannels allowing the creation of multi-step assays. Further, specific bases can be designed for pre-packaging reagents, cryopreserving cell-suspensions, or preparing user-specific reagents, thereby making the KOALA platform a modular technology for a wide range of biochemical and cell-based assays.

Microfluidic design

The transfer of fluid from the base to the lid leverages capillary forces to provide a reliable and passive flow generation in the microchannels. In brief, when the lid is placed on the base, a post located at the input of the microchannel in the lid contacts a microwell with reagents in the base, and a post located at the output of the microchannel in the lid contacts an absorbent pad in the base (Fig. 2A). The variance in height between the posts accounts for the difference between the depth of the wells and the pads. Furthermore, the system was designed to (1) ensure that good fluidic connection is made between the lid and base parts, and (2) to ensure that the fluidic connection can be severed at the right time to leave a precise amount of fluid in the microchannels at the end of the operation.

In order to create a robust fluidic connection between the microchannels and the wells, we designed a connection system that eliminates the risk of capturing air bubbles upon application of the lid on the base. The posts placed at the input and the output of the microchannels have a horseshoe shaped cross-section such that air bubbles can escape when the post contacts the fluid in the microwells situated in the base. Further, the horseshoe shaped cross-section allows capillary force to pull the fluid into the channel and into contact with the absorbent pad.

Secondly, once the fluidic connection is initiated both on the inlet side (in contact with the microwell) and the outlet side (in contact with the absorbent pad), the capillary phenomenon must cease before the entire contents of the channel gets emptied into the absorbent pad. To

accomplish this the horseshoe shaped design enables an air gap to form, severing the fluidic connection between the channel and the absorbent pad (Fig. 2B). An additional design consideration needs to be validated to ensure that the fluid preferentially remains in the microchannel. For this to occur, the capillary pressure, $\Delta P_{\text{channel}}$, generated by the fluid in the channel (and providing the driving force to keep the fluid in the channel) must be higher than the capillary pressure, ΔP_{post} , generated in the horseshoe post (and providing the driving force to empty the channel). Both pressures, $\Delta P_{\text{channel}}$ and ΔP_{post} , can be written as a function of θ , the contact angle of the fluid, γ , the surface tension of the liquid, w_c and h_c , the width and height of the channel respectively, and d_p the depth of the horseshoe (ESI):

$$\Delta P_{\text{channel}} = 2 \gamma \left(\frac{\cos(\theta)}{w_c} + \frac{\cos(\theta)}{h_c} \right) \quad (1)$$

$$\Delta P_{\text{post}} \leq 2 \gamma \frac{\cos(\theta)}{d_p} \quad (2)$$

$$\Delta P_{\text{channel}} > 2 \gamma \frac{\cos(\theta)}{d_p} \geq \Delta P_{\text{post}} \quad (3)$$

Microfluidic operation

During the operation of the KOALA device (placing the lid on a base containing microwells) the fluid in all the microchannels contained in the lid is replaced simultaneously (Fig. 2C). This aspect is advantageous for time-sensitive cell-based experiments, as every microchannel is treated at the exact same time. Thus, phenomena such as evaporation do not affect some channels more than others. Simultaneous fluid transfer also allows straightforward scaling of the number of channels, as the time requirement is constant. Furthermore, we show that cell suspensions can be flowed into the microfluidic device and that cells can be cultured for extended periods of time. As the volume of the microchannels is minute ($\sim 2 \mu\text{L}$), we have previously found that evaporation may have a strong impact on cell culture.²⁵ Evaporation was mitigated by placing the lid onto a base containing blank media during the incubation stage. Using this approach, we observed that BEAS-2B cells were viable after 24 hours culture, as shown by a live/dead stain. For cultures lasting more than 24 hours, the culture media can be replaced using a fresh base. In order to create advanced assays integrating multiple fluid handling steps, such as cell fixing or staining, we quantified the reliability and repeatability of the fluidic replacements. The data shows that 24 fluid replacements were performed on 12 channels before a failure was observed, with one channel failing on the 25th replacement. Noticeably, 24 sequential replacements are sufficient for developing most cell-based assay.

Cryopreservation and thawing

We have developed cryopreservation methods that enable the freezing and thawing of cells in a KOALA-base, enabling the creation of entirely pre-packaged cell-based assays. Microfluidic platforms have been reported allowing the removal of cryopreservation media from a cell suspension by leveraging the rapid diffusion at small scales, though these methods do not integrate freezing and storing.^{26,27} Furthermore, these methods require complex fabrication features, specialized microfluidic equipment, and do not allow easy access to the thawed cell suspensions as they are enclosed within the microfluidic device.

The KOALA platform allows the pre-packaging, freezing, and thawing of cell-suspensions. A key feature is the removal of the cytotoxic cryopreservation fluid added to the cell suspension prior to freezing.²⁶ In this embodiment, we developed a KOALA-base containing microwells with a micro-porous membrane on the bottom (Fig. 3A). Cell suspensions are prepared in the desired cryopreservation media, placed in the wells, sealed, and stored in a -80°C freezer or liquid nitrogen until ready for use. Before use, the base is removed from the freezer, and the cryopreservation fluid, typically DMSO, is removed through dialysis, by adding warm culture media to the chamber adjacent to the wells. The dialysis process leverages the rapid diffusion timescales enabled by microfluidics as the height of liquid in the well is in the order of tens to hundreds of micrometres. To further accelerate the process, the base can be placed on a rocker or rotated from one side to another, in order to increase the mixing of the media in the dialysis chamber (Fig. 3B). We have characterized the dynamics and efficiency of the dialysis process by monitoring the extraction of fluorescent dye (Alexa 488) into PBS (Fig. 3C). The results reveal that after 6 minutes of dialysis ~97% of the dye is extracted. As the diffusion coefficient of DMSO is higher than Alexa488, the result suggests that this timeframe allows the removal of a sufficient amount of DMSO for healthy cell culture. For higher extraction efficiency, the dialysis can be performed twice, consecutively. To prove the effectiveness of the device, we froze various cell lines (3T3, BEAS-2B, HEK-293, and HUVEC) for at least 72 hours in a solution containing 10% DMSO, performed the dialysis, and cultured cells for 24 hours in KOALA-microchannels. The data indicates a greater than 70% viability for each cell type, which is on par with the viability obtained when preparing the cells using traditional methods (centrifuge, eppendorf tubes, etc..) and culturing them in the microchannels (Fig. 3D).

The KOALA approach for cell cryopreservation is user-friendly and scalable, as multiple wells are prepared simultaneously and each can contain a different cell line / strain. More importantly, this method allows the freezing and thawing of only the required amount of cells for each microchannel. We envision this to be a critical aspect for cell-based assays using primary, patient, or stem cells, as a large number of these KOALA-bases can be loaded from the same sample and used at later dates without requiring the thawing of the whole sample. Additionally, this may also allow researchers to go back to the same batch of cells, as tens/hundreds of these KOALA-bases can be prepared from the same original cell sample.

Integrated cell-based KOALA assays

Using the KOALA microchannels and the KOALA cryopreservation lid, we demonstrate that the KOALA platform enables the creation of entirely self-contained cell-based assays. Noticeably, the enabling aspect of a KOALA approach was demonstrated previously for applying a diffusive source onto a microchannel for neutrophil migration applications²⁸. A typical immunohistochemistry readout was chosen to illustrate the potential of the KOALA assay. HELA cells were frozen in a KOALA base, thawed, and cultured in KOALA microchannels. After 24 hours in culture, a series of treatments using KOALA-bases was performed in order to wash, fix, permeabilize, and stain the cells in culture. We show that the KOALA platform allows the use of various reagents critical for cell assays, such as paraformaldehyde, surfactant, live cell stains, small molecule stains, and antibody-based stains (Fig. 4). The KOALA platform thus raises the possibility of developing standardized and optimized in-vitro phenotype screens in which the researcher could screen a mutant cell strain or disease model for a whole series of phenotypes and morphology features all at one time, without having to prepare and optimize each combination of antibodies and assay reagents. The potential impact for this technology is to reduce the time required for identifying and characterizing the functional role of genes of interest, by discarding those

with little or no phenotype of interest. Further, the assay described here was performed by a cell-biology researcher that did not have an engineering background. This emphasizes the underlying goal of the KOALA assay, which is to provide a simple user-friendly assay that can be used by an audience that is not versed in microfluidics and medical engineering.

While the KOALA platform is efficient for streamlining assay steps by pre-packaging reagents and cell lines, some users may want to customize one or more steps of the assay. An obvious option is to provide the user with an empty base, in which each well can be loaded with a custom solution. To facilitate such workflows, we demonstrate a KOALA-base that allows the user to load a reagent of interest in one pipetting step (ESI). This approach allows the allocation of user-inputted fluid into set volumes, and the preparation of this liquid for subsequent interfacing with the KOALA workflow. More advanced assays can also be performed by changing the microchannel configuration in the lid. For example we show that a chemotaxis and gradient-generation device can be created (ESI). These examples display the modularity of the KOALA approach to create a variety of cell-based assay that can be performed in a simple, inexpensive, and rapid way.

Conclusions

We present a novel microfluidic technology for handling fluids in microfluidic platforms that enable the exchange of fluids between two open microfluidic parts: a base and a lid. This technology, dubbed Kit-On-A-Lid-Assay (KOALA) technology, enables the creation of entirely self-contained assays that can be stored, shipped, and operated without any specialized equipment. Further, we developed methods to freeze and thaw cell suspensions with nearly zero dead volume. Using these methods, we show the ability of creating cell-based assays in which all reagents are pre-packaged, and that require no equipment other than an incubator. This enables streamlined and standardized invitro cell based assays that use very little reagents as only what is needed is thawed. The significant time and material/reagent savings are particularly important for primary patient samples as well as immunocytochemistry applications. For these, the KOALA allows the researcher to prepare a large batch of assays and use only the amount required for each assay. The KOALA technology has the potential to be a broadly applicable platform for creating user-friendly in-vitro cell-based assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure. 1.

Schematic of the KOALA assay. A lid containing microchannels is put in contact with a base containing pre-packaged reagents. Upon contact the fluid in the base flows into the microchannels replacing the fluid it previously contained. The KOALA platform is modular and multiple bases with specific functions can be developed for the cryopreservation of cell suspensions, the shipping and storing of reagents, or the rapid custom loading of reagents for instance

Figure. 2.

A. Schematic of the KOALA platform. B. Illustration of the operation of the KOALA platform using red food colorant for the initial filling, and blue food colorant for the subsequent fluid replacement. C. Operation of the KOALA platform. After the lid is mated with the base, an absorbent pad drives the flow, causing the replacement of the fluid within the channels. Once the input microwell is empty, the flow stops and the lid can be removed from the base. D. Design consideration of the KOALA microchannels. The posts on either side of the microchannels are designed to enable capillary flow, and thus create fluidic contact with the absorbent pad. When the microwell is empty, the dimension of the horseshoe relatively to the channel is such that the fluid stays pinned within the channel and an air bubble extends into the post, breaking the fluidic connection, and thus stopping the flow of fluid.

Figure. 3.

A. Schematic of the cell cryopreservation embodiment where cells are contained in a well enclosed by a tape and a microporous membrane. B. The device is rocked back and forth to accelerate dialysis for replacing the freezing media with fresh media, after which the tape is removed and the lid is placed on the base for seeding cells C. Quantification of the dialysis of Alexa488 dye in PBS (left). After 6 minutes ~97% of the dye was removed from the wells. Demonstration of dialysis using blue food colorant (right) D. Viability of various cell lines through the KOALA freezing and thawing process compared to that of the traditional freezing and thawing process. Microscopy images of the live/dead stain for the HEK cell line, after KOALA process, are shown.

Figure. 4.

Using a series of lids, as whole cell-based assay can be pre-loaded into the KOALA platform. Here BEAS-2B cells were loaded in a cryopreservation base, and an immunostaining assay was loaded in a series of pre-packaged reagent bases. After cell loading and culture in a KOALA lid, each step of the immunostaining assay was performed by thawing the specific base required, and applying a specific lid onto the microchannel culture. Fluorescent microscopy was used to image the assay performed. All 12 channels of the KOALA lid were treated simultaneously at each application of a new base, removing the need to prepare each reagent individually and treating each channel individually.