# Rapid on-chip apoptosis assay on human carcinoma cells based on annexin-V / quantum dot probes

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**Abstract**

Despite all the efforts made over years to study the cancer expression and the metastasis event, there is not a clear understanding of its origins and effective treatment. Therefore, more specialized and rapid techniques are required for studying cell behaviour under different drug-based treatments. Here we present a quantum dot signalling-based cell assay carried out in a segmental microfluidic device that allows studying the effect of anti-cancer

drugs in cultured cell lines by monitoring phosphatidylserine translocation that occurs in early apoptosis. The developed platform combines the automatic generation of a drug gradient concentration, allowing exposure of cancer cells to different doses, and the immunelabelling of the apoptotic cells using quantum dot reporters. Thereby a complete cell-based assay for efficient drug screening is performed showing a clear correlation between drug dose and amount of cells undergoing apoptosis.

**KEYWORDS:** Quantum dots, apoptosis, modular microfluidics, drug screening, optical detection.

## 1. Introduction

Drug discovery is a field of research in constant development due to the need of new and improved drugs to treat different kinds of disorders, diseases or illnesses. Up to date, several methods for drug development have been reported (Koel and Kaljurand, 2006). The development of new strategies to study the effect of specific drugs on biological samples are of crucial importance to verify the drug function on human cell lines before conducting the clinical trials (Lledo-Fernandez and Banks, 2011)' (Peng and Chiou, 1990).

One of the most important fields in drug development research is related to cancer treatment (Rodwell, 2013)' (Suggitt and Bibby, 2005)' (Lieberman et al., 2001). Most anticancer drugs such as Camptothecin (CAMPT) induce apoptosis (Lin et al., 2013)' (Han et al., 2009), with some common characteristics (endonucleolytic cleavage of DNA, chromatin condensation, caspases activity, phospholipids translocation, etc.), which can be used to develop different methods for cell apoptosis detection by the recognition of a specific apoptotic marker (Sellers and Fisher, 1999). Several strategies to detect apoptotic cells have been reported being the detection of phosphatydilserine (PS), a phospolipid located in the inner leaflet of the plasma membrane (PM) that translocates to the outer one at early stages of apoptosis, one of the most common methods currently in use.

Detection of PS through the specific phospholipid-binding protein Annexin V (AnnV) has been widely used for diagnosis (Schutters and Reutelingsperger, 2010)' (Van Tilborg et al., 2010) and as an imaging tool to monitor the apoptotic cell death progression when AnnV is conjugated to a fluorescent dye (Andree et al., 1990)' (Vermes et al., 1995). This non-invasive technique for the cell is a good

alternative for drug screening assays. However, one of the main problems of AnnV-based fluorescent probes is the limited photostability of the organic dyes conjugated to it and the scarce diversity of commercially available AnnV-conjugated dyes, to avoid spectral overlapping. These problems can be solved using Quantum Dots (QDs) as fluorescent reporters instead of standard organic dyes (Resch-Genger et al., 2008).

QDs are already commercially available due to their high demand given their unique optical properties and great potential in diagnostic imaging with particular interest in cancer diagnosis (Algar and Krull, 2010). We have already reported the advantages of QDs over organic dyes for labelling intracellular structures through double or triple immunocytochemistries, showing higher fluorescence intensity, narrower bandwidth and higher photostability (Montón et al., 2009) and we have as well demonstrated the efficient employment of QDs as labels in microarray technology for either protein (Morales-Narváez et al., 2012) (Alzheimer's disease biomarker screening) or bacteria (Morales-Narváez et al., 2013) detection (E. coli detection in a digital like response via fluorescence quenching with graphene oxide). Thereby, the use of QDs conjugated to AnnV provides a unique candidate to monitor apoptosis in time-lapse imaging due to their high photostability (Le Gac et al., 2006).

QD-based labelling strategies can improve conventional technologies but, modifications in the assay platform such as the use of microfluidics would allow the development of a cheaper and more efficient method (Vannoy et al., 2011) by down-scaling the cell environment, making biological interactions more dynamic (microflows), and decreasing sample, reagents and time of analysis (Janasek et al., 2006).

Microfluidic devices are basically known as integrative platforms to carry out diverse and complex experiments (Whitesides, 2006). Although many researchers are proposing highly integrated and sophisticated platforms (Du et al., 2013; Jang et al., 2011), the complexity of the experiments for non-experienced personnel can be a drawback due to their multiple components. However, the use of modular configurations, also called "building blocks" (Hill et al., 2016), brings the opportunity to couple different kinds of chips depending on the requirements of each experiment in a versatile and simple way, resulting in a large number of combinations (Yuen, 2008; Yuen et al., 2009). The main idea is that several microfluidic components can be connected in a motherboard to perform a larger integrated system (Skafte-Pedersen et al., 2013), or to mimic organs on a chip for drug screening under different physiological conditions (Loskill et al., 2015).

DNA detection (Hsieh et al., 2012; Yazdi et al., 2013), protein immunoassays (Diercks et al., 2009; Tekin and Gijs, 2013), and cell-based experiments (Hajba and Guttman, 2014), based on microfluidic platforms have been widely reported in the literature. Focusing on cell studies using microfluidics, several platforms for cytotoxicity evaluation and drug discovery have also been described (El-Ali et al., 2006; Jedrych et al., 2011). However, the above mentioned platforms have been used to study drug toxicity in cells by analysing their morphology or viability ex-situ mainly using organic dyes, which are not stable and not sensitive enough for long-term studies (Kim et al., 2011; Wang et al., 2015).

Here, we present a more specialized assay in which the translocation of PS to the outer leaflet of the plasma membrane in apoptotic cells is specifically labelled by an AnnV-QD conjugate, all done within microfluidic modules, giving more insight on the apoptotic process. The anticancer drug CAMPT is used as model to induce apoptosis and QDs

conjugated to AnnV to reveal the PS translocation in cells undergoing apoptosis upon exposition to CAMPT. To achieve the drug test in human carcinoma cells we carry out the assay in a segmental microfluidic device in two phases: i) we culture the cells into the chip intended for apoptotic cell detection (CELL chip) and then we connect it with the concentration gradient generator chip (CGG), previously reported by Jeon et al. (Jeon et al., 2000) to expose the cells to different CAMPT doses; ii) the cells previously exposed to different CAMPT doses are incubated with the AnnV-QD conjugates prepared using a mixer chip (MIX). The AnnV-QD labelling performed in a microfluidic platform proves to be an effective method to quantify cell apoptosis through specific recognition of PS (Figure 1B).

## 2. Results

### *Optimisation of the CELL chip*

We established a sterilisation method for the CELL chip, made of glass and polydimethylsiloxane (PDMS), and an appropriate number of cells to seed in it to obtain optimal results. On the one hand, we checked different methods of sterilisation: i) chips filled with 70 % ethanol, emptied immediately after and followed by UV light exposure for 1 h after removing the ethanol, ii) chips filled with 70 % ethanol and exposed to UV light for 1 h, and iii) chips filled with 70 % ethanol until use (no UV light exposure). According to our experiments, the sterilisation methods involving UV light exposure resulted in low cell attachment and growing, probably due to the creation of free radicals produced by the UV-cleavage of the polymer backbone in the PDMS. The presence of free radicals might affect the cell growth due to oxidative stress which can modify both cells and cell products leading to cell apoptosis, protein modification, cross-linking and precipitation.(V. Lobo, A.

Patil, A. Phatak, 2010). Therefore the most suitable method in terms of cell culture growth 24 h post-seeding was a single-step of filling it with ethanol 70% until use (Supporting Information Figure S1). On the other hand, we analysed the number of cells growing in each chamber of the sterilised chip (1, 2, or 3 in Figure 1A, i) to ensure that the cells were uniformly distributed in the CELL chip. We designed the CELL chip with oval-shaped chambers and channel ramifications to obtain a uniform distribution of cells and to avoid a possible cell detachment caused by high flow, which is very important for obtaining reproducible results during microscope image acquisition (Figure S2). With this design, we found no differences in cell number among the chambers (Figure 1A, ii), therefore each chamber is considered a replicate in the final assay. In addition, we seeded different number of cells in different chips to find the optimal concentration of cells after 24 h incubation (data not shown). We chose to seed 120,000 cells per chip to almost reach a cell monolayer in the chambers at the moment of inducing the apoptosis (24 h post seeding).

***Optimisation of the CGG chip***

To optimise the performance of the CGG chip we evaluated the flow rate and duration of the reagent injection. We simulated the real composition of the solutions to be used in the final assay: supplemented cell culture medium (Minimum Essential Medium, MEM) was introduced by one of the inlets and a combination of MEM, dimethyl sulfoxide (DMSO, CAMPT diluent) and trypan blue (to simulate CAMPT drug and to visualize the mixture) was introduced through the other one. To evaluate the gradient of concentrations obtained with the CGG chip, we took microscope images in the outlets (Figure 1B, i) and we further processed them with ImageJ software to obtain the relative intensities of colour (Supporting Information Figure S3) which would be related to a specific solution concentration

introduced in the inlets. According to theoretical predictions for such design (Supporting Information Figure S4), the relative concentrations in the outlets should be 0, 0.125, 0.5, 0.875 and 1, corresponding to 0, 2.5, 10, 17.5 and 20 µM of CAMPT. We compared the experimental values obtained at different flow rates and times (Supporting Information Table 1) with the predicted theoretical values (Figure 1B, ii) and we found that a 10 µL/min flow rate during 3 min resulted in the closest to theoretical given concentrations at the end of each outlet. The experimental concentrations corresponded to 0, 4, 11, 16 and 20 µM respectively. Considering that range a good start point for gradual doses of CAMPT we used this setup to test the platform performance for a full drug screening assay. We as well carried out repeatability and reproducibility studies by repeating measurements in the same chip and in three different chips, respectively (Supporting Information Figure S5) and we found negligible differences between measures, thus demonstrating the functionality of the CGG chip in generating a gradient of concentrations.

***Optimisation of the MIX chip***

Conjugation of AnnV-biotin with Streptavidin-QDs is performed in the first section of the chip (C sector -conjugation-, Figure 2C, i) whereas AnnV-QD is mixed with the $Ca^{2+}$ enriched medium for cell incubation in the second section (M sector -mixing-, Figure 1C, ii). To optimise the performance of the MIX chip we first evaluated the C sector by introducing streptavidin-QDs at a concentration of 120 nM through inlet 1 (Figure 1C) and AnnV-biotin through inlet 2 (Figure S6, Supporting Information). Fluorescence images were taken at certain points (C0, C50, C100 and C150 cm) from the beginning of the inlet path of C sector in order to evaluate the fluorescence intensity in several Regions of Interest (ROIs) with the aim to calculate

the mixing factor which reflects the homogeneity of the solution and thus the best conditions to perform the AnnV-QD conjugation. We tested several flow rates and injection times (Supporting Information Table 2). We found that a flow rate of 5 µl/min during 12 min were the optimal parameters to obtain a mixing factor value of almost 1 at the end of the C sector of the chip (Figure 1C, i). We also carried out repeatability and reproducibility studies for the C sector and we observed negligible differences between measurements or chips (Supporting Information Figure S7a).

We proceeded similarly to evaluate the M sector of the MIX chip (Figure 1C, ii); we introduced AnnV binding buffer through inlet 3. We acquired fluorescence images along the channel path in M sector (M0, M50, M100 and M150 cm: see, inset images in Figure 1C, ii) and we determined the mixing factors at each of these points. We found the best compromise between the duration of the assay (6 min) and the obtaining of appropriate mixing factors at a flow rate of 20 µL/min (Figure 1C, ii). As for the C sector, we evaluated repeatability and reproducibility for M sector of the chips and we found no significant differences between repeated measurements neither in a same chip nor in different ones (See Supporting Information Figure S7b).

***Screening the apoptotic effect of CAMPT on human adenocarcinoma cells***

Once the three modules were separately optimised, the suitability of the modular platform to carry out a real cell-based assay was demonstrated by performing three sequential steps. First, the cells were seeded in 5 different CELL chips and incubated overnight to proliferate in a $CO_2$ incubator. Second, after incubation each of the 5 CELL chips was connected to an outlet of the CGG chip followed by introduction of 20 µM CAMPT (diluted in DMSO) and 1X MEM through the two different inlets of the CGG chip (Figure 2A). As a result, each

CELL chip was exposed to a different dose of CAMPT ranging from 0 to 20 µM using optimal flow rates and times (Table 1) (Figure 2A ).

Then CELL chips were disconnected from the CGG chip and placed in the incubator for 4 h in order to allow the induction of apoptosis in the cell cultures. The third step of the assay was to label positive-apoptosis cells with AnnV-QD probes (Figure 2B). To achieve this, biotinylated AnnV and streptavidin-QD were introduced through the two inlets (1 and 2) of the C sector of the MIX chip followed by the introduction of AnnV binding buffer through the third inlet (3) located in the M sector of the MIX chip in order to dilute the AnnV-QD probe in the appropriate binding buffer. We sequentially connected the MIX chip to each of the CELL chips in order to let the probe interact with the cells. We incubated the CELL chips with the AnnV-QD probe solution for 30 min in order to label the translocated PS in apoptotic cells, followed by a washing step of supplemented MEM for 7 min at a flow rate of 4 µL/min to remove unbound AnnV-QD. Each CELL chip exposed to a different dose of CAMPT was analysed by fluorescence microscopy in order to determine the percentage of apoptotic cells. Three areas per chamber for each chip were selected to acquire bright field images (Figure 3A, i) and fluorescence images (Figure 3A, ii) for further analysis.

The images were processed to establish the apoptosis ratio for each CAMPT dose exposure (0, 4, 11, 16 and 20 µM). As expected, the higher the concentration of CAMPT used to induce apoptosis the higher the number of cells labelled with AnnV-QD (apoptotic cells). In order to determine the number of apoptotic cells upon different drug concentrations, we calculated the ratio between the number of positive cells (apoptotic cells labelled with AnnV-QD) upon the total number of cells (apoptotic and non-apoptotic cells). Figure 3B shows the dose-response curve of cell cultures exposed to specific doses of CAMPT (0; 4;

11; 16; and 20 µM) obtained from the image analysis. Each point represents the mean value obtained from the quantification of three different chambers and the error bars show the standard deviation between those measurements. A certain amount of apoptosis (10-20%) was found for chips incubated with 0 µM CAMPT corresponding to the basal level of apoptosis within the cell culture. Two replicates of this experiment were carried out in different days and results obtained were similar (Supporting Information Figure S8) showing the good reproducibility of the method.

## 3. Discussion

The results shown here indicate an appropriate correlation between the dose of CAMPT and the relative number of apoptotic cells labelled with AnnV-QD probes. As a proof-of-concept, we have demonstrated the applicability of the platform to carry out cell-based assays for drug screening with a low standard deviation (from 3 to 8 %). Considering the factors that a cell based assay for drug development should feature, such as sensitivity of detection and reagent stability (Riss, 2005), an important advantage of the developed system is the use of QD instead of fluorescent dyes to reveal apoptosis. QDs are one of the most robust bioassay labels to carry out long optimisations and quantifications which require high sensitivity and stability of signal that other labels such as dyes cannot provide. Their compatibility in biological media, long-term stability and resistance to photobleaching make them ideal tools to monitor real time events, as we have shown, obtaining sensitive and reliable measures of apoptosis. Moreover, the versatility of QDs offers the possibility to evolve this work in different ways to fulfil a specific goal depending on the bioanalytical/bioassay scenario. For instance, CELL chips can be fabricated with integrated electrodes and electrochemical assays can be performed using the

same QD-based probes thanks to the use of redox properties of QDs (Medina-Sánchez et al., 2015). Proof of that is the electrochemical-based microfluidic platform we have reported as an efficient alternative to detect very low levels of a relevant biomarker for diagnostics (Medina-Sánchez et al., 2014). Therefore, an important advantage of the presented QD mediated monitoring is the possibility to be also used in a dual optical/electrical detection (given the QD properties) that may allow a fast quantitative monitoring of the apoptosis process induced by a chemical stimulus. In this way the effect of certain doses of an anticancer drug would be detected electrochemically in-chip as it has been recently demonstrated in batch mode (Montón et al., 2015).

The developed platform combines the versatility of QDs with the advantages of modular microfluidics: each chip can be modified, replaced by another one or removed to obtain a suitable system for each new application. The obtained results demonstrate that the combination of QDs with a modular microfluidic platform gives rise to a robust and sensitive cell assay for drug discovery applications. In addition this leads to significant both time and reagent saving due to the in-chip preparation of the reagents (drug doses, labelling probe conjugation) and due to the small volumes required to perform the assay. For example, experiments performed in the presented modular microfluidic system use 4 times less volume of CAMPT stock solution (1 µL of stock solution is sufficient to prepare all working solutions in CGG chip) comparing with common techniques based on multiple well plates. Similarly, smaller volumes of QDs, cells suspensions and buffers are used in microfluidic chips than in plate-based assays. Moreover, this cell-based assay performed in microfluidic chips is characterized by a shorter duration of the experiment (at least 40% using our platform) and by fewer manual steps that may introduce variability in the system. For instance, if a multiple well plate is necessary to carry out the same or a similar assay

several additional steps can be applied. For example, the conjugation protocol would include various washing and separation steps, while in microfluidics this step is reduced and simplified by flowing a buffer through the channel, reducing time and avoiding possible damage of the cell culture. Regarding the preparation of CAMPT doses, these should be manually and individually prepared in the case of a multiple well plate based assay. The use of the proposed microfluidic chip drastically reduces errors in manipulation and cell contamination in addition of offering a robust and easy to use system even for use out of a specialized laboratory and even by non-professional users.

The novelty of this work relies not only on the use of an optimised modular platform to carry out a full drug screening assay without manual steps (from drug doses and labelling to apoptosis detection) but also on the high accuracy of the assay as shown in the reproducibility of the results. The functionalities of the chips we used in this work have been already described and similar geometries have been used separately for different purposes. However their combination to carry out a complex assay in a simple, fast and cost-effective manner has not been reported until now. So far, reported lab-on-chip platforms for drug screening or imaging purposes have been used in disconnected steps so either drug concentration preparation, labelling or both of them have been done manually (Kim et al., 2011; Zhao et al., 2013). Moreover, the versatility of the modular platform we presented will allow a number of different types of assays to be performed, other than drug screening, opening the doors to many other applications.

## 4. Conclusions

In this work, we presented a versatile modular microfluidic platform that performs optical drug screening assays. As test bed, we focused on the detection of apoptosis in cancer cells, induced by the presence of the pro-apoptotic drug camptothecin. In particular, we combined geometrically different chips to achieve three main functionalities: the generation of a drug gradient concentration, the functionalisation of QDs with AnnV, and the optical detection of apoptotic HeLa cells. Our platform successfully showed a clear correlation between drug dose and amount of cells undergoing apoptosis. Compared to traditional methods used for drug screening, our platform offers improved ease of use (decreasing the number of manual steps), speed (reducing the overall assay time by 40%), miniaturization/portability and versatility. Considering that this QDs/modular microfluidic bioassay platform can be easily adapted to specific requirements by redesigning the geometries of the chips, the probes or the target cells, we believe that our system can meet the needs of laboratories looking for a versatile methodology to evaluate new drugs during preclinical stages.

## ASSOCIATED CONTENT

Supporting Information containing supporting Figures and Tables

## Notes

The authors declare no competing financial interest.

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**Figures**

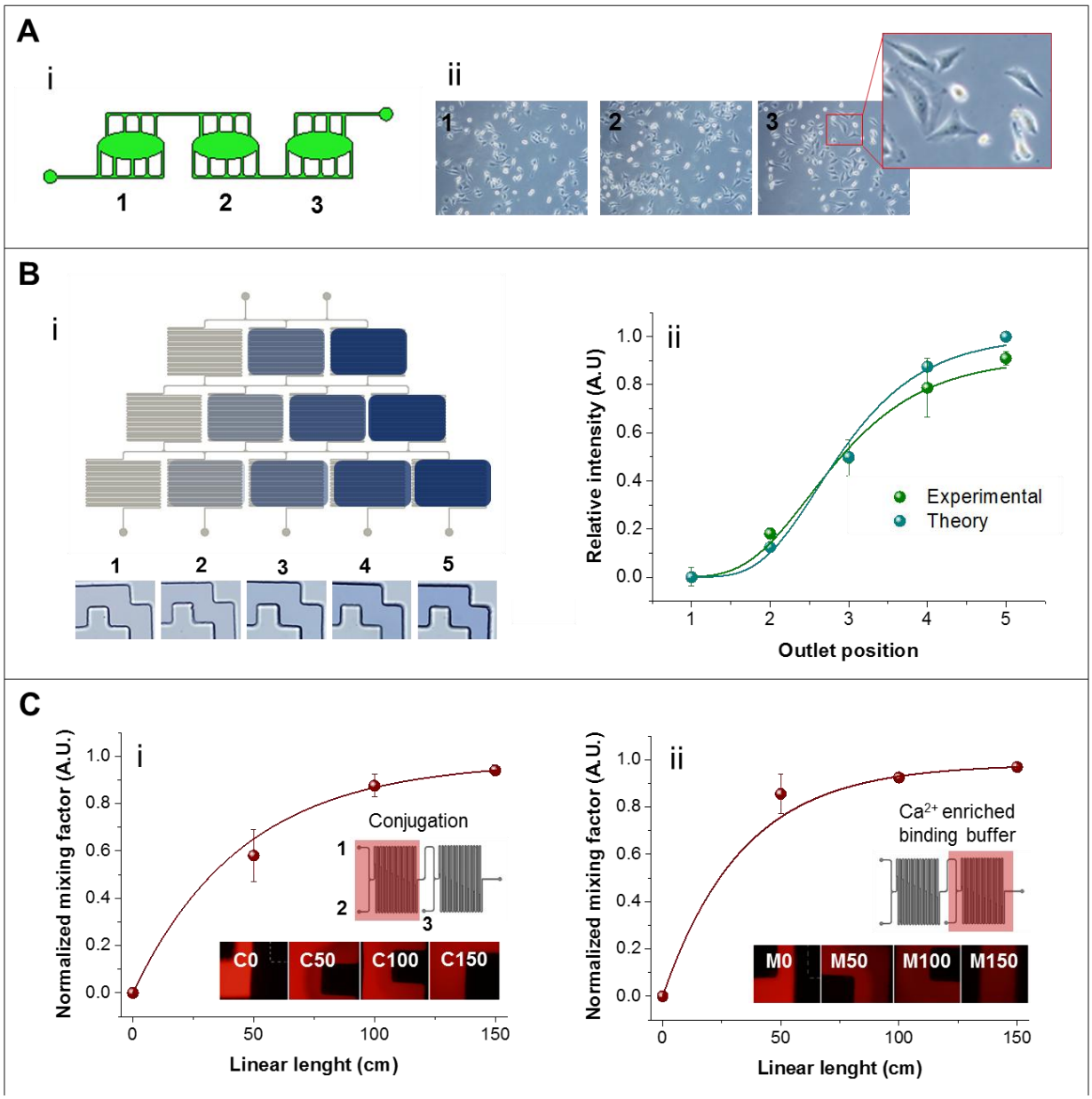


Figure 1

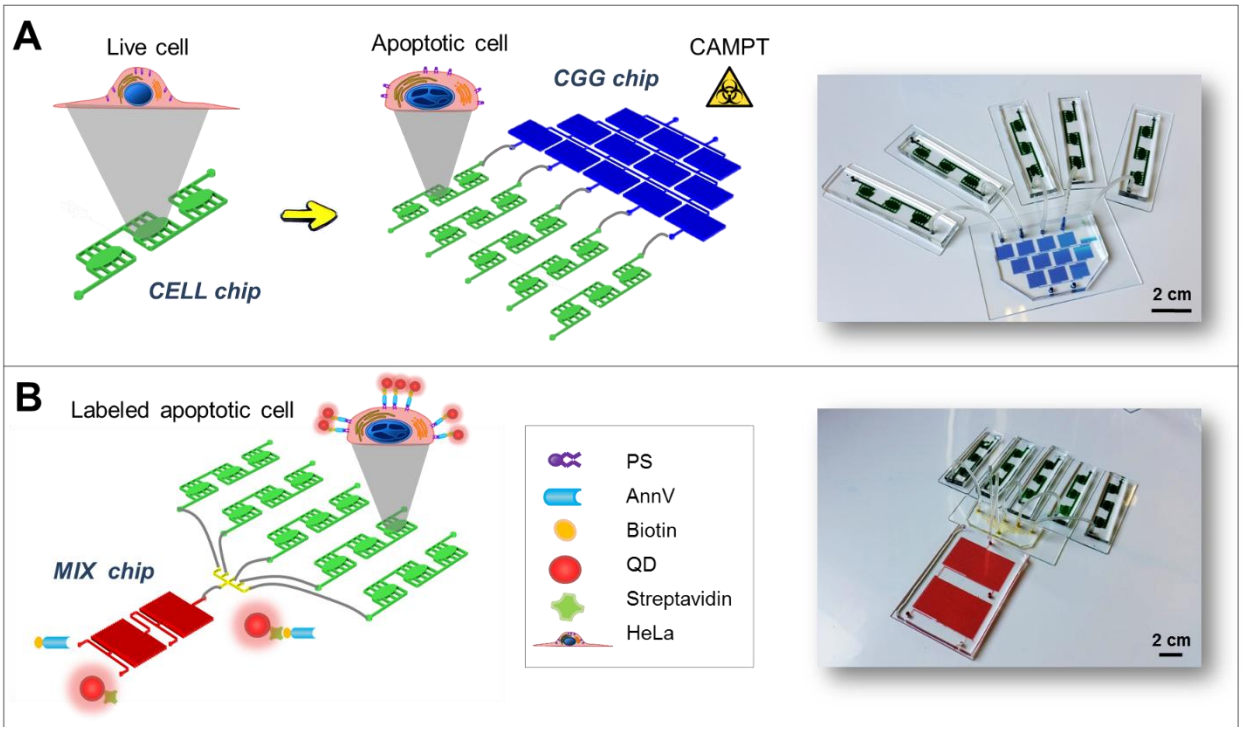
A
Live cell
Apoptotic cell
CAMPT
CGG chip
CELL chip
2 cm
B
Labeled apoptotic cell
MIX chip
PS
AnnV
Biotin
QD
Streptavidin
HeLa
2 cm

Figure 2

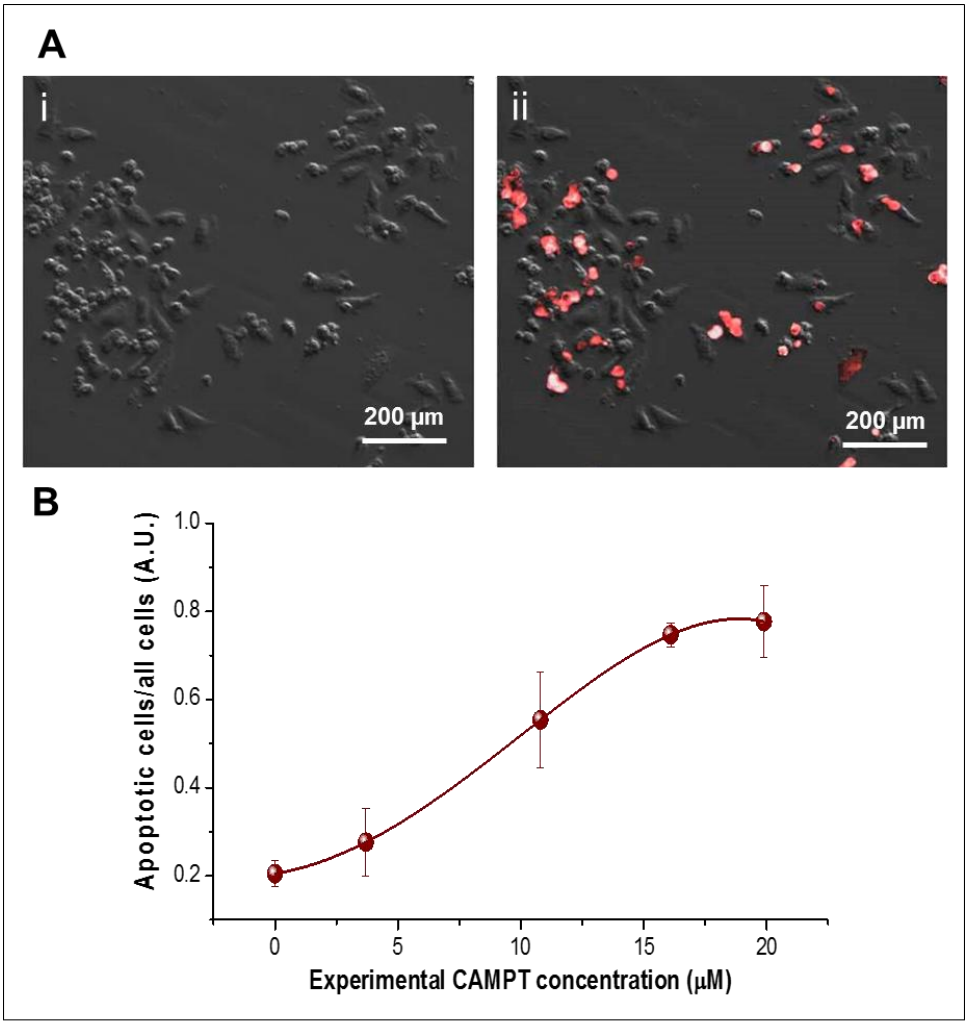
A
i
200 μm
ii
200 μm
B
Apoptotic cells/all cells (A.U.)
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
Experimental CAMPT concentration (μM)

Figure 3

**Tables**

**Table 1. Flow rate and time settings to perform the full assay**

| | ***CGG chip*[a]** | ***MIX chip (C sector)*[b]** | ***MIX chip (M sector)*[c]** | ***CELL chip*[d]** |
|---|---|---|---|---|
| ***Flow rate (µl/min)*** | 10 | 5 (inlet 1)<br>5 (inlet 2) | 10 (outlet C sector)<br>10 (inlet 3) | 4 |
| ***Time of injection (min)*** | 3 | 8 | 4 | 5 |

*[a]Generation of CAMPT concentration, [b]Conjugation of AnnV and QD, [c]Mixing with AnnV binding buffer, [d]Injection of AnnV-QD labeling into CELL chips.*

**Figure captions**

**Figure 1. Segmental chips optimisation.** (A) CELL chip optimisation: i) schematic of the CELL chip with the three chambers where cells are seeded for analysis, and ii) cells uniformly distributed in the three chambers of the same chip 24 h post-seeding. (B) Optimisation of concentration gradient generator: i) schematic of the CGG chip with the corresponding outlet images (optical images of the trypan blue dilutions at the end of the channel of each outlet), and ii) comparison between theoretical and optimal experimental concentration data resulted from the obtained solutions at the outlets (0, 2.5, 10, 17.5, 20 µM and 0, 4, 11, 16, 20 µM, respectively), and (C) optimisation of the MIX chip: i) graph of mixing factors obtained from images taken at C0, C50, C100 and C150 cm of the channel path in the C sector (conjugation of AnnV and QDs), and ii) Graph of mixing factors calculated along the M sector of the chip from images taken at M0, M50, M100 and M150 cm, where conjugated AnnV-QDs are mixed with the $Ca^{2+}$ enriched binding buffer.

**Figure 2. Modular microfluidic platform for apoptosis monitoring**. (A) Human carcinoma cells (HeLa) are cultured in the CELL chip composed by three identical chambers. Cells growing in the CELL chip are exposed to different CAMPT doses obtained through the concentration gradient generator chip (CGG). In this stage, cells will start translocation the PS to the outer leaflet of the plasma membrane. (B) In a second step a mixer chip (MIX) with two identical areas is in charge, first, of the conjugation of biotinylated AnnV with streptavidin-QD and, second, the mixture of AnnV-QD conjugates with the binding buffer containing the $Ca^{2+}$ required for the specific binding with the translocated PS. The MIX chip is then connected to the CELL chip in order to incubate cells with the probe and to label the apoptotic cells. Photographs of the set of chips used in the two-stage assay are shown next to the schematics: (A) fluidic interconnection between the CGG chip where the CAMPT doses are generated and the respective CELL chips where cells are grown, (B) fluidic interconnection of the MIX chip, where the AnnV-QD conjugation takes place, with the CELL chips exposed, in the previous step (A), to different doses of CAMPT. Labelling is done in this step.

**Figure 3. Apoptosis induction by different CAMPT doses**. (A) Example of images obtained from the chips after incubation with CAMPT and labelling with AnnV-QD: i) typical bright field image of a selected area of the chamber, ii) fluorescence image of the same selected area overlapped with the bright field image. (B) Graph showing the dose-response curve corresponding to the ratio of apoptotic cells/total cells (QD-labelled cells/total number of cells) in relation to the CAMPT concentration they were incubated with. Standard deviation bars show the repeatability results between the three different chambers of a CELL chip.

**Supplementary Material**

**Click here to download Supplementary Material: Supporting Information.docx**

***Highlights (for review)**

- A modular microfluidic platform combined with AnnV/QD probes is designed to perform drug screening assay.

- Human carcinoma cell lines are used as a target and apoptosis is detected upon an anti-cancer drug exposure.

- Our system shows dose-response correlation being easier to use, faster and versatile than traditional drug screening methods.