Screening and high-throughput platelets assays

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Abstract

High-throughput assays are important biological research tools but are rarely utilised for platelet research. However, screening compounds for efficacy against a physiologically relevant cellular response in primary cells such as platelets can be an advantageous approach to compound screening and drug development. In this section we describe a panel of three high-throughput microtitre plate assays designed for platelets that can be used as the basis for compound screening, or be modified and used individually to increase throughput in platelet research labs. The platelet adhesion assay has the lowest requirement for platelet numbers and is therefore capable of the greatest throughput and so is suggested as the primary screen used to identify hits. A second screen against the ‘gold standard’ of platelet function, aggregation, is used to confirm and further characterise hits. Finally, a Ca^{2+} assay is used for initial mechanistic characterisation to begin the process of elucidating the mode of action of hit compounds.

Key words: Platelet, High-throughput, High-Content, Screening, Ca^{2+}, Adhesion, Aggregation
1. Introduction

Screening on the basis of clinically relevant functions using primary cells is referred to as physiological or phenotypic screening [1] and, by identifying compounds that exert a desired clinical effect on a target tissue, bypasses the need for initial target identification and screening using a cell line expressing a target protein [2]. Two functions of platelets that are relevant to their roles in thrombosis and haemostasis are their ability to adhere to proteins of the subendothelial matrix and to form aggregates. Mechanistic characterisation of compounds that are able to modulate these platelet functions should follow ‘hit’ identification to elucidate their mode of action. Cytosolic Ca\(^{2+}\) is a master regulator of platelet function and is common to nearly all platelet signalling pathways [3] and is therefore a versatile signalling output that can be used for initial mechanistic characterisation.

This chapter describes a panel of high-throughput techniques to characterise the effects of compounds on three aspects of platelet function; adhesion, aggregation and Ca\(^{2+}\) signalling. The miniaturised platelet assays measure these outputs in microtiter plates and may be applied in sequence to screen compound libraries and to give comprehensive characterisation of ‘hits’ that modulate platelet function. Alternatively, the methods may be adapted and utilised individually to reduce costs, increase throughput and facilitate a pharmacological approach to platelet research.

Platelet adhesion assays are commonly performed on glass coverslips coated in matrix proteins such as collagen. Our approach utilises 96-well microtitre plates, but the assay could easily be miniaturised for use in 384-well plates given the correct setup. The rationale behind using the platelet adhesion assay as the primary high-throughput screening test is the very low number of platelets required per test, at \(1 \times 10^6\) platelets per well in a half-area 96-well plate or just \(5 \times 10^5\) per well of a 384-well plate. Such low requirements are conducive to screening of compound libraries that may contain thousands of molecules. The challenges of the approach lie in data collection and analysis, as every well must be imaged and analysed to give a quantitative output of platelet adhesion and morphology. However, automated imaging platforms and bespoke analysis packages are now
common and so capturing and analysing thousands of images is no longer the insurmountable challenge it once was.

Platelet aggregometry has remained the gold standard in platelet function testing since the method was published by Gustav Born in 1963 [4]. Our plate-based aggregation assay was adapted from the method published by Lordkipanidze et al [5] and gives only an end-point measurement rather than the real-time aggregation trace generated by Born aggregometry. This means that only a single value is generated per condition tested giving a simplistic but easily interpretable read-out of platelet aggregation. Two advantages of the plate-based aggregation assay are that both the assay itself and the analysis are extremely quick.

Ca$^{2+}$ is a master regulator of platelet function, indeed elevation of cytosolic [Ca$^{2+}$] is mediated downstream of almost all platelet activators and so it is also a point of signal integration. Measurement of cytosolic Ca$^{2+}$ is a commonly utilised assay tool within the pharmaceutical industry and so specialised plate readers with automated liquid handling are similarly well established and also achieve good results when used to assay platelet responses. Our assay utilises half-area 96-well microtitre plates, but systems that enable rapid measurements of 384-well plates are also compatible with screening platelet responses. Ca$^{2+}$ measurements are performed in real time and are therefore potentially more time consuming than other techniques described in this chapter. However, real time Ca$^{2+}$ measurements reveal details of signalling dynamics that may be useful and can be used to inform quantitative systems pharmacology.

High-throughput methodologies are increasingly being adopted within academia and while few academic labs have access to robotics and automated systems, facilities have been established that can give academic researchers access to high-throughput screening (HTS) platforms, compound libraries and specialists with expertise in HTS [6]. In addition to compound screening high throughput methodologies facilitate a pharmacological approach to the study of platelets by enabling concentration-response relationships to be defined quickly and easily. Estimation of IC$_{50}$,
EC$_{50}$ and E$_{\text{max}}$ values from platelet concentration response data, as well as other more complex pharmacological analysis have been outlined by Hourani [7]. Any attempts at compound screening and medicinal chemistry must include such pharmacological characterisation of compounds. Finally, high throughput methodologies facilitate testing of many different combinations of biologically active compounds, and therefore have many practical applications in research.
2. Materials

2.1. Buffers and solutions

1. Tyrode’s buffer: 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 0.2 mM Na₂HPO₄, 12 mM NaHCO₃, 5.5 mM D-glucose, [pH 7.4]

2. Acid citrate dextrose (ACD): 29.9 mM trisodium citrate, 113.8 mM D-glucose, 72.6 mM NaCl, and 2.9 mM citric acid [pH 6.4]

3. Sodium citrate buffer: 10 mM trisodium citrate

2.2. Reagents and equipment

1. Half-area, clear, flat bottom 96 well microtiter plates (for aggregation assay)

2. Half-area clear, flat bottom 96 well microtiter plates (for adhesion assay)

3. Black, flat bottom half area 96-well microplates (for Ca²⁺ assay)

4. Heated orbital plate shaker (capable of shaking at 1,200 rpm)

5. Fluorescence microplate reader

6. Absorbance microplate reader

7. Confocal microscope

8. Essentially fatty acid free bovine serum albumin

9. 10% formalin solution

10. 1mg/ml 3,3′-dihexyloxacarbocyanine iodide (DiOC₆)

11. Fura-2 AM

3. Method

3.1. Blood collection

1. Obtain human blood from consenting healthy volunteers who are free of anti-platelet medication and who have not taken painkillers (aspirin, ibuprofen or paracetamol) within 10 days, via venesection of the antecubital vein. Collect the blood into 3.8% (w/v) sodium citrate.

3.2. Test compound preparation
1. Prepare test compounds in 96-well ‘V’ or ‘U’-bottomed plates, deep-well plates may be used if volumes are sufficiently large

2. Test compounds should be diluted in Tyrode’s buffer to 5 X final assay concentration. For example, for initial screening it may be appropriate to test compounds at 100µM, as so an intermediate dilution to 500µM should be prepared.

3. If a concentration range of test compound is to be tested, prepare half-log dilutions at 5 x final concentrations (e.g. 500, 150, 50, 15, 5µM etc.) in Tyrode’s buffer and include a vehicle control containing an equal concentration of solvent. The volume test-compound solution prepared should be equal to the volume required for all experiments plus a ‘dead volume’ of at least 20%.

4. High-throughput adhesion assay

4.1. Washed platelet preparation

1. Add acid citrate dextrose (ACD) to a final concentration of 12.5% (v/v) and then centrifuge whole blood at 100g for 20 minutes and collect platelet rich plasma (PRP) into falcon tubes. Centrifuge PRP at 350g for 20 minutes, discard the supernatant and gently re-suspend platelet pellet in Tyrode’s buffer at a concentration of 2e⁷ platelets / ml

4.2. Adhesion assay plate preparation

1. Using a multichannel pipette, coat wells of 96-well, half area, clear bottom microtitre plate (referred to as ‘assay plate’) with 40µl of adhesive receptor ligand solution of choice for 1 hour at 37°C. Suitable proteins and peptides include collagen (100µg/ml), fibrinogen (100µg/ml), CRP-XL (10µg/ml) and GFOGER (10µg/ml).

2. Aspirate ligand solution and wash once with Tyrode’s buffer

3. Pipette 40µl of 1% BSA into assay plate and incubate for 1h at 37°C
4. Aspirate BSA solution and wash three times with Tyrode’s buffer, if plate is not going to be used immediately, add 40µl of Tyrode’s buffer and use a plate sealer or lid to cover the plate and store at 4°C for no more than 24h

4.2.2. Adhesion Assay

1. Transfer 10µl of test compound solution to each well of the assay plate using a multichannel pipette, ensure that at least one well contains vehicle only, as all other conditions will be compared to the vehicle-treated sample

2. Transfer washed platelet suspension to a reservoir and use a multichannel pipette to add 40µl to each well of the assay plate

3. Cover and incubate for 45 minutes at 37°C

4. Aspirate and discard washed platelet suspension and wash well once with Tyrode’s buffer, do not aspirate or dispense directly onto the centre of the well as this can dislodge adhered platelets.

5. Add 40µl of 10% formalin solution to each well and incubate for 10 minutes at room temperature

6. Aspirate and discard formalin solution and wash once with Tyrode’s buffer

7. Add 40µl Tyrode’s buffer with 4µg/ml DiOC₆ and incubate for 20 minutes at room temperature (there is no need to wash off the DiOC₆)

8. Either image immediately or store at 4°C for no more than 24h

4.2.3. Imaging and analysis

1. Either a microscope system with an automated, motorised stage or a high-content imaging platform is highly recommended for image acquisition

2. Fluorescence imaging should be performed using a 20X objective lens (note 1) using excitation at 488nm and measuring emission at 550nm

3. Capture images with a resolution of at least 1024 x 1024 so that morphological characteristics of platelets can be distinguished
4. Images should be captured in the centre of every well and exported as an image series to simplify analysis.

5. Many different image analysis packages are available and the exact method to identify, count and measure individual platelets will vary but usually involves:
   A) creating a ‘threshold’ that identifies platelets on the basis of fluorescence intensity (see note 2) and can be used to calculate % surface coverage which is the simplest output from the spreading assay (figure 1)
   B) using the binary image generated by the ‘threshold’ to perform object or particle measurements, whereby characteristics of platelets (such as number and spread area) are quantified (Figure 2A)
   C) The open source image analysis software, ImageJ or Fiji (Fiji Is Just ImageJ) can be used to analyse spreading data and a macro is included in the notes for this section (note 3), however, users are encouraged to gain familiarity with this software to ensure accurate results are achieved
   D) Once measurements of platelet area have been generated, scoring based on platelet area can be performed (figure 2)

4.3. High-throughput aggregation assay

4.3.1. Platelet preparation

1. Either:
   A) For PRP, centrifuge whole blood at 100g for 20 minutes and collect PRP into falcon tubes (also collect 50µl of platelet poor plasma (PPP) for use as a ‘blank’ per assay plate by centrifuging 60µl PRP at 10,000g for 2 minutes and aspirating 50µl of supernatant)
   B) For washed platelets, add acid citrate dextrose (ACD) to a final concentration of 12.5% (v/v) and then centrifuge whole blood at 100g for
20 minutes and collect PRP into falcon tubes. Centrifuge PRP at 350g for 20 minutes, discard the supernatant and gently re-suspend platelet pellet in Tyrode's buffer at a concentration of \(2 \times 10^8\) platelets / ml.

4.3.2. Aggregation assay

1. Agonists should be prepared at 10 X final concentration is Tyrode’s buffer. For examples, if stimulation with 1µg/ml collagen (note 4) is required, prepare a sufficient volume of 10µg/ml collagen solution.

2. Using a multichannel pipette, transfer 5µl of test compound to each well of a half-area, flat-bottom, clear 96-well microtitre plate (referred to as ‘assay plate’).

3. Decant washed platelets or PRP into a reservoir and transfer 40µl into each well of the assay plate, avoid creating bubbles by using reverse pipetting (note 5) and incubate at room temperature for 5 minutes.

4. Add 40µl of Tyrode’s buffer or PPP plus 10µl of Tyrode’s buffer to one well of the assay plate to be used as a ‘blank’.

5. Add 40µl of washed platelets or PRP to one well and add 10µl of Tyrode’s buffer, this will be the ‘resting’ sample.

6. Pre-heat plate shaker to 37°C and then securely clip assay plate onto the plate shaker.

7. Quickly and carefully add 5µl of 10 X agonist into the remaining wells of the assay plate (avoid bubbles), an electronic multichannel pipette with multi-dispense function makes this process quick and easy.

8. Shake plate at 1,200 rpm for 5 minutes.

9. Measure absorption (any wavelength of visible light should work) using a plate reader within 10 minutes.

4.3.3. Analysis

1. Convert absorbance to % light transmittance (% LT) using the following equation:
\[
% LT = 10^{-\text{Abs}} \times 100
\]

2. Then convert \% LT to \% aggregation using this equation:

\[
% \text{Aggregation} = \frac{100}{\text{Resting} - \text{Blank}} \times x - \text{Blank}
\]

Where ‘resting’ and ‘blanks are the \% LT value for the resting and blank samples respectively and \(x\) is the \% LT value of a test sample.

3. If a concentration range of each test compound was tested, plot concentration response curves using curve fitting software to estimate \(IC_{50}\) and \(E_{\text{max}}\) values (figure 3).

5. High-throughput \(Ca^{2+}\) assay

5.1.1. Preparation of fura-2 loaded washed platelets

1. Add acid citrate dextrose (ACD) to a final concentration of 12.5\% (v/v) and then centrifuge whole blood at 100g for 20 minutes and collect PRP into falcon tubes.

2. Add fura-2 AM or other \(Ca^{2+}\) dye (note 6) for a final concentration of 2\(\mu\)M and incubate at 30\(^\circ\)C for 1h.

3. Centrifuge PRP at 350g for 20 minutes, discard the supernatant and gently re-suspend platelet pellet in Tyrode’s buffer at a concentration of 4\(\times 10^8\) platelets / ml.

4. Store platelets at RT (note 7) and protect from light by wrapping falcon tube in aluminium foil.

5.1.2. \(Ca^{2+}\) Measurements

1. Prepare agonist and test compound plates as described in the previous section, add \(CaCl_2\) or EGTA at 10X final concentration to test compounds if assay needs to be performed with or without the contribution of store operated \(Ca^{2+}\) entry respectively (note 8) or omit these if the assay is to be performed under ‘nominally \(Ca^{2+}\) free’ conditions (note 9).
2. Turn on fluorescence plate reader 1h in advance of experiment to ensure it is at temperature.

3. Store assay plates at 37°C (in plate reader if possible) to enable them to equilibrate (note 10).

4. Load 10µl of test compound (containing CaCl₂ or EGTA) into as many wells as can be read simultaneously.

5. Add 80µl of fura-2 loaded platelets into the same wells.

6. Incubate at 37°C for 3 minutes.

7. Load next column / plate with test compounds and incubate at 37°C (this will allow these samples to reach 37°C while the other samples are measured).

8. Use a measurement protocol with the following settings:
   
   A) Excitation at 340nm and 380nm.
   
   B) Emission at 510nm.
   
   C) Measure each well at least once every 4 seconds (note 11).
   
   D) 10µl of agonist injected at highest speed (see note 12) 20 seconds after measurement begins (Figure 4).
   
   E) Measure for a total duration of at least 2 minutes (see note 11).
   
   F) Data can be displayed as a ratio of the fluorescence emission intensity excited by 380nm over 340nm, alternatively the [Ca²⁺] can be quantified using the method described by Ohlmann et al.[8].
6. **Notes**

1. Large images (acquired with a lower magnification objective lens) enable more accurate measurements by giving larger sample area but can compromise resolution and must be optimised for the system used for image acquisition.

2. Thresholding must initially be performed manually but then should be not altered for consistency.

3. Basic FIJI macro for analysis of spreading data:

   ```java
   run("Set Measurements...", "area mean area_fraction stack display add redirect=None decimal=3");
   //run("Threshold...");
   setAutoThreshold("Huang dark");
   run("Convert to Mask", "method=Huang background=Dark");
   run("Analyze Particles...", "size=20-200 display clear summarize stack");
   ```

4. Collagen is a versatile agonist in the context of platelet function screening because both adhesion to collagen and collagen-evoked aggregation are highly dependent on kinase signalling as well as signalling evoked by secreted secondary mediators such as ADP and TxA₂. If a screening compound affects any of these processes, they will cause measurable inhibition collagen-mediated platelet function.

5. Reverse pipetting, whereby the volume aspirated into the pipette tip is greater than the volume to be dispensed, is critical to avoid creation of bubbles in PRP when performing the aggregation assay. Bubbles cause major artefacts when light transmittance is measured. To reverse pipette; push the pipette plunger to the ‘second stop’ and the carefully aspirate liquid until the plunger is back in the starting position. Transfer liquid to the recipient well by pressing the plunger down to the ‘first stop’. The liquid remaining in the tip can be discarded, returned to the original well or another transfer of the same liquid can be performed without dispensing the residual liquid.

6. Many non-ratiometric Ca²⁺ dyes, such as fluo 4 and fluo 4 can be used to measure intracellular Ca²⁺ in platelets and excitation and emission filters for the appropriate wavelengths are more
likely to be included with non-specialist plate readers. However, agonist addition artefacts cannot be compensated for by non-ratiometric dyes and can complicate data analysis.

7. Storage of Fura-2 loaded platelets at RT is preferable to storage at 37°C as leakage of Ca²⁺ dyes is temperature dependent, therefore warm to 37°C only immediately prior to measurement

8. If it is desirable to perform Ca²⁺ measurements experiments in the presence of extracellular Ca²⁺ or EGTA, these should be combined with test compounds and pre-incubated with platelets for no longer than 5-10 minutes to avoid spontaneous platelet activation or depletion of intracellular Ca²⁺ stores respectively.

9. It should be noted that unless a Ca²⁺ chelator is added to assay buffer, the buffer cannot be truly free of Ca²⁺ due to contamination of other salts with Ca²⁺-containing salts and is instead referred to as ‘nominally Ca²⁺ free’

10. Ca²⁺ measurements are extremely sensitive to temperature so it is critical to ensure proper equilibration to 37°C prior to agonist stimulation in order to achieve reproducible results

11. Ca²⁺ release can occur very rapidly, especially when evoked by GPCRs, and so care should be taken to ensure that a reading is taken from each well at least every 4 seconds. However, some responses (e.g. to collagen) occur slowly and total measurement time may need to be increased to capture the peak of the response.

12. As Ca²⁺ assays performed in microtitre plates cannot be performed under stirring conditions, mixing of agonists with the platelet suspension is dependent on the speed of agonist injection. Agonist injection speed should therefore be set to a high setting to achieve complete mixing.
13. References

Figure 1. Measurements of platelet surface coverage on fibrinogen. Washed platelet were treated with inhibitors at a range of concentrations and then incubated at room temperature for 45 minutes in fibrinogen coated wells of a 96-well plate and then fixed and stained with DiOC₆. A) Representative images of fibrinogen adhered platelets treated with integrin $\alpha_{IIb}\beta_3$ antagonist, integrillin or vehicle. B) Platelet adhesion quantified in terms of % surface coverage and plotted against inhibitor concentration and fitted using non-linear regression. Only integrillin is able to ablate adhesion of platelets to fibrinogen while cangrelor, dasatinib and ibrutinib are partial inhibitors.
Figure 2. High content analysis of spreading on different coatings. Washed platelet were treated with ibrutinib at a range of concentrations and then incubated at room temperature for 45 minutes in fibrinogen, CRP-XL or collagen coated wells of a 96-well plate and then fixed and stained with DiOC₆. Wells were imaged and then the area of individual platelets was estimated and used to ‘bin’ platelets into categories corresponding to the presence of the following morphological characteristics; Lamellipodia, filopodia or adhered only. A) Representative images of platelets fitting into these categories and binary images used to estimate their area. Numbers of platelets in each category were plotted against [ibrutinib] for platelets adhered to B) CRP-XL, C) collagen and D) fibrinogen. High content analysis highlights important differences in the way that ibrutinib modulates platelet adhesion and spreading on different adhesive surfaces.
Figure 3. Test compound titrations and log IC₅₀ values. Washed platelets were treated with a range of inhibitor concentrations (100µM to 100nM at half-log intervals). Platelets were stimulated with 1µg/ml collagen and shaken for 5 minutes. Log IC₅₀ values were estimated from non-linear regression analysis, although concentration ranges of some compounds could be adjusted in subsequent experiments. Stimulating with a low concentration of collagen is useful for screening as
aggregation under these conditions is highly dependent on tyrosine kinases such as SFKs and Syk as well as the contribution of receptors activated by secreted agonists such as ADP. Consequently inhibition caused by dasatinib (SFK inhibitor), entospletinib (Syk inhibitor) and MRS2179 (P2Y1 antagonist) as well as several other inhibitors with diverse functions is easily detected.
Figure 4. Simultaneous Ca$^{2+}$ measurements stimulated by a range of CRP-XL concentrations. Fura-2 loaded washed platelets were stimulated with CRP-XL (between 10µg/ml to 0.01 µg/ml at half-log intervals) in a 96-well plate. Agonist was injected into 8-wells and fluorescence measured simultaneously for 200s.
Figure 5. Inhibitor combinations to study relative contributions of secreted secondary mediators to CRP-XL evoked Ca\(^{2+}\) signalling and regulation by Btk, Syk and PI3K. Fura-2 loaded washed platelets in the presence or absence of secondary mediator inhibitor cocktail (100µM MRS2179, 1µM Cangrelor and 10µM indomethacin) were treated with a range of concentrations of A) Ibrutinib, B) PRT062607 or C) Alpelisib (between 10µM to 0.01 µM at half-log intervals) and stimulated with 1 µg/ml CRP-XL in a 96-well plate. Agonist was injected into 8-wells and fluorescence measured simultaneously for 300s. Ibrutinib and PRT062607 inhibit Ca\(^{2+}\) signalling with equal potency in the presence or absence of the inhibitor cocktail, while Alpelisib is a more potent inhibitor of the portion of Ca\(^{2+}\) signalling contributed by secreted secondary mediators.
Figure 6. Pipetting into microtiter plates. When pipetting into microtitre plates A) angle the pipette tips into the corner of the well and make contact with one of the walls and avoid B) touching the pipette tip at a right-angle to the bottom of the well which may block the opening or C) failing to touch the edge of the well which may cause drops to be retained on the pipette tip.