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BRIEF REPORT

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# Flow cytometric kinetic assay of calcium mobilization in whole blood platelets of bottlenose dolphins (*Tursiops truncatus*)

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## Abstract

Marine mammals may suffer alterations in platelet function and hemostasia due to multiple pathologies, environmental conditions (including stress) or exposure to different contaminants that induce platelet activation. Detecting early alterations in platelet function in these animals could be an especially relevant diagnostic tool in these species because they typically do not show signs of weakness or disease until the pathology is in advanced state, in order to avoid attracting predators in natural conditions. The study of early markers of platelet activation is relevant for the detection, monitoring and therapy of inflammation and hemostasis disorders. Flow cytometry provides a convenient method to evaluate platelet activation by following the kinetics of intracellular $Ca^{2+}$, using sensitive fluorescent indicators that can be loaded into intact cells. In order to study intraplatelet $Ca^{2+}$ mobilization in marine mammals, we have adapted a kinetic assay of human platelet activation to study platelet activation in whole-blood samples of bottlenose dolphins (*Tursiops truncatus*) using the $Ca^{2+}$-sensitive dye Fluo-4AM and a clone of the platelet-specific antibody CD41-PE that recognizes dolphin platelets. This no-wash, no-lyse protocol provides a simple and sensitive tool to assess in vitro the time course and intensity of signal-transduction responses to platelet agonists under near-physiological conditions. The adaptation of this technique to marine mammals represents a methodological advance for basic and clinical veterinary applications but also for general environmental studies on these species.

**KEYWORDS**
ADP, marine mammals, platelet activation, signal transduction, veterinary medicine

## 1 | INTRODUCTION

Platelets are small non-nucleated blood cells present only in mammals, their main physiological function being to regulate intravascular

Alicia Martínez-Romero and José-Enrique O'Connor contributed equally to this work.

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hemostasia by preventing interstitial bleeding [1]. However, this process may be deregulated, leading to hemorrhage or thrombosis in many clinical situations, including congenital platelet disorders [2] and acquired conditions, such as hypertension [3], diabetes mellitus [4], leukemia [5] and neurological [6] and neurodegenerative [7] diseases. On the other hand, under acute stress conditions, released catecholamines, such as epinephrine and norepinephrine, may exert activating effects on platelets [8]. Focusing on marine mammals, they can suffer gas embolism during diving, which has been related to platelet activation in other species [9–11]. In another context, environmental pollutants, including heavy metals [12, 13] and polychlorinated biphenyls [14] have also been shown to induce platelet activation.

The study of early markers of platelet activation is relevant for the detection, monitoring and therapy of disorders of hemostasis [15]. Platelet-activating signals, including ADP, thrombin and other biological modulators trigger the release into the cytosol of $Ca^{2+}$ stored in mitochondria, granules or dense tubular system. Such $Ca^{2+}$ mobilization is a signal-transduction event that precedes shape change, aggregation, granule secretion and expression of platelet procoagulant activity [16]. Flow cytometry provides a convenient method to evaluate platelet activation by following the kinetics of intracellular $Ca^{2+}$, using sensitive fluorescent indicators that can be loaded into intact cells [17]. Previously our group developed a fast and sensitive flow cytometric procedure using the $Ca^{2+}$-sensitive dye Fluo-3AM and the platelet-specific antibody CD41 conjugated to phycoerytrin (PE) to determine the kinetics of intracellular $Ca^{2+}$ mobilization in whole-blood platelets with minimal manipulation of the samples [18, 19]. Several independent groups have applied this method in studies of cardiovascular risk in human patients [20–26] and rhesus monkeys [27].

Much like humans and terrestrial mammals, marine mammals may suffer alterations in platelet function and hemostasia due to multiple pathologies, diving disturbances, stress conditions or exposure to environmental contaminants also found in the sea that induce platelet activation [11, 28–30]. Such alterations, even when platelet count is normal, can lead to thromboembolic or hemorrhagic disorders [31, 32]. Detecting early alterations in platelet function is especially relevant in these species, as they do not show signs of disease until the pathology is already advanced, to minimize signs of weakness that could attract potential predators [33]. For this reason, routine measurement of platelet function in animals under human care may be a very useful tool for the early diagnosis and monitoring of hemostasis alterations, improving preventive veterinary medicine in aquariums.

In order to study intraplatelet $Ca^{2+}$ mobilization in marine mammals, we have adapted the kinetic assay previously designed by our group [18, 19] to study platelet activation in whole-blood samples of bottlenose dolphins (*Tursiops truncatus*) using the $Ca^{2+}$-sensitive dye Fluo-4AM and a clone of the platelet-specific antibody CD41-PE that recognizes dolphin platelets. Fluo-4 is similar in structure and spectral properties to Fluo-3, but it has brighter fluorescence emission when excited by argon-ion laser, a high rate of cell permeation, and a large dynamic range for reporting $Ca^{2+}$ [34].

This no-wash no-lyse protocol provides a simple and sensitive in vitro tool to assess the time course and intensity of signal-transduction responses to platelet agonists under near-physiological conditions. Moreover, in the clinical setting, whole-blood techniques have obvious advantages to avoid artifactual platelet activation and allow the maintenance of near-physiological conditions.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Twenty bottlenose dolphins (*Tursiops truncatus*) under human care were included in the study. Fifteen individuals (six males, nine females) were from the Oceanogràfic Aquarium of the City of Arts and Sciences (Valencia, Spain) and five animals (one male, four females) were from the Mundomar Aquarium (Benidorm, Spain). Mean age was 18, 1 year (range: 3–38 years). All the experimental procedures have been approved by the Animal Care and Welfare Committee of the Oceanogràfic Aquarium.

### 2.2 | Blood sampling

In order to facilitate blood extraction, these animals were previously trained to present voluntarily the ventral surface of the caudal fin. Blood was drawn directly through a butterfly needle 21G into a tube containing 1 ml 135-mM $Na_3$-citrate and the samples were kept in the dark at room temperature and analyzed within 2 h after extraction in the Cytomics Technological Service, Príncipe Felipe Research Center (Valencia, Spain). The average blood platelet count of the group was 50,800 ± 4, 200 platelets/μl (range: 22,746–93,400).

#### 2.2.1 | Reagents and solutions

Antibody CD41-PE, clone P2 was from Beckman Coulter (Cat. No: A07781). Fluo-4AM (Life Technologies, Cat # F14201) was prepared as 1 mM stock solution in DMSO, aliquoted and kept at −20°C. Adenosine 5′-diphosphate (ADP) was from Sigma-Aldrich (Cat # A5285). The stock solution was 10 mM in PBS, aliquoted and kept at −20°C. Modified Tyrode's Buffer (137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM $Na_2HPO_4$ and 10 mM HEPES, pH 7.4) was prepared weekly and stored at 4°C. Before starting each experiment, 0.35% BSA and 5.5 mM glucose were added and the buffer kept at room temperature until use.

### 2.3 | Sample preparation for platelet activation

Citrated whole blood was diluted 1:10 in modified Tyrode's buffer and incubated with 5 μM Fluo-4AM at 37°C and 5% $CO_2$ for 15 min in the dark. To identify platelet population, 25 μl of whole blood loaded with

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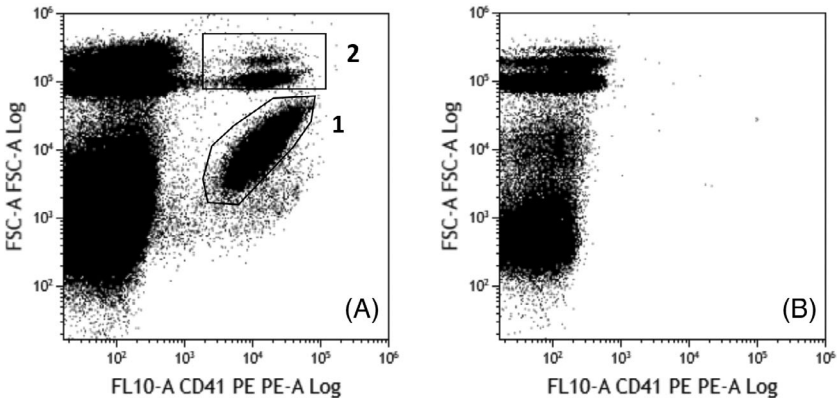


**FIGURE 1** Identification of platelets in whole blood samples. (A) Identification of platelets based on CD41-PE staining and forward scatter signal. Free platelets (region 1) are identified as $CD41^{+}$/$FSC^{low}$ events. Platelets coincident with erythrocytes or leukocytes are identified in region 2 as $CD41^{+}$/$FSC^{high}$ events. (B) Negative control, showing an unstained whole blood sample

Fluo-4AM were incubated with 5 μl of CD41-PE (clone P2, Beckman Coulter) for 15 min at room temperature in the dark. Then, each sample was diluted with 500 μl modified Tyrode's buffer containing BSA and glucose, and transferred to a 1.5 ml Eppendorf vial.

### 2.4 | Flow cytometry setup

All the experiments were performed on a CytoFLEX S Flow Cytometer (Beckman Coulter) using the cytometer-interfaced CytExpert software (Beckman Coulter). The flow cytometer was set up to measure forward scatter (FSC) and side scatter (SSC) signals, Fluo-4 fluorescence (FL1, exc 488 nm/em 525 nm), CD41-PE fluorescence (FL10, exc 561 nm/em 585 nm). All the signals were set to logarithmic amplification. Time was included as a cytometric parameter with stop condition at 10 min. Off-line data analysis and display were performed using CytExpert or Kaluza Software (Beckman Coulter).

### 2.5 | Kinetic analysis of platelet activation

To study the intraplatelet $Ca^{2+}$ mobilization in dolphins, we have adapted the kinetic assay previously described by our research group in human samples [18, 19]. After initiating sample acquisition in the cytometer, the baseline of Fluo-4 fluorescence in single platelets was registered for 20–50 s. At this time, appropriate amounts of the platelet agonist ADP were pipetted directly into the sample vial and mixed gently with a Pasteur pipette, while the cytometer continued aspiring the sample. Data acquisition was stopped after 10 min of the start.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Identification of platelets in dolphin whole-blood samples

To identify specifically platelets in dolphin whole blood, and based on our previous experience with human whole blood platelets [18, 19] CD41-PE Clone P2 (Beckman Coulter, Cat. No: A07781) was found to label specifically platelets in dolphins, as shown in Figure 1.

As for human whole blood under similar experimental conditions [18, 19], single platelets can be identified by their specific expression of CD41 and distinct scatter signals, considering as single platelets the events with lower FSC Log and positive for CD41-PE (Figure 1A), while those with highest FSC signals are considered as platelets bound-to or coincident with erythrocytes and leukocytes (Figure 1A). An unstained sample was used as negative control (Figure 1B).

### 3.2 | Flow cytometric detection of intraplatelet $Ca^{2+}$ mobilization in platelets activated with ADP

As we previously showed for humans [18, 19] intraplatelet $Ca^{2+}$ mobilization as a response to the platelet agonist ADP may be assessed in dolphin whole-blood following the changes in Fluo-4 fluorescence in a fluorescence vs time graph, as exemplified in Figure 2 for a sample stimulated with 24 μM ADP. This concentration was chosen based on previous titration experiments in our original kinetic method in human whole blood [18, 19].

As it is preceptive, single free platelets were identified (Figure 2A) and aggregates were discarded according to FSC-height versus FSC-area values of CD41-positive events (Figure 2B). The baseline of Fluo-4 fluorescence was registered during 50–60 s. At this time, 24 μM ADP (Figure 2C) was added directly to the sample vial and mixed with a Pasteur pipette while the cytometer continued aspirating the sample. After adding the platelet agonist, the run continued and data were recorded up to 10 min. Since the rise in $Ca^{2+}$ induced by ADP happens in a $Ca^{2+}$-free buffer (modified Tyrode's buffer, described in Materials and Methods section), it reflects $Ca^{2+}$ mobilization from internal stores. The subsequent decrease in Fluo-4 fluorescence indicates re-uptake of cytosolic $Ca^{2+}$ into intraplatelet stores or extrusion across plasma membrane, consistent with well-established data [15, 16] and our own experience with the original kinetic method in human whole blood [18, 19].

### 3.3 | Assessment of the kinetic assay of intracellular $Ca^{2+}$ mobilization in activated platelets

Providing numerical indicators of the $Ca^{2+}$ mobilization kinetics is important for characterizing both this dynamic, transient process [18,

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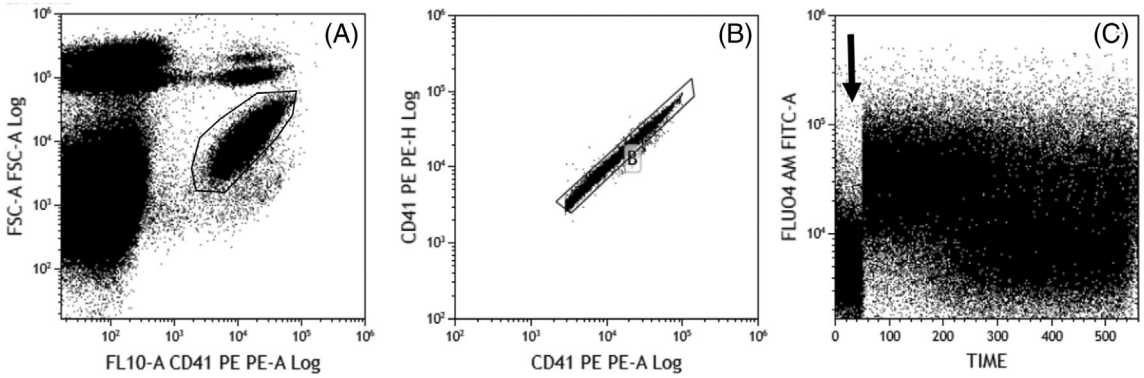


**FIGURE 2** Kinetic analysis of platelet activation of intracellular $Ca^{2+}$ mobilization in activated platelets. (A) Gating of free platelets as $CD41^{+}$/forward scatter $(FSC)^{low}$ events. (B) Aggregate exclusion by FSC-height versus FSC-area of $CD41^{+}$ events. (C) Kinetic plot of $Ca^{2+}$ mobilization in platelets: the baseline of Fluo-4 fluorescence is registered during 50–60 s. At the time point indicated by the arrow, 24 μM adenosine 5′-diphosphate was added directly to the sample vial and the run continued and data were recorded up to 10 min.

19] and assessing its reproducibility. For this purpose, we took advantage of the analytical capabilities of the flow cytometric software. As described above (Figure 2), our kinetic assay involves gating on single platelets, which conditions a Fluo-4 fluorescence versus Time dot plot.

For extracting numerical parameters of this type of plot, we define analytical regions covering the whole length of the fluorescence axis along the Time axis that is time-windows. In the real-time experiment, the cytometer software automatically displays the selected statistical data for each time window as it is filled with events. The original time-windows can be modified after the run, if necessary, for more accurate numerical analysis. For each region, we registered the mean time (in seconds) and the mean fluorescence intensity (MFI) of Fluo-4, and these numerical values were exported for creating diverse kinetic plots using standard graph software (Figure 3). Accordingly, in the kinetic dot plot shown in Figure 3A, we established up to eight time-windows to follow Fluo-4 fluorescence intensity upon activation with ADP. Point "1" defines the baseline fluorescence of Fluo-4, reflecting the platelet $Ca^{2+}$ level at rest. Region "2" marks the peak of Fluo-4 fluorescence after platelet activation, i.e. the maximal level platelet $Ca^{2+}$ induced by the agonist. Region "8" contains the last period of the 10-min acquisition. Regions "3" to "7" follow the changes in Fluo-4 fluorescence over the time.

In this way, we generated numerical descriptors relevant to $Ca^{2+}$ mobilization after ADP addition in whole-blood platelets. Since most of these indicators are described as ratios, they provide normalized indicators that allow to assess the reproducibility of the assay and to compare the kinetics of $Ca^{2+}$ mobilization in different specimens or under different conditions.

Thus, as summarized in Table 1, we calculated several kinetic parameters related to Fluo-4 MFI that is, reflecting the concentration of intracellular $Ca^{2+}$ revealed by Fluo-4. These parameters included the Ratio of Peak MFI to Baseline MFI; the Ratio of Endpoint MFI to Baseline MFI; the differential (Δ) between Peak MFI and Baseline MFI and Δ between Endpoint MFI and Baseline MFI. On the other hand, we also defined relevant parameters related to the kinetics of $Ca^{2+}$ mobilization, namely the delay between ADP addition until Peak fluorescence, and the Recovery slope that is, the slope of the

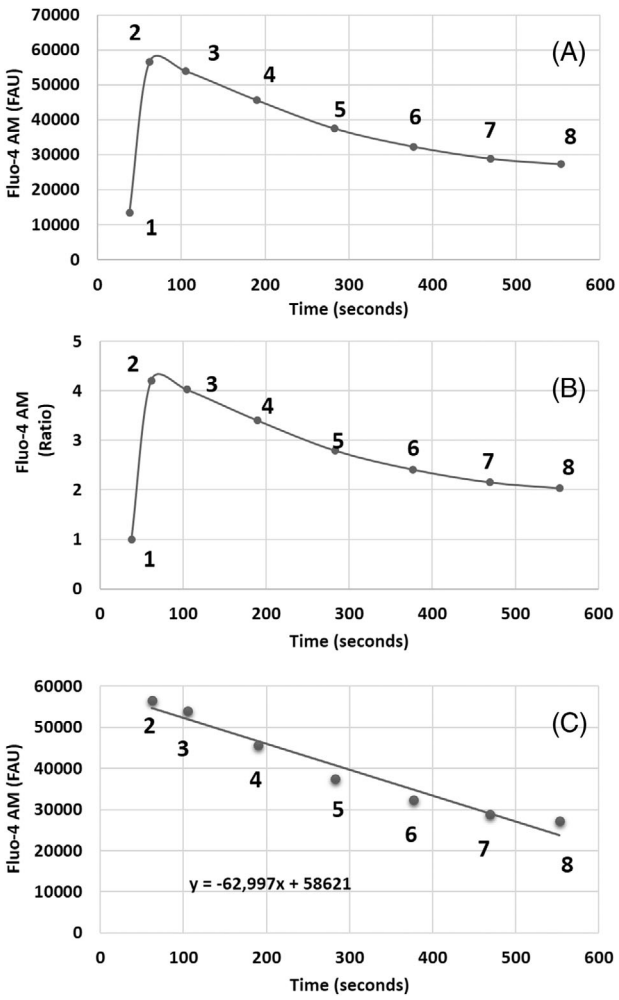


**FIGURE 3** Numerical descriptors for assessing the kinetic assay of intracellular $Ca^{2+}$ mobilization in activated platelets. (A) Standard Fluo-4 versus time dot plot with the eight time points extracted to record Fluo-4 mean fluorescence intensity changes, expressed in arbitrary units (FAU). (B) Normalized graph representing the evolution of Fluo-4 fluorescence expressed as the ratio of fluorescence intensity at each time point over the baseline fluorescence prior to adenosine 5′-diphosphate (ADP) addition. (C) Regression equation for the recovery of intracellular $Ca^{2+}$ based on mean Fluo-4 fluorescence over time. Representative data calculated using FlowJo software.

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**TABLE 1** Parameters relevant to the flow cytometric analysis of $Ca^{2+}$ mobilization after ADP addition in whole-blood platelets of bottlenose dolphins under human care. Data are the mean ± SEM ($n = 20$). The numerical parameters are obtained from cytometric data, as shown in Figure 3 and described in the text

| Parameters related to Fluo-4 MFI | | | | Parameters related to $Ca^{2+}$ kinetics | |
|---|---|---|---|---|---|
| Ratio peak to baseline | Ratio endpoint to baseline | ΔPeak to baseline | ΔEndpoint from baseline | Delay since ADP (s) | Recovery slope |
| 3.4 ± 0.2 | 1.9 ± 0.1 | 34,040 ± 3675 | 12,954 ± 2, 091 | 19.6 ± 1.3 | −50 ± 7 |

fluorescence-decrease curve, related to the kinetics of $Ca^{2+}$ disappearance from platelet cytosol [18, 19]. As the tabulated data show, all the normalized indicators have a relatively low dispersion within the group of animals studied, as their SEM indicate. On the other hand, absolute values such as the delay between ADP addition until Peak fluorescence or the MFI differentials could be expected to show more interassay variability, as they might be affected by methodological factors, such as sample flow rate or dye-to-cell proportion. However, SEM values of these indicators also show that this assay has a good reproducibility, when carefully performed.

Altogether, the results presented here show the applicability to bottlenose dolphins under human care of a fast and sensitive flow cytometric assay designed to determine the kinetics of intracellular $Ca^{2+}$ mobilization in whole-blood platelets with minimal manipulation of the samples [18–27]. The adaptation of this technique to marine mammals represents a methodological advance for basic and clinical veterinary applications but also for general environmental studies on those species. Indeed, in previous works on dolphins, platelets have been studied mainly from hematological or morphological points of view [35], while in killer whales or elephant seals, platelet activation has been studied by aggregometry, membrane lipid composition or thromboxane production [31, 32]. All these experimental procedures require centrifugation, washing or fixation, which impose artifactual conditions to the sample. On the contrary, our protocol provides a simple and sensitive tool to assess in vitro the time-course and intensity of signal-transduction responses to platelet agonists under near-physiological conditions, and should be broadly applicable to studies of platelet reactivity in marine mammals.

Listmode files exemplifying the experiments reported in the manuscript are publicly available in Flow Repository https://flowrepository.org/id/FR-FCM-Z5XT.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## PEER REVIEW

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## SUPPORTING INFORMATION

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