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METHODOLOGICAL ARTICLE

A novel ex vivo approach for measuring plasminogen activation upon established plasma clots

Jason S. Palazzolo PhD[1] | Robert L. Medcalf PhD[2] | Christoph E. Hagemeyer PhD[1] | Be'eri Niego PhD[1]

[1]NanoBiotechnology Laboratory, Australian Centre for Blood Diseases, Central Clinical School, Monash University, Melbourne, Victoria, Australia

[2]Molecular Neurotrauma and Haemostasis, Australian Centre for Blood Diseases, Monash University, Melbourne, Victoria, Australia

Correspondence
Christoph E. Hagemeyer and Be'eri Niego, NanoBiotechnology Laboratory, Australian Centre for Blood Diseases, Central Clinical School, Monash University, Melbourne, Vic. 3004, Australia.
Emails: christoph.hagemeyer@monash.edu (CH); beeri.niego@monash.edu (BN)

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Abstract

Background: The fibrinolytic system plays a critical role in maintaining hemostasis. Central to fibrinolysis is the degradation of fibrin by plasmin, produced in the circulation following the activation of plasminogen by plasminogen activators (PAs). Accurately measuring the plasminogen activation rate is vital for the understanding of fibrinolytic processes, particularly in the context of thrombolysis. Yet, due to the insoluble nature of fibrin, in vitro and ex vivo investigations of PA-mediated plasminogen activation have proven challenging. As researchers frequently adopt soluble fibrinogen fragments and/or alter the experimental system beyond what is physiologically relevant, they limit the validation and interpretation of their findings. Here, we present a novel, high-throughput assay for measuring plasminogen activation rates on natural, plasma-derived fibrin that optimally simulates in vivo conditions.

Method: Human plasma was used as the source of plasmin(ogen) and fibrin(ogen). "Halo-shaped" plasma clots were produced in a 96-well plate using a thrombin-containing clotting mixture, ensuring that an optically compatible and plasma-free center is maintained in each well. Subsequent additions of a plasmin chromogenic substrate and different PAs were followed by absorbance measurements over time to extract the corresponding enzyme kinetics information.

Results and Discussion: Validation experiments demonstrated the capability of our approach to accurately model fibrin-dependent and -independent plasminogen activation as well as sensitively detect variations in plasminogen and fibrinogen plasma levels.

Conclusion: This assay allows a straightforward, yet powerful, measurement of plasminogen activation rates on established plasma clots, with the capability of properly assessing fibrin- and non–fibrin-dependent plasminogen activation by various therapeutic PAs.

KEYWORDS
chromogenic substrate, fibrinogen, fibrinolysis, plasminogen, plasminogen activation, thrombolysis

Essentials

- Current assays to study fibrin-dependent plasminogen activation are limited in design.
- We developed a novel assay that uses a halo-shaped plasma clot to improve in vivo simulation.
- Validation studies could discern the unique properties of several plasminogen activators.
- The method detected variations in plasma proteins, including fibrinogen and plasminogen.

1 | INTRODUCTION

The fibrinolytic system plays an essential role in the maintenance of hemostasis.[1] Central to fibrinolysis is the degradation of fibrin by plasmin, where plasmin is produced following the activation of zymogenic plasminogen by plasminogen activators (PAs).[2] In vitro and ex vivo assays have long been used to study plasminogen activation by several PAs, both fibrin dependent, like tissue-type plasminogen activator (t-PA) and desmoteplase,[3] and non–fibrin dependent, like urokinase plasminogen activator (u-PA).[2] Measuring the rates of plasminogen activation has provided important insights into how PAs influence and are being influenced by different physiological and pathological environments.[4]

From a therapeutic perspective, PAs are routinely administered in thrombolytic therapy.[5] Specifically in the context of thrombolysis for acute ischemic stroke (AIS), recombinant t-PA (rt-PA) has been a front-line pharmacological intervention since the mid-1990s.[6] Therefore, studying the rates of rt-PA–mediated plasminogen activation could serve as a powerful tool toward improving the existing therapeutic regimen for AIS. Given that t-PA significantly drives plasminogen activation only when cofactored with fibrin,[7] the correct simulation of this physiological reality critically requires an intact fibrin polymer. Nevertheless, given the insoluble nature of fibrin, attempts to model its presence in vitro and ex vivo have proven challenging.

Although enzymatic activity assays have been developed to simulate fibrin cofactoring with t-PA, they often harbor limitations that hinder interpretation of findings.[4] Fibrin-mimicking substitutes include soluble fibrinogen products, such as cyanogen bromide–digested fibrinogen fragments.[3,8] Alternative methods employ plasminogen embedded within a fibrin matrix prepared from purified fibrinogen that is rendered artificially transparent with the aid of high NaCl concentrations.[9] Meanwhile, recent advances in the field have adopted a fluorogenic plasmin substrate to overcome the opacity of fibrin clots, as described by Miszta et al.[10] Notably, the investigations of plasminogen activation with PAs embedded *within* plasma clots limit the interpretation of outcomes, particularly in the context of clinical thrombolysis, where PAs are only ever added after clot formation.

In the instances described above, plasmin generation is detected by the plasmin chromogenic substrate S-2251. Although these assays serve the purpose of measuring t-PA–mediated plasminogen activation in the presence of a fibrin-like cofactor, they often involve modifying the reaction system beyond what is physiologically relevant. Furthermore, the solid and opaque nature of the fibrin polymer presents with a practical challenge when designing these assays, since an optically compatible surface and/or sufficiently transparent milieu is required to allow the undisturbed passage of light for assessment by either absorbance or fluorescence detection methodologies.

In this report, we describe a simple and novel methodology for measuring plasminogen activation on natural fibrin, with improved physiological relevance. Derived from the whole blood clot degradation assay described by Bonnard et al.,[11] termed the "halo assay", our method uses plasma as the source of both fibrin(ogen) and plasmin(ogen). When combined with a thrombin-containing clotting mixture, the plasma is formed into a halo-shaped fibrin clot around the perimeter of a well in a 96-well plate, leaving a plasma-free and optically compatible well center that is ideal for light-based assays. Once formed, different PAs together with the amidolytic substrate S-2251 were added to the clot and analyzed over time to determine rates of plasminogen activation in the presence of a subject's own native fibrin. Specifically, we present validation studies that demonstrate the capacity of our assay to discern differences between two fibrin-dependent PAs, rt-PA (alteplase; a second-generation PA) and tenecteplase (TNK; a third-generation rt-PA variant), in addition to the non–fibrin-dependent urokinase (u-PA).

2 | METHOD

The experimental use of human plasma was approved by the Monash University Human Research Ethics Committee (Project 67/15). Blood from healthy volunteers was collected using a 19-gauge butterfly needle into syringes containing 3.2% sodium citrate (blood: anticoagulant ratio = 9:1 [v/v]). All volunteers signed informed consents before blood collection and did not take any medications that could have affected hemostasis in the 10 days leading up to their blood donation. Within 30 min of blood collection, the blood was centrifuged at room temperature at 180 relative centrifugal force (RCF) with the rotor acceleration/deceleration set at 4/0 (out of 10), respectively (Allegra X-15R Centrifuge; Beckman Coulter). Following centrifugation, the platelet-rich plasma (PRP) was collected using low-shear pipetting to minimize any platelet activation. The isolated PRP was centrifuged at room temperature for 7 min at 1700 RCF with the rotor acceleration/deceleration set at 10/0, respectively, to produce platelet-poor plasma (PPP). The PPP supernatant (referred to as "plasma" hereafter) was collected carefully to avoid disturbing the platelet pellet and stored at −80°C.

A clotting mixture was freshly prepared containing 24 U/ml thrombin (Human Plasma, High Activity; EMD Millipore) and 400 mM $CaCl_2$ buffered with 4-(2-hydroxyethyl)-1-piperazine

ethanesulfonic acid (HEPES) buffer (25 mM HEPES and 137 mM NaCl). The final concentrations after mixing with plasma was 4 U/ml thrombin and 66.6 mM $CaCl_2$. A 5-µl droplet of the clotting mixture was pipetted onto the bottom edge of the well in a flat-bottomed, transparent, polystyrene, tissue culture–treated, sterile 96-well plate. The plasma was then left untreated, or supplemented *prior* to the clotting reaction with either 1 or 2 µM of human glu-plasminogen (Hematologic Technologies), or 1 or 2 mg/ml of human fibrinogen (Enzyme Research Laboratories). Equal volumes of HEPES buffer were added to the nontreated samples (naïve plasma). All plasma samples were then diluted 1:2 with HEPES buffer, allowing conservation of the plasma volumes used. Next, using a P100 pipette, 25 µl of diluted plasma was used to form a halo-shaped plasma clot around the bottom edge of each well (Figure 1A).

To achieve a circular-shaped clot while homogenously mixing the clotting mixture with the plasma, the same technical approach as described by Bonnard et al.[11] was followed. In brief, the plasma-filled pipette tip was first used to spread the clotting mixture droplet around the entire bottom perimeter of the well (around the edge), then the plasma was slowly dispensed. Due to the fluidic cohesion effect, the plasma followed a circular pattern to produce a halo-shaped clot. For nonclotted plasma samples, the same procedure was followed; however, the clotting mixture was replaced by HEPES buffer. The plate was then sealed and incubated at 37°C for 1 h (Figure 1A).

Once the clots were formed, 70 µl of a 1:1 ratio of S-2251 (Chromogenix) and either alteplase (rt-PA; Boehringer Ingelheim; dialyzed against 0.4 M HEPES), TNK (Boehringer Ingelheim) or u-PA (U-FRAG; Bharat Serums and Vaccines) was added to the clotted plasma samples. The final concentrations for both reagents in the reaction were 0.5 mM S-2251 and 30 nM for all PAs. The plate was then immediately placed into a 37°C preheated FLUOstar Omega microplate reader (BMG Labtech), and absorbance at 405 nm was read in 1 min intervals (10 flashes per read) over 150 min. No automated plate shaking was performed throughout the experiment duration (Figure 1B). Alongside the tested plasma samples, negative controls (plasma free, where plasma was replaced by HEPES buffer) were included. The absorbance values from the negative controls were subtracted at each time point before plasminogen activation rate calculation. *Data analysis:* Using Prism version 9.0 software (GraphPad Software), the raw absorbance values were plotted against time and all absorbance values greater than 0.5 were excluded. The remaining data were fitted to a second-order polynomial (quadratic) nonlinear regression ($Y = A + B \times X + C \times X^2$), and the resulting equation was twice differentiated to yield the rate of plasminogen activation ($2 \times C$).[3]

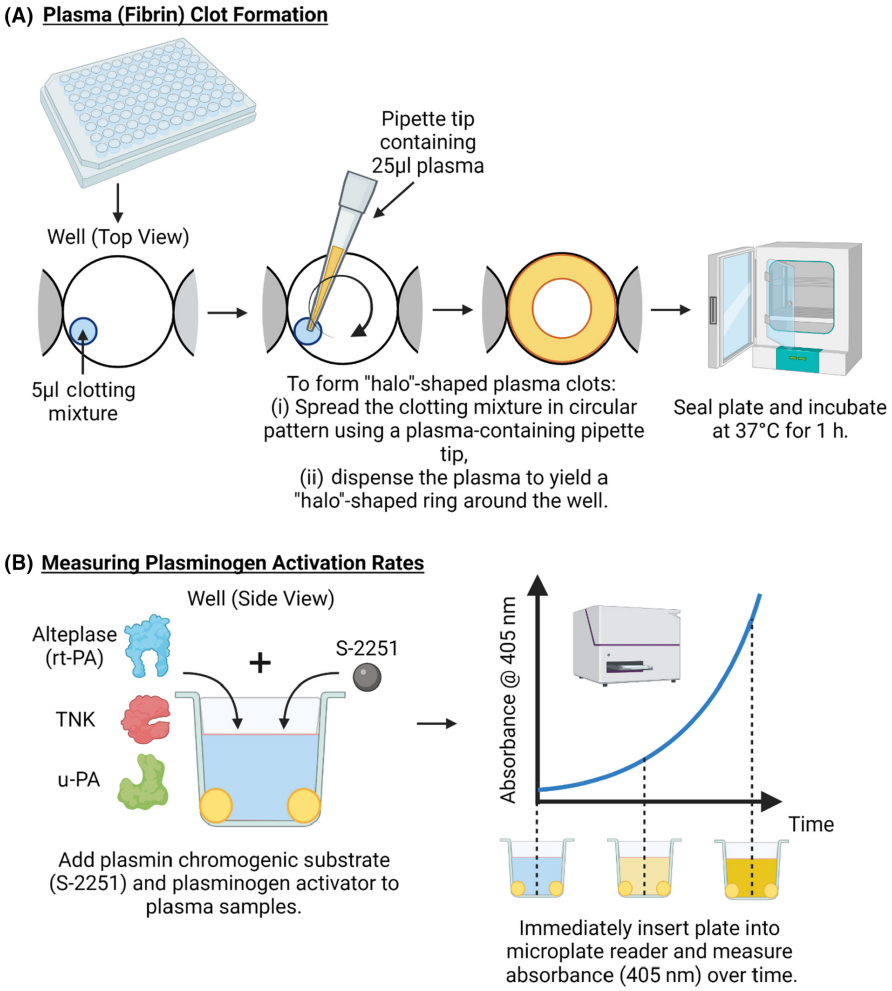

FIGURE 1 Schematic representation of the "plasma halo" assay protocol to measure plasminogen activation rates ex vivo on natural fibrin. Illustration of the assay protocol for the formation of the halo-shaped plasma (fibrin) clot (A) and the measurement of detectable plasminogen activation (B). Figure created with BioRender.com. Abbreviations: rt-PA, recombinant tissue-type plasminogen activator; TNK, tenecteplase; u-PA, urokinase plasminogen activator

2.1 | Statistical analysis

All data are presented as mean ± standard error of the mean. All statistical analysis was performed using GraphPad Prism version 9.0 software. Comparisons among three groups or more were performed by two-way ANOVA with uncorrected Fisher's least significant difference post hoc test. Direct comparisons between two groups were performed by Student's *t* test (paired, two-tailed). A *p*-value of <0.05 was considered significant.

3 | RESULTS AND DISCUSSION

Our validation studies first aimed to demonstrate the capacity of the "plasma halo" assay to measure and compare the plasminogen activation profiles of alteplase, TNK, and u-PA on clotted plasma, (i.e., in the presence of natural fibrin) and nonclotted plasma. These investigations were then extended to determine the capability of this method to detect variations in the levels of (exogenously supplemented) plasminogen and fibrinogen. When testing alteplase (Figure 2), the rates of plasminogen activation were found to be significantly higher on clotted plasma in comparison to nonclotted plasma ($p < 0.01$). Plasma supplementation with 1 and 2 μM of plasminogen (Figure 2A–C) resulted with significant increases in alteplase-mediated plasminogen activation rates on clotted plasma, relative to nonclotted plasma ($p < 0.0001$). Interestingly, plasma supplementation with 1 and 2 mg/ml of fibrinogen (Figure 2D–F) also led to significant increases in alteplase-mediated plasminogen activation rates exclusively on clotted plasma. Furthermore, supplementation with 2 mg/ml of fibrinogen also resulted in significantly greater plasminogen activation rates than both naïve and 1 mg/ml of fibrinogen-supplemented plasma ($p < 0.05$). Notably, these findings well aligned with the expected fibrin dependency of alteplase, where we anticipated that an increased fibrin cofactor levels would drive enhanced plasminogen activation rates.[7] Our assay therefore allows for an accurate simulation of fibrin and its cofactoring role in fibrin-dependent plasminogen activation.

In addition to alteplase, TNK was also studied as another fibrin-dependent PA[12] (Figure 3). Consistent with the results observed with alteplase, TNK achieved significantly higher plasminogen activation rates on clotted plasma relative to nonclotted plasma. TNK also responded in a similar manner to alteplase following plasminogen supplementation of plasma (Figure 3A–C), whereby increased plasminogen levels yielded significantly elevated plasminogen activation rates ($p < 0.01$). Interestingly, TNK-mediated plasminogen activation rates in the presence of nonclotted plasma proved to be smaller than alteplase, confirming that TNK is more dependent upon fibrin than alteplase. Indeed, TNK is known to offer increased dependency upon fibrin as compared to fibrinogen in comparison to alteplase toward plasminogen activation.[12,13] In line with this, TNK showed a significantly higher plasminogen activation rate with as little as 1 mg/ml of fibrinogen supplementation when compared to naïve clotted plasma (Figure 3D–F; $p < 0.05$), whereas alteplase required 2 mg/ml of exogenous fibrinogen to reach a similar level of

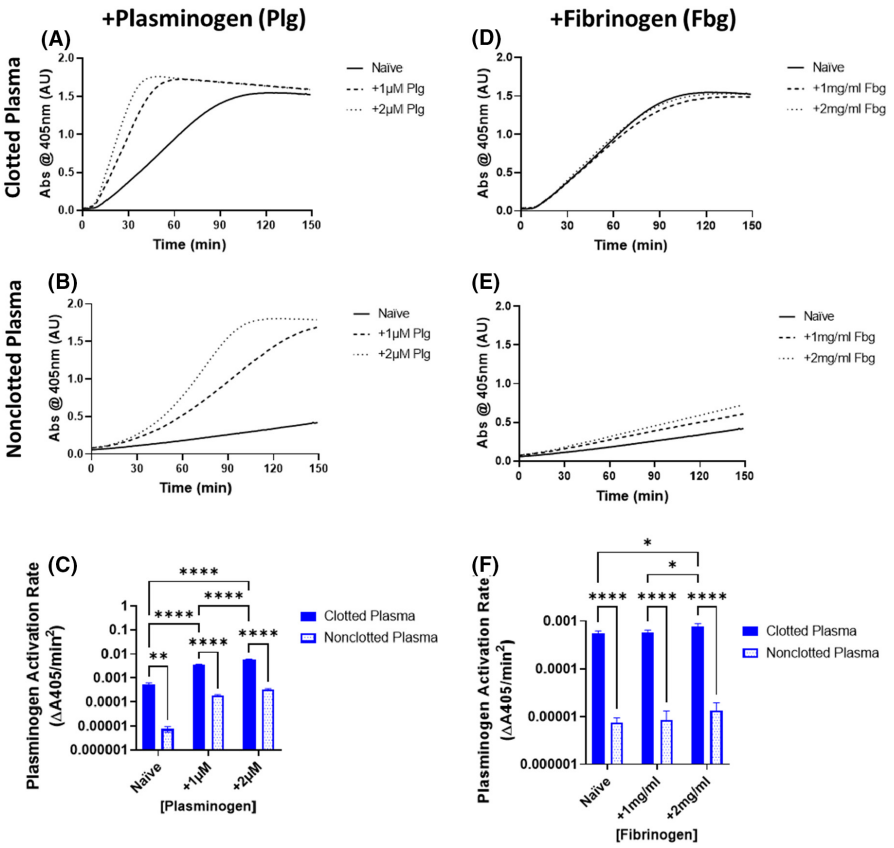

FIGURE 2 Use of the novel "plasma halo" assay to measure alteplase (rt-PA)-mediated plasminogen activation on plasma-derived fibrin. (A–D) Representative S-2251 accumulation curves (resulting from plasmin activity) after addition of rt-PA onto halo-shaped clotted plasma (A, B) as well as nonclotted plasma (C, D), comparing naïve plasma to plasma supplemented with plasminogen and fibrinogen. (E, F) Quantification of the plasminogen activation rates with plasminogen (E) and fibrinogen (F) plasma supplementation. $n = 5$. Two-way ANOVA with Fisher's least significant difference post hoc test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. Abbreviations: rt-PA, recombinant tissue-type plasminogen activator

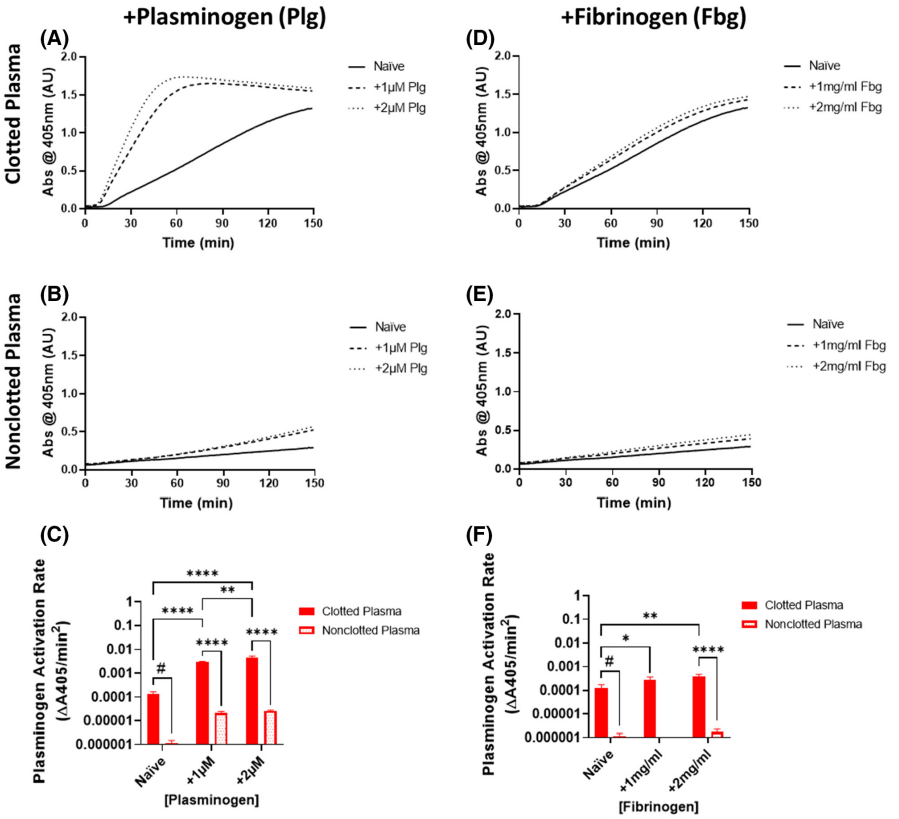

FIGURE 3 Use of the novel "plasma halo" assay to measure tenecteplase-mediated plasminogen activation on plasma-derived fibrin. Representative plasminogen activation profiles on clotted plasma (A, B) as well as nonclotted plasma (C, D) with naïve plasma compared to plasma supplemented with plasminogen and fibrinogen. Quantitated plasminogen activation rates from clotted and nonclotted plasma with plasma supplemented with plasminogen (E) and fibrinogen (F). $n = 5$. Two-way ANOVA with Fisher's least significant difference post hoc test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. Student's t test; $\#p < 0.05$

significance (Figure 2F; $p < 0.05$). This finding is consistent with the increased fibrin dependency of TNK for mediation of plasminogen activation.[12,13] Interestingly, TNK also yielded consistently lower plasminogen activation rates to those measured with alteplase under identical conditions. These findings correlate with those first reported by Keyt et al.,[12] where TNK was associated with less enzymatic activity overall than rt-PA in plasma clots. The ability of our novel plasma halo assay to accurately demonstrate even fine details in the mode of operation of these fibrin-dependent PAs highlights the power and value of this methodology in fibrinolysis research.

u-PA was finally applied to the assay as a reference for a non–fibrin-dependent PA (Figure 4). As anticipated, the measured plasminogen activation rates with u-PA were comparable across clotted and nonclotted plasma (in contrast to the observations with the fibrin-dependent alteplase and TNK). Our findings showed that the plasminogen activation rates of u-PA were dependent on plasminogen concentrations (Figure 4A–C). Surprisingly, at 2 μM of supplemented plasminogen, the measured plasminogen activation rate in nonclotted plasma was significantly greater than when measured in clotted plasma. This was the only occasion throughout the study where plasminogen activation was more robust in nonclotted plasma than on clotted plasma. Fibrinogen supplementation of the plasma (Figure 4D–F) induced significantly higher u-PA–mediated plasminogen activation on clotted plasma, in direct correlation to the supplemented fibrinogen concentration relative to plasminogen activation rates measured on naïve plasma ($p < 0.05$). This observation could be explained by an anticipated conformational change of plasminogen following increased engagement with fibrin,[14,15] which causes the plasminogen molecule to adopt a more relaxed and activatable conformation.[14,15]

From a practical perspective, our presented assay overcomes the flaws associated with the anticipated variations with using human plasma as the assay's source of plasmin(ogen) and fibrin(ogen). To this end, the quality of human plasma samples may be impacted by different circumstances of collection, such as being isolated from hemolyzed red blood cells or from an individual having recently consumed a high-fat meal. These events result in the plasma adopting varying degrees of red or white coloring, respectively. By accommodating for a clear and unperturbed passage of light through the experiment's solution, via the halo-shaped clot, our described assay remains unaffected by those aforementioned outcomes. Meanwhile, the alternate assay designs (described earlier in this article) are often adversely impacted by the varying degrees of plasma quality, which in turn affect any experimental readouts and can limit the interpretation of results.

Moving forward, future directions of this work aim to investigate potential clinical applications of this assay, including as a diagnostic measure. Given the use of human plasma, prospective uses of this assay may include studying plasma collected from patients of various diseases where the fibrinolytic system is impacted, such as thrombotic diseases or cancer.[1] Additionally, this described assay provides the versatility to offer applications in preclinical investigations as well, such as studying plasma collected from animals used for in vivo investigations.

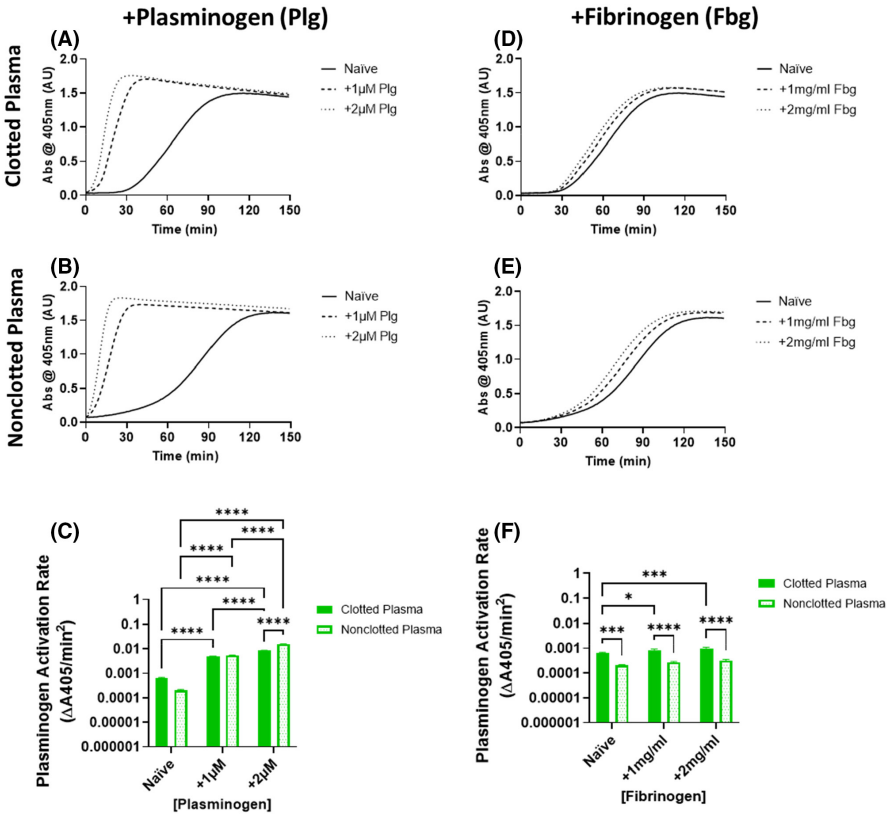

FIGURE 4 Use of the novel "plasma halo" assay to measure u-PA–mediated plasminogen activation on plasma-derived fibrin. Representative plasminogen activation profiles on clotted plasma (A, B) as well as nonclotted plasma (C, D) with naïve plasma compared to plasma supplemented with plasminogen and fibrinogen. Quantitated plasminogen activation rates from clotted and nonclotted plasma with plasma supplemented with plasminogen (E) and fibrinogen (F). $n = 5$. Two-way ANOVA with Fisher's least significant difference post hoc test; $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$. u-PA, u-PA, urokinase plasminogen activator

4 | CONCLUSION

In this study, we present a newly developed, robust, and simple methodology for measuring plasminogen activation rates on established clots ex vivo using natural, plasma-derived fibrin as the ultimate cofactor. We demonstrated that this assay could accurately reflect the main features of fibrin- (alteplase and TNK) and non–fibrin-dependent (u-PA) PAs, and importantly allow the interaction between an exogenously added PA and its cofactor plus substrate to take place only after the clot has formed (similar to the clinical therapeutic scenario). Our validation studies further showed that our novel approach could detect variations in plasminogen and fibrinogen plasma levels. Given the ex vivo nature of our assay, there is the future prospect of using this approach to measure plasminogen activation rates from individual patient plasma samples for various purposes, for example, as a potential diagnostic tool or as a future approach for personalized medical care.

AUTHOR CONTRIBUTIONS

J.S.P. formed the study concept, developed the assay, planned and performed all experiments, analyzed and interpreted the data, generated the figures, and drafted the manuscript. R.L.M. provided critical expert advice and assisted with the interpretation of the experimental results. C.E.H. and B.N. supervised the work and provided critical input in drafting the manuscript.

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RELATIONSHIP DISCLOSURE

The authors have no conflicts of interest to declare.

ORCID

Jason S. Palazzolo https://orcid.org/0000-0001-5731-1246
Robert L. Medcalf https://orcid.org/0000-0001-6885-3892
Christoph E. Hagemeyer https://orcid.org/0000-0002-7114-4023
Be'eri Niego https://orcid.org/0000-0001-6714-9131

TWITTER

Jason S. Palazzolo @PalazzoloJason
Christoph E. Hagemeyer @cehagemeyer

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