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Abstract. We report a new technique to measure coagulation dynamics on whole-blood samples. The method relies on the analysis of the speckle figure resulting from a whole-blood sample mixed with coagulation reagent and introduced in a thin chamber illuminated with a coherent light. A dynamic study of the speckle reveals a typical behavior due to coagulation. We compare our measured coagulation times to a reference method obtained in a medical laboratory. © 2011 Society of Photo-Optical Instrumentation Engineers (SPIE). [DOI: 10.1117/1.3573813]

Keywords: blood dynamics; coagulation; prothrombin time; International Normalized Ratio; multiple scattering analysis; speckle correlation.

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1 Introduction

Coagulation is an important part of the haemostasis process corresponding to the cessation of blood loss from a damaged vessel. The coagulation mechanism implies a complex series of cascading enzymatic reactions involving proteins, referred to as coagulation factors. In order to diagnose disorders in these coagulation factors, different biological tests have been developed (prothrombin time, activated partial thromboplastin time, activated cephalin clotting time, etc.). Coagulation disorders can lead to an increased risk of haemorrhage or thrombosis. Moreover, according to the International Self-Monitoring Association of Oral Anticoagulated Patients (ISMAAP), nearly four million people are under oral anticoagulant therapy in Europe and need to monitor their coagulation time daily to self-adjust the dosage of their treatment and thus maintain the optimal therapeutic range.

Blood coagulation leads to many changes in chemical and physical properties of blood; consequently, many different techniques have been developed in order to detect clot formation. Indeed, many methods have been used to monitor changes in blood viscosity (surface plasmon resonance,^{1,2} quartz crystal microbalance,^{3,4} capillary migration,^{5,6} ultrasounds^{7–9}), surface tension,¹⁰ optical properties,^{11,12} and electrical properties,¹³ among others.

Historically, two main techniques were used in order to measure blood clotting time:¹⁴ the tilt-tube method, where the tube containing a blood sample is tilted back and forth until a fibrin network is formed, and the wire-loop technique, where a wire loop is passed through the mixture until a clot forms and adheres to the loop. These two protocols were done manually but have since been replaced by automated methods. Most systems cur-

rently measuring the clotting time use one of the two following principles: the immobilization of a moving probe immersed in the sample when a clot is formed^{15,16} or the change in optical density of the mixture when coagulation has occurred.^{11,17}

We report a new technique to measure coagulation times relying on the detection of the blood cell immobilization while being trapped into the clot as coagulation occurs. The need for blood cells in the sample urges one to work with whole blood, hence, simplifying the sample preparation. Therefore, by avoiding the extraction of plasma, our novel method appears to be simpler and faster than the other classical techniques.

1.1 Prothrombin Time and International Normalized Ratio

The prothrombin time (PT) is defined as the necessary time for citrated plasma to clot in the presence of tissue thromboplastin and calcium chloride. Actually, when blood is drawn from a donor, it is immediately mixed with sodium citrate, which binds to the calcium of whole blood in order to prevent coagulation and allows transport to the laboratory and preparation steps of the sample (i.e., centrifugation). Thus, to counteract the action of the anticoagulant in citrated plasma, calcium ions must be added to the sample, while the addition of tissue thromboplastin triggers the coagulation cascade to occur. Most techniques used to obtain PT produce results from plasma samples at 37°C.

Most of the time, PTs are used in comparison to a PT_{reference} measured on a pool of healthy donors. Moreover, different coagulation reagents (i.e. human, rabbit or rat thromboplastins) will not have the same sensitivity; the PT ratio will therefore be affected by the reagent used. In order to standardize the PT ratio, the International Sensitivity Index (ISI) has been developed to characterise each batch of reagent. This allows the calculation of the International Normalized Ratio (INR), which minimizes the

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Fig. 1 (a) Schematic and (b) image of the transmission setup, where the sample is placed between the laser and the camera. (c) Typical speckle image recorded on a whole-blood sample.

differences between laboratories (and between thromboplastin reagents). The INR is defined as the ratio of the patient's PT to a normal control PT scaled by ISI,

$$\text{INR} = \left(\frac{\text{PT}_{\text{patient}}}{\text{PT}_{\text{reference}}} \right)^{\text{ISI}}. \quad (1)$$

The INR usually ranges between 1 for healthy patients and >7 for highly pathological samples; we emphasise that hemorrhagic risks appear at >5 . The PT or quick time is dedicated to the screening of extrinsic coagulation pathway, including detection of deficiencies in coagulation factors (i.e. factor II, V, X, etc.). It is also frequently used to monitor the course of oral anticoagulant. An estimated 800 million PT/INR assays are performed annually worldwide.¹⁸

1.2 Principle of Multiple Scattering Dynamics

The speckle pattern is the figure of multiple scattering resulting from the illumination by a coherent light, of a high number of diffusing objects randomly distributed.¹⁹ For example, when illuminating a suspension of diffusing particles with a spatially coherent light (e.g., a laser), each particle diffuses the light, and the constructive or destructive interferences between the diffused rays form the speckle image.^{20,21} In the case of a blood sample, as the cells are unbounded, their residual motions due to the migration in the chamber induce a constantly changing speckle figure, exhibiting a “swarminglike” behavior. When the blood clot forms, the cells are immobilized leading to a speckle image fixed in time.

In conventional multiple dynamic light scattering, the basic idea is to illuminate a sample with a laser and to measure the temporal fluctuations in the resulting speckle pattern of scattered light. In a typical experiment, the setup is arranged in such a way that the intensity of a single speckle is measured as a function of time, hence, recording a strong fluctuation signal.²² Here, we choose another approach consisting in imaging a lot of speckle by using a charge-coupled device (CCD) camera as a multispeckle light detector.²³

We propose a new way to measure this immobilization time characteristic of the coagulation phenomenon through dynamic analysis of speckle patterns. The setup developed to allow the measurement of the correlation coefficient will be described, and the results obtained will be discussed.

2 Material and Methods

2.1 Samples

Blood samples were collected in tubes containing sodium citrate ($V_{\text{blood}}/V_{\text{sodium citrate}} = 9/1$) in order to prevent sample clotting. Whole blood samples were obtained from healthy donors from EFS Rhône-Alpes (France) and patients admitted at the Edouard Herriot Hospital of Lyon (France).

2.2 Experimental Protocol and Setup

Neoplastine® (Stago Diagnostic, Asnières-sur-Seine, France) a commercial mix of thromboplastin and calcium chloride is used to initiate the coagulation phenomenon. 20 μL of Neoplastine is added to 10 μL of whole blood. After homogenisation, the solution is introduced by capillary forces in a fluidic chamber located in the light path between the camera and the laser. The material composing the chambers needs to be transparent; that is why they are made of two glass slides spaced by some double-coated tape of calibrated thickness (Nitto Denko, Osaka, Japan).

The typical size of the chamber is not critical; it has just been adapted to the volume of the sample to analyze, here $10 \times 20 \text{ mm}^2$ suitable for 30 μL of solution. On the contrary, the thickness of the chambers has been carefully selected. In a thick chamber, the resulting speckle figure is well defined because of a high number of cells involved in the multiple scattering; on the other hand, whole blood tends to absorb and diffuse light when passing through thick samples.²⁴ Thus, we need to compromise between too thin (40 μm) and too thick (800 μm) chambers (experimental results not shown here). In the end, 160-μm thick double-coated tapes have been used to set the height of the chamber.

The experimental setup is presented in Figs. 1(a) and 1(b): the light source and the detector are located on opposite sides of the sample. Indeed, the coherent light of a laser (Power Technology, Arkansas, USA) of low-power (10–20 mW) passes through the chamber containing the blood sample. Different wavelengths were investigated: 490, 690, and 980 nm. Transmission measurements not reported here showed that 490 and 980 nm light were absorbed by whole blood and that 690 nm was optimal as expected, considering the absorption spectrum of whole blood (minimum between 650 and 700 nm).²⁵

The resulting speckle figure is received on the CCD of a webcam (Motic China group, Hong Kong, China), without any ob-

jective, which has been slightly shifted compare to the light path in order to avoid dazzling the detector. We checked the speckle statistics (i.e., the adequacy between the speckle's size and the size of a pixel on the CCD chip). Indeed, in order to maximize the signal-to-noise ratio, we need to image as many speckles as possible. On the other hand, if speckles are too small (smaller than the typical size of a pixel), then the optical contrast is diminished; therefore, it is important to adjust the speckle size. The size s of the speckles formed on the CCD chip is given by the relation $s \approx \lambda d/a$,²³ where λ is the wave length of the light (690 nm), d is the distance between our sample and the CCD chip (~ 15 cm), and a can be assimilate to the diameter of the laser beam (3–4 mm). Therefore, we can estimate the typical diameter of the speckles to be $\sim 50 \mu\text{m}$. The CCD chip used to perform our experiments is composed of 640×480 pixels, each pixel occupying $13 \mu\text{m}^2$, approximately ($3.6 \times 3.6 \mu\text{m}$). We can conclude that each speckle is defined by 150 pixels or so, and that the total image shows >2000 speckles. Thus, the images obtained thanks to our experimental setup are suitable and representative of the sample we want to study.

The movies are triggered on the laptop connected to the webcam, simultaneously with the mixing of the two reagents, and captured at a frame rate of 20 fps for 4 min and split by Matlab to 1 fps. Each movie is then analyzed by the correlation of two consecutive images, and the blood clot formation is highlighted by the cells' immobilization. All these operations (blood homogenisation with Neoplastin, capillarity introduction of the sample in the microfluidic chamber, and recording) are performed at room temperature. The total experimental time, including the correlation calculation, is 7 min.

2.3 Image Analysis

In order to determine when the cells are immobilized, the differences between two consecutive images must be quantified. The correlation coefficient R , between these two images is calculated according to the following formula:²⁶

$$R = \frac{\sum_{i=1}^N (I_i - \bar{I}) \times (J_i - \bar{J})}{\sqrt{\sum_{i=1}^N (I_i - \bar{I})^2} \times \sqrt{\sum_{i=1}^N (J_i - \bar{J})^2}}, \quad (2)$$

where N is the total number of pixels, I_i and J_i (with $i = [1; N]$) are pixel intensity values of an image and the previous or following image respectively, $\bar{I}$ and $\bar{J}$ are the mean intensity values for the images I and J , respectively.

We calculate the derivative of R , the correlation coefficient as follows:

$$\frac{dR}{dt} = f(t), \quad (3)$$

After smoothing dR/dt in order to eliminate electronic and optical noises, we define the coagulation time (CT) as the time corresponding to the maximum of the derivative.

2.4 Reference Method

The automated analyzer MDA II (Trinity Biotech, Bray, Ireland) of the laboratory of haematology of the Edouard Herriot Hospital has been used as a reference technique; it measures the light intensity transmitted through the sample. The measurements were run according to the recommendations of the manufacturer (Trinity Biotech, Bray, Ireland): the analyser works with

Fig. 2 Addition of Neoplastine to the blood sample at T_0 triggers the coagulation dynamics. After a first phase corresponding to the homogenization of the solution in tube (phase I), the sample is introduced in the chamber by capillary forces, leading to blood cells motion (phase II). Upon coagulation the cells motion suddenly slows down (phase III), before their complete immobilization in the clot (phase IV).

$50 \mu\text{L}$ of anticoagulated (i.e., calcium-free) plasma, withdrawn from 5 mL of centrifuged whole blood (sodium citrate as anticoagulant), mixed with $50 \mu\text{L}$ of Neoplastine. The measurement is carried out at 37°C and lasts 25 min (20 min for the centrifugation of the whole-blood sample and 5 min in the analyzer). The differences between the reference technique (MDA II) and our multiple scattering analysis (MSA) method are summed up in the Table 1.

3 Results

3.1 Results on Coagulation Time

3.1.1 Coagulation dynamics

We present here results obtained for a whole-blood sample. Figure 2 shows the correlation coefficient versus time; different phases of the phenomenon arise from this graph. The beginning of the recording corresponds to the addition of Neoplastine to the whole-blood sample; it defines the T_0 of the coagulation dynamics.

During the first 7 s or so, the chamber is empty while the reagent is being mixed to the blood sample in a tube; the corresponding speckle figure produced by the glass chamber is fixed, leading to a constant correlation coefficient close to 1 ($R = 0.93$); this is called phase I.

Phase II corresponds to the introduction of the sample in the chamber by capillarity and the resulting convection. Once set in motion, the blood cells induce changes to the corresponding speckle image, leading to the sudden drop of correlation coefficient ~ 7 s and the persistence of the low value of R , close to 0.

During phase III, the blood clot forms. As coagulation occurs, the viscosity of the sample rises, slowing down the blood cells motion and consequently the swarminglike behavior of the speckle. This corresponds to the sharp increase of the correlation coefficient of ~ 30 s. Finally, the blood cells get confined in

Table 1 Mean characteristics of the automated MDA analyzer and our MSA technique.

	Reference method	MSA
Principle of measurement	Transmittance	Speckle correlation
Sample volume	50 μL of plasma	10 μL of whole blood
Temperature	37°C	Room temperature
Replicate	1	3
Range	10–60s	25–150s
Duration of measurement	20 min centrifugation + 5 min analyser = 25 min	7 min
Repeatability	CV <5%	CV <10%

the solid clot and completely stop their motion; the consequent immobilisation of the speckle is shown by the establishment of the plateau ($R = 0.85$); this is phase IV.

We can note that during phase I, although the chamber is empty, $R \neq 1$ ($R_I = 0.93$), which is explained by electrical and optical noises in the system. However, in phase IV, the final

value R_{IV} tends to be close to R_I , hence showing that the blood clot traps the cells and immobilizes them completely.

The temporal derivative dR/dt is obtained and represented as a function of time [Figs. 3(a2) and 3(b2)]; dR/dt is smoothed in order to rub out the optical noise and variations due to sample introduction in the chamber. In fact, as illustrated in Fig. 3(b1),

Fig. 3 Correlation coefficient (1) and its derivative (2) versus time for two patients (a) and (b); the thick black line represents the smoothed data. Measured coagulation times are designed by CT and are reported on the variation of correlation coefficient for two different patients: a normal patient (a), CT = 37 s and a pathological patient (b), CT = 91 s.

Fig. 4 In the absence of calcium chloride, coagulation cannot take place. Variation of the correlation coefficient versus time, with (open circles) and without calcium chloride (crosses) for the same blood sample. The inset shows a close-up on the first 200 s.

phase II can be perturbed by the way the sample is introduced in the chamber (introduction of bubbles, local defect on the surface, etc.) and by modification of blood physical properties (surface tension or viscosity due to the first steps of the coagulation cascade). These variations have an impact on the evolution of the derivative of the correlation coefficient as a function of time as shown by Fig. 3(b2).

Without smoothing the data, the CT would be measured at ~ 24 s, which would be inexact. The CT is defined as the time at which the smoothed dR/dt reaches a maximum value; here, it is equal to 37 and 91 s for patients (a) and (b), respectively, which is what is expected knowing the PT values from the laboratory analysis. The technique is thus robust and does not depend on the completion phase.

The technique repeatability has been evaluated by the realisation of 20 replicates of the coagulation time measurement on the same sample coming from a healthy donor. The results show a standard deviation of 1.54 s on a CT of 33 s, leading to a coefficient of variation of 4.5%. Our technique therefore exhibits a good repeatability.

3.1.2 Coagulation versus control experiment

In order to highlight the typical behavior of the sample during the coagulation phenomenon, we performed the same measurements with a “control” solution, which cannot coagulate. Indeed, without the addition of calcium chloride (replaced by water), the anticoagulant used to stabilize the blood samples blocks the coagulation cascade in spite of the addition of thromboplastin. Figure 4 shows the results obtained with and without calcium chloride. The experiment is performed on a control sample, which maintains the same dilution, viscosity, and optical characteristics as regular samples. The video acquisition has been extended to 15 min.

We can see that in the absence of coagulation, the correlation coefficient stays constant and is equal to zero for >4 min; whereas for the same sample mixed with thromboplastin and sodium chloride, R suddenly increases to 0.8 at ~ 37 s. Nevertheless, in the absence of coagulation after 350 s, R starts to increase slowly and finally reaches similar values as in the case of coagulation ($R_{IV} \sim 0.9$). In absence of clot formation, the

Fig. 5 Variation of correlation coefficient R versus time for a plasma (crosses) and a whole-blood (solid circles) samples coming from the same donor.

blood cells are free to sediment and to immobilize at the bottom of the chamber, leading to the increase in correlation coefficient.

Even for highly pathologic cases (corresponding to INR of >5), coagulation occurs within 150 s after Neoplastine addition to the blood sample. Consequently, we can conclude that in our experimental conditions, sedimentation of the blood cells will not affect our determination of coagulation time.

3.1.3 Plasma and whole-blood samples

Both plasma and whole-blood samples coming from the same donor have been tested. The plasma sample obtained by a weak centrifugation of the whole blood (15min at 150 G at room temperature) has been prepared in order to preserve most of the platelets (smallest blood cells) in the sample. The results are presented in Fig. 5; the coagulation times measured on the plasma and the whole-blood samples have been founded to be 29 and 31 s, respectively.

However, we note that the variation of correlation coefficient during the coagulation phenomenon is larger for whole blood than for plasma. This can be explained by the difference in the amount of cells contained in both samples (i.e., by collecting only the plasma, $>90\%$ of the blood cells have been discarded).

Hence, the determination of coagulation time on the whole-blood sample is preferred because it is simpler and faster in terms of sample preparation (no need to centrifuge) and it gives a better detection due to the large dynamical variation of correlation coefficient.

3.2 Validation of Coagulation Time Determined on a Pathological Range and Comparison to a Reference Method

We performed a study in collaboration with the Hospices Civils de Lyon at the Laboratory of Haematology of the Edouard Herriot Hospital, Lyon, France, in order to compare our technique

Fig. 6 Comparison of the CT measured with our technique versus the PT obtained with the MDA II. Error bars represent twice the standard deviation of the triplicates.

to the routine laboratory automation technique. Blood was analyzed with both techniques: our MSA and the transmittance measurements of the MDA II. Two parameters were obtained from the analyzer: the PT and the INR. In this section, we present the results of the study performed on 65 patients.

3.2.1 Prothrombin time measurements

Each sample was run in triplicate, and the mean CT was calculated, as well as the standard deviation (st) and the coefficient of variation (cv). Figure 6 presents the CT measured by our MSA as a function of the PT obtained by the reference technique. We show that the CT increases proportionally to the PT; the linear regression is in pretty good agreement with the experimental data ($R^2 = 0.94$).

However, the slope is not equal to 1. This difference can be explained by the fact that the MDA II is working at 37°C, whereas our measurements are performed at room temperature. Taking into account the enzymatic reactions in the coagulation cascade, and the fact that enzymes work better at 37°C, we can conclude that PT is lower than CT. Our setup allows the determination of the prothrombin time of a whole-blood sample. The precision of the measurement is good on the whole range explored.

The correct determination of the coagulation dynamics covers the normal as well as a large part of the pathological range (PT between 18 and 40 s). For very high PT, however, we note that the results are slightly underestimated. We remind the reader that these values correspond to highly pathological cases; regarding the low number of samples concerned, it is difficult to conclude on a possible weakness of the technique.

The cv (calculated on the triplicates) has been estimated on the whole range explored, including these last highly pathologic samples; it appears that most of the measurements have a cv of <10%. Although only one measurement per sample has been performed on the routine laboratory automation, a coefficient of variation of the MDA II measurements is estimated as 5% according to the manufacturer.

3.2.2 Effect of blood hematocrit

We have also investigated the sensitivity of our technique regarding to the hematocrit of the blood samples. Hematocrit is

Fig. 7 Effect of the hematocrit on the CT measured with our technique versus the PT obtained with the MDA II. The solid circles, open squares, solid diamonds, and open triangles represent donors with an hematocrit respectively, <31%, between 31 and 36%, between 36 and 40%, and >40%.

the volume fraction of red blood cells in whole blood. Figure 7 presents CT measurements versus PT for different ranges of hematocrit: the solid circles, open squares, solid diamonds, and open triangles represent hematocrits of <31%, between 31 and 36%, between 36 and 40%, and >40%, respectively. The CT measured with our MSA method does not seem to be impacted by the hematocrit of the patient. In fact the CT values are fairly dispersed regardless of the value of the patient's hematocrit.

3.2.3 International normalized ratio calculations

Finally, we have estimate the ratios $INR_{CT} = (CT_d/CT_{ref})^{ISI}$ calculated from the CT measurements according to Eq. (1). The CT_{ref} was chosen to be the coagulation time of a known healthy donor measured with our setup; here, it is equal to 34 s. The Neoplastin used for our experiments came from the same batch that the one used by the MDA II; for this particular thromboplastin reagent the ISI value was given by *Stago Diagnostic* to be 1.3.

The ratios were compared to the INR given by the analyzer; the results are presented in Fig. 8.

We can see from Fig. 8 that INR_{CT} measured by our technique is in good agreement with the INR obtained by the laboratory

Fig. 8 INR_{CT} measured with our method versus the INR obtained with the MDA II.

Fig. 9 Mean light intensity versus time for a healthy donor. We report on the graph the four phases that were detailed earlier.

apparatus, even though there is a difference in temperature between our setup and the analyzer protocol. The linear correlation has a good regression coefficient $R^2 = 0.93$, and the slope is close to 1, which is an excellent result. The regulation of the temperature does not appear to be necessary to obtain INR measurements. Finally, our method allows the determination of the INR with a good sensitivity.

4 Discussion

4.1 Light Intensity Measurements

It is interesting to note that we can couple our MSA technique with light intensity or contrast measurements as shown by Piederrière et al.¹² However, our experimental setup is different from the one of Piederrière et al.¹² because our camera is shifted compared to the direct light path. Thus, the measurements of diffused light intensity cannot be directly compared to their results on contrast variation.

Figure 9 represents the variations of $\bar{I}$, the mean light intensity measurement versus time for a healthy donor, and the

Fig. 10 Coefficient of correlation (1) and mean light intensity (2) versus time for two different pathologic samples (a) and (b). The coagulation times estimated with the speckle dynamical analysis technique are reported on the figure tanks to the dashed lines.

Fig. 11 Mean light intensity as a function of time, with (crosses) and without (open circles) calcium chloride, for the same blood sample.

four phases defined earlier. Initially when the chamber is empty (phase I), the mean light intensity is constant and low due to the weak light diffusion by glass, ~ 7 on a gray-scale value ranging between 0 and 255 (0 being black and 255 being white). Then when the sample is introduced in the fluidic chamber by capillary migration, $\bar{I}$ suddenly increases to 170 before decreasing slowly (phase II). It is known²⁷ that upon blood coagulation, the optical density of the blood increases leading to a decrease of the light transmitted through the sample. During blood clot formation (phase III), $\bar{I}$ reaches a plateau ~ 47 s, highlighting the stabilization of the sample optical properties. Once coagulation has occurred (phase IV), the mean light intensity stays constant.

We can see in Fig. 10 that coagulation is associated with the establishment of a plateau for the mean light intensity. Once the blood clot is formed, the variation of optical density of the sample stops leading to the establishment of a constant value for $\bar{I}$. We can note that there is good agreement between the establishment of the plateau and the coagulation time determined by the correlation method as illustrated in Figure 10 for two patients with different CT.

In order to highlight the behavior of the sample during the coagulation phenomenon, the same measurement is realised with a control solution that cannot coagulate, as detailed previously. In Fig. 11, we represent the variations of the mean light intensities associated with the measurements of the correlation coefficients presented in Fig. 4. In the absence of coagulation (i.e., in absence of calcium chloride), $\bar{I}$ reaches a maximum and drops slowly, ~ 67 s. Then due to cell sedimentation, it increases back around 283 s before reaching a constant value of 116 at ~ 400 s, which is consistent with the beginning of the stabilization of the correlation coefficient associated (see Fig. 4). On the contrary, in presence of calcium chloride, the coagulation leads to the stabilization of the mean light intensity at 30 s.

The increase in optical density of blood during coagulation is indisputable for plasma samples as reported in the literature, but is a lot less detectable for whole-blood samples. Thus, during

the coagulation process of a whole-blood sample, the variation of the mean light intensity is $<16\%$ while the variation of the correlation coefficient is $\sim 80\%$.

5 Conclusion

We have reported a simple, novel method of determining the coagulation time of whole-blood samples based on the analysis of the sudden stop of the resulting speckle swarminglike behavior. This technique performs on whole-blood samples, which simplifies the sample preparation by avoiding the common plasma separation. We obtained good agreement between our measurements and the prothrombin time measured by the medical laboratory, as well as a very good direct determination of the INR. Both parameters are obtained with a fair repeatability ($cv < 10\%$). This technique can be applied to the determination of any coagulation times [activated cephalin clotting time or activated kaolin clotting time, etc]. The use of the convenient “reflection setup” allows the coupling of this simple setting with an agitation unit, for the purpose of integrating the mixing of the dried reagents with the blood sample *in situ* in the fluidic chamber.

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