# BLOOD-TO-BLOOD IMMUNOLOGICAL COMPATIBILITY TEST

## *A possibility with fluorescent immuno-biochips*

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Abstract: One of the most feared transfusion accident is the haemolytic reaction. A majority of countries impose a compatibility test before each transfusion, at the patient's bedside or in laboratory. Regardless of the test performed, it does not prevent human errors and nothing ensures an "error free" procedure. Complete crossmatch is the only test ensuring a complete blood compatibility between donors and patients. It relies on the direct or indirect detection of agglutinations which occur when the patient's plasma is mixed with the red cells to be transfused. It requires extracting plasma. The work described here will help avoid all the immunologic incompatibilities by the use of a compatibility test without plasma extraction. It relies on an immuno- biochip technology in a microfluidic environment with fluorescence detection. This position paper presents preliminary results obtained with artificial samples together with comments on the state of industrial competition and the new device market positioning.

# 1 INTRODUCTION

In France every year, more than 3 million labile blood products, 80% of which were red blood cells (RBC), are given to more than 550 000 patients (ANSM 2016). French health institutions have seen a rise in deliveries of RBC of + 26.3% in total between 2000 and 2014 (EFS, 2014). In 2016, according to the haemovigilance report by ANSM (National Agency for Drug Safety), 6780 adverse effects in recipients (AER) related to transfusion were reported. In total, 255 adverse effects in receivers related to transfusion were recorded due to immunological incompatibility. The situation seems to be similar in countries with the same level of safety as France.

In most countries, a crossmatch (a compatibility test between blood for transfusion and the receiver's blood) is carried out in a laboratory prior to transfusion. The current techniques for carrying out a crossmatch are either manual, with blood reagents and samples being mixed in tubes or being placed on gel columns before centrifuging, or automated. As far as these automated systems are concerned, the analyzers may only be used in a laboratory and the analyses are time consuming which can delays the delivery of the blood product. This test is even avoided in some emergency situations.

Currently, the final pre-transfusion test at the patient's bedside consists mainly of an identity check (identity of the red cell concentrate (RCC) and identity of the patient). This method cannot guarantee that there will be no transfusion accident because 50% of the reported adverse effects are due to human error, most of them being "wrong blood in tube" (SHOT 2011). Therefore, in spite of increasingly effective safety systems, it is currently impossible to eliminate entirely the risks due to human error, both in the laboratory and at the time of the transfusion.

These errors highlight the need for a pre-transfusion analyzer which does not require pre-treatment (plasma extraction, and/or centrifugation) in order to limit human manipulation and subsequent errors.

Some research was carried out in order to make this process ultimately safe. They are mainly based on gel agglutination techniques which requires plasma extraction (Cid et al. 2006; Longston et al. 1999) or SPR (Malomgre et al. 2009; Quinn et al. 2000; Houngkamhang et al. 2013). More recently, Long-Range Surface Plasmon-Polaritons have been suggested to detect selectively captured red blood cells using specific surface chemistry (Krupin et al. 2014). This system can capture red blood cells in a sample with a very low cell concentration. The initial dilution of the sample is carried out in a buffer with a controlled refractive index. Blood samples must then be prepared which is not an improvement compared to current crossmatch. Test plates and image processing have also been proposed for phenotyping blood groups (Ferraz et al. 2010). Micro fluidics coupled with optical fibers are reported in (Ramasubramanian et al. 2009). A while ago, we proposed the use of functionalized biochips for ABO and rhesus compatibility tests (Charrière et al. 2015, Wacogne et al. 2017).

Except the gel agglutination method which requires samples preparation, the other techniques could allow for blood grouping but not a direct compatibility check between the RCC to be transfused and the patient. The idea developed in this position paper arose from the fact that incompatible antibodies present in the patient's blood bind to red cells to be transfused and may lead, more or less rapidly, to the lyses of RCC.

Therefore, our proposal is to use a single biochip onto which red cells to be transfused are trapped. Subsequently, patient's whole blood is applied onto the biochip and possibly present irregular antibodies react with the RCC. Finally, fluorescent anti-IgGs antibodies are used in order to allow rapid optical detection. Using such an architecture allows detecting any erythrocytic immunological incompatibilities without extracting patient's plasma. In this way, this very rapid test can be performed by nonspecifically trained people, with a reduced and non prepared receiver's blood sample.

This principle will be described in part 2 of this communication together with the actual biological model used to demonstrate a first proof of concept. A description of the experimental set-up and results obtained using fluorescence spectroscopy will be the subject of part 3. In part 4 and in line with the scope of a position paper, we will comment on the state of industrial competition and the new devices market positioning.

# 2 GENERAL PRINCIPLE AND ACTUAL IMMUNOLOGICAL MODEL

## 2.1 Biochip principle

The method we propose is depicted in figure 1 in the case of non-compatible transfusion. Red cells from the RCC to be transfused are applied to the biochip previously grafted with antibodies directed to a high-incidence antigen (figure 1(a)). After rinsing, a RCC layer is formed at the biochip surface.

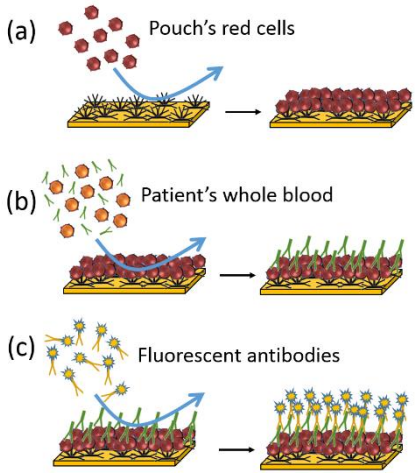


Figure 1: Principle of the blood-to-blood immunological compatibility test biochip.

Patient's whole blood is directly injected onto this surface (figure 1(b)). Antigen/antibody reactions occur when RCC exhibit an antigen which is complementary of an antibody present in the patient's blood (irregular IgGs in this case). After rinsing, elements which have not reacted with the red cells layer are evacuated.

Then, a solution of fluorescent anti-IgGs antibodies is applied to the biochip (figure 1(c)). These antibodies react with the patient's irregular antibodies forming a fluorescent layer at the biochip's surface. The latter is eventually detected using conventional fluorescence techniques.

In this example, irregular IgGs have been considered. However, incompatibility may be due to presence of IgM in the patient's whole blood (case of the ABO incompatibility). In this case, and this is not shown in this proof of concept communication, fluorescent anti IgMs antibodies should be employed. In order to address any incompatibility situation, the ideal sensor should be adapted to the presence of both IgGs and IgMs. A way of doing would be to use a mixture of fluorescent IgGs and IgMs antibodies. The

fluorescent labels could be chosen in such a way that both exhibit the same excitation wavelength but different emission spectra. Here, a simple fluorescence spectrum fitting would furthermore indicate which kind of immuno-incompatibility arose.

## 2.2 Immunological model

In this model, RCC are captured on the biochip by means of anti Kell IgMs (figure 2). The KEL:2 antigen (cellano) is present at the red cell surface of about 99.6% of the population. Therefore, for this proof of concept, the anti Kell IgMs are considered universal. In the future, we will have to find a red cell capture method which also works for the remaining 0.4% of the population.

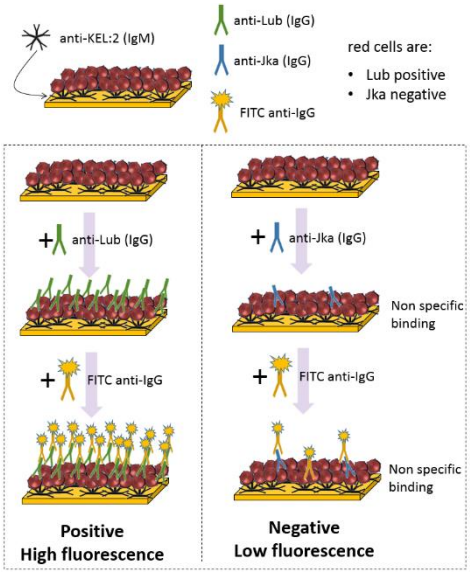


Figure 2: Immunological model used in this proof of concept.

In this example, red cells are Lub positive and Jka negative. For the positive test, a solution of irregular anti-Lub IgG antibodies is applied to the RCC immobilized on the biochip. In the same manner, a solution of anti-Jka antibodies is used for the negative test. When antigen-antibody reactions occur, antibodies bind to the red cells surface.

After rinsing, FITC coupled anti-IgGs antibodies are applied to the biochips. They react with the irregular antibodies possibly present at the RCC surface. In a positive test, a large amount of FITC coupled antibodies is present and a strong fluorescence signal is detected. On the contrary in a negative test, only non-specific interactions occur leading to a weak fluorescence signal.

For these experiments, commercially available irregular antibodies were used (Bio-Rad). Further experiments will be conducted with whole blood.

# 3 EXPERIMENTAL SET-UP AND PRELIMINARY RESULTS

## 3.1 Biochemical reactions

Immunological reactions described in figure 2 were performed using the fluidic system of our SPRi apparatus (SPRi-Plex imager, Horiba Scientific) at 37°C with a MLB2 (Bio-Rad) running buffer. Biochips were prepared according to the process described in (Charriere et al. 2015) without RSA saturation. Anti-Kell IgMs (dilution 1/10 in acetate buffer, 10 mM and pH 4.5) were grafted onto biochips.

200 µL of pouch's red cells were injected onto the biochips at 20 µL/min. After rinsing, 200 µL, irregular antibodies (anti-Lub and anti-Jka, 1/10 in MLB2, BioRad) were injected at 20 µL/min. FITC anti-IgGs (Sigma) were injected (200 µL, 20µL/min, 1/25 in MLB2).

Complex red cells / antibodies were finally fixed using a 0.5% glutaraldehyde solution. Biochips were wetted with MLB2 buffer and protected with cover slits before fluorescence measurements.

## 3.2 Fluorescence measurement set-up

FITC was excited at 488 nm using an Oxxius 488-50-COL-PP laser. Excitation light was injected into a fluorescence beam-splitter as shown in figure 3.

This beam-splitter (Doric Lenses) is equipped with excitation and emission filters together with a dichroic mirror (respectively FF01-488/10-25, BLP01-488R-25 and FF500-Di01-25×36). Fluorescence spectra were recorded using a QE-Pro spectrometer (Ocean Optics).

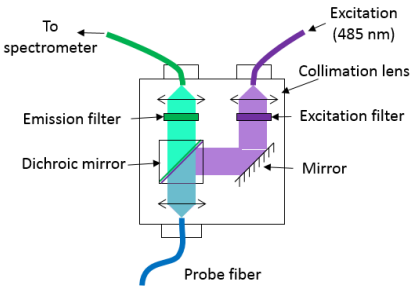


Figure 3: Fluorescence beam-splitter used in these experiments.

## 3.3 Additional fluorescence

This kind of beam-splitter is usually designed to be used with optical fibres. Because we use it in an open beam configuration, additional fluorescence signals are observed due to multiple reflexions in the beam-splitter (figure 4). This additional fluorescence is mainly generated by the epoxy resin used to glue optical elements in the beam-splitter.

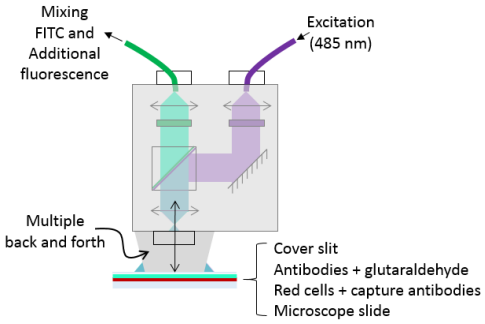


Figure 4: Origin of the additional fluorescence.

The shape of the additional fluorescence was recorded and used as explained in the next section.

## 3.4 Experimental results

Overall, 18 measurements were performed with 2 demonstration biochips (7 random places on the positive biochip and 11 on the negative). Fluorescence spectra were recorded as above mentioned. In order to isolate the contribution of the FITC signal from the whole recorded spectrum a simple fitting was employed. Indeed, we recorded the shape of the additional fluorescence and we know the shape of the FITC emission spectrum. Fitting the experimental spectra with these 2 shapes allows separating the contribution of the FITC from the contribution of the additional fluorescence. Figure 5(a) shows an example of spectrum recorded with the positive biochip.

In this figure, the green shape corresponds to the FITC while the grey shape correspond to the additional fluorescence. It can be seen that the fitting efficiently reproduces the recorded spectra. The feature reported on the right of the spectra represents the standard deviation of the difference between the experimental spectrum and the fitted one. It is an estimation of the fitting accuracy. It is clearly observed that the signal due to the FITC (about 10000 levels) is much greater than the signal of the additional fluorescence.

In figure 5(b), we present a spectrum obtained with the negative biochip. Here the shape of the additional fluorescence is clearly visible.

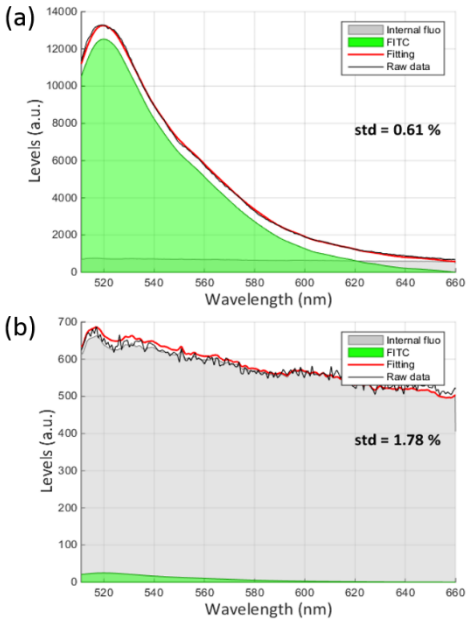


Figure 5: Fluorescence spectra of: (a) positive biochip, (b) negative biochip.

As previously mentioned, 18 spectra were recorded. Table 1 summarizes the levels of FITC and additional fluorescence obtained at each places of the biochips.

Table 1: Summary of the fluorescence levels obtained with the 18 measurements on 2 biochips (1 positive, 1 negative).

| Type | Spectra N° | FITC | Add. | std |
|---|---|---|---|---|
| Positive | QEP003631_11 | 12525 | 757 | 0.61 |
| | QEP003631_12 | 7470 | 766 | 0.39 |
| | QEP003631_13 | 8419 | 702 | 0.64 |
| | QEP003631_14 | 11261 | 797 | 0.37 |
| | QEP003631_15 | 12355 | 732 | 0.58 |
| | QEP003631_16 | 13935 | 702 | 0.7 |
| | QEP003631_17 | 1784 | 575 | 2.27 |
| Negative | QEP003631_02 | 0 | 666 | 1.79 |
| | QEP003631_03 | 0 | 697 | 1.72 |
| | QEP003631_04 | 198 | 697 | 1.31 |
| | QEP003631_05 | 198 | 697 | 1.31 |
| | QEP003631_06 | 270 | 657 | 1.78 |
| | QEP003631_20 | 577 | 672 | 1.15 |
| | QEP003631_21 | 537 | 668 | 1.51 |
| | QEP003631_22 | 8 | 664 | 1.65 |
| | QEP003631_23 | 24 | 663 | 1.78 |
| | QEP003631_24 | 6 | 732 | 1.73 |
| | QEP003631_25 | 74 | 750 | 2.06 |

From these data, it can be seen that the level of additional fluorescence is relatively constant (700±50 levels). Also, FITC levels of the positive biochip is always much larger (except for the QEP003631_17

spectrum). It is however possible to define a threshold above which a biochip is considered positive. This is shown in figure 7 where a threshold at 1300 levels is reported.

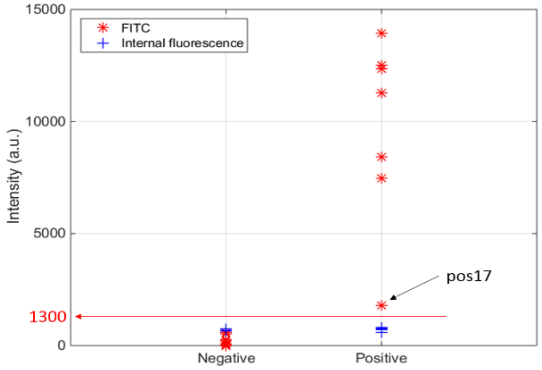


Figure 7: Definition of a positive threshold.

This figure shows that the experimental set-up can be drastically simplified as a simple photodiode can be used to detect positive biochips. This would make the final device cost-effective and would ensure its use in a large number of situations.

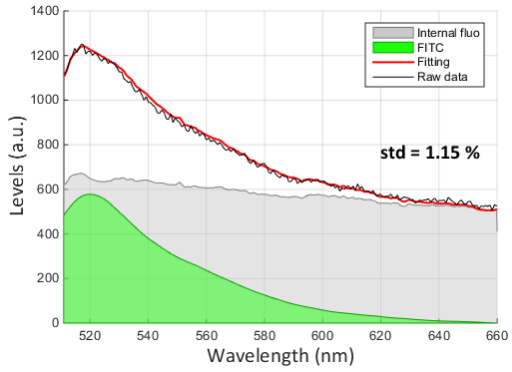


Figure 8: Extracting a weak FITC response using spectral fitting.

However, the use of fluorescence spectroscopy may prove to be extremely useful in the case of weak antigen-antibody affinity or weak concentration of irregular antibodies. In some cases indeed, even a very low antibody concentration can lead to severe consequences for the patient (for example the anti-Jka). The interest of fluorescence spectroscopy is illustrated in figure 8 which reproduces results obtained with the QEP003631_20 spectrum. This demonstrates that a simple fitting can extract a weak level of FITC signal from a spectrum possibly recognized as negative. Importantly, it must be noted that figure 8 does not represent a weak affinity/avidity situation but a case of non-specific interaction at the place where the spectrum was recorded.

To conclude this experimental section, this work represents the preliminary results demonstrating that the immuno-biochip technology can be used to perform a complete blood compatibility test without plasma extraction. Further experiments are still required with a large range of irregular antibodies before considering a clinical trial on a larger scale.

# 4 MARKET POSITIONING

In countries with a safe transfusion system (80% of wealthy countries and 60% of averagely wealthy countries), the compatibility check is carried out either in the laboratory or at the patient's bedside, or both in some cases.

In the laboratory, ABO grouping of RCC and the patient are carried out by analysers (Bio-Rad, Diagast, HTZ, Dia Pro, Grifols). The current techniques for carrying out a crossmatch or an irregular antibody screening are either manual, with blood reagents and samples being mixed in tubes or being placed on gel columns before centrifuging (e.g. Across Gel® Cross Match, from Dia Pro or ID-Card 50531 from Bio-Rad), or automated (e.g. the Qwalys analysers from Diagast). The analysers require additional time-consuming manual operations, (blood centrifugation for example) which may increase the risk of errors. Furthermore, they are oversized for technical platforms or small size laboratories.

To mitigate these risks, some countries, including France, have formalised a final ABO compatibility check at the patient's bedside (ABO compatibility charts from Bio-Rad, Diagast). This check requires qualified and regularly trained staff, is limited to ABO compatibility and cannot prevent certain human errors in terms of allocation, realization or interpretation.

During the work presented here, a market research carried out by a specialist firm and companies with a potential interest, found that the biochip technology, is of major interest. There is nothing similar in this enormous market. This market research also showed that the final control of just ABO compatibility on a biochip would not currently be sufficient to penetrate the world market because of the practices in place in most countries. The companies we approached strongly advised increasing the added value of this biochip by broadening its application to carry out crossmatch, what we did. Given this international perspective, the two initial patents we published (Pazart et al. 2001-1, 2011-2) were also broadened and recorded in Europe, North America and in “BRIC” countries.

# 5 CONCLUSION

We have presented the proof of concept of a biochip potentially able to perform a blood-to-blood immunological compatibility test in a simple fluidic environment. It relies on a single biochip onto which red cells to be transfused are trapped. Subsequently, patient's whole blood is applied onto the biochip and possibly present incompatible antibodies react with the RCC. Eventually, fluorescent anti-IgGs antibodies are used in order to allow rapid optical detection.

Fluorescence spectroscopy experiments showed that irregular antibodies can easily be detected. Here, we used solutions of irregular antibodies. The next steps will consist in multiplying the types of irregular antibodies and to perform experiments with whole blood obtained from donors. Potentially, a simple photodiode based detection can be used. This would allow using a compact device which can be used either by trained or non trained medical staff. However, the use of fluorescence spectroscopy in a more complex set-up can probably lead to the detection of weak but potentially dangerous incompatibilities.

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