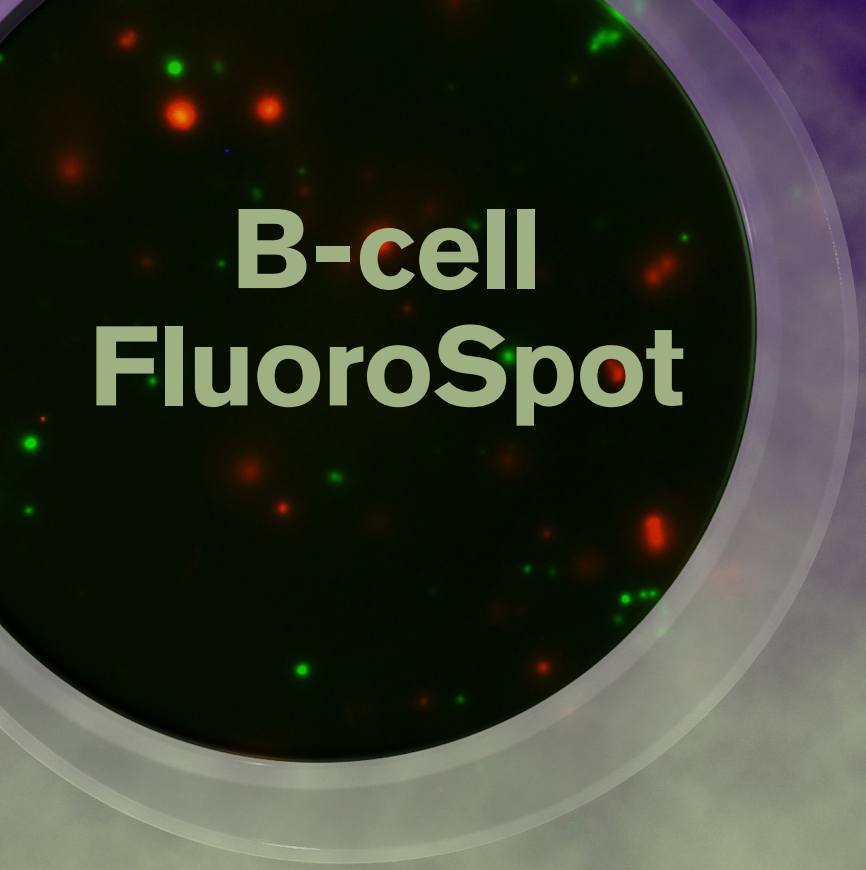

B-cell FluoroSpot

B-cell ELISpot

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B-cell ELISpot

While there are a multitude of assays designed to measure antibody reactivity and specificity (e.g. ELISA, immunoblot, flow cytometry) only a few focus directly on the antibody-secreting cell (ASC). One of these is the B-cell ELISpot, first described in 1983 [1,2]. With this method one can, in a highly sensitive manner and at the cellular level, identify and enumerate both the total number of ASC in a sample and those secreting antibodies to a specific antigen. This way it can provide information not easily obtainable using other methods and has, for instance, been used to demonstrate the presence and frequencies of long-term memory B cells in the blood [3].

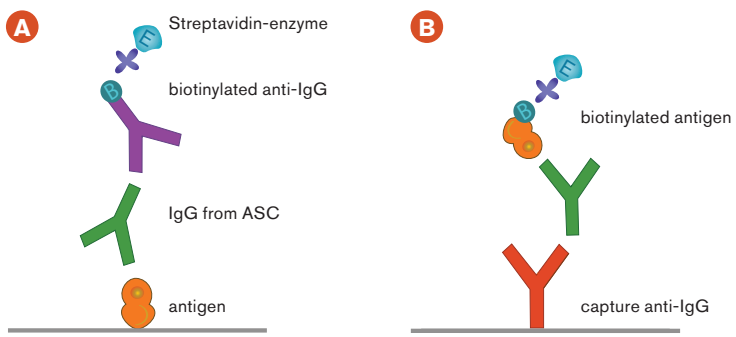
To meet the need for optimized and validated reagents we have developed ELISpot kits to be used for analyzing B cells secreting antibodies of different isotypes in human, monkey and mouse samples. We have also established an improved protocol in which the antigen is used for detection instead of immobilized to the ELISpot plate as in the conventional assay.

Two ways to perform the B-cell ELISpot

Since first described, the B-cell ELISpot has been performed in essentially the same way with only minor modifications. As outlined in Figure 1A, the antigen is first immobilized to the membrane-based bottom of an ELISpot plate. This is followed by the addition of a cell preparation containing the B cells to be tested. After suitable incubation time, allowing sufficient secretion and binding of antibodies to the coated antigen, bound antibodies are detected by the sequential addition of secondary reagents. These usually comprise a biotinylated anti-Ig (Immunoglobulin) antibody followed by enzyme-conjugated Streptavidin. Finally, the assay is developed by the addition of a precipitating substrate and the spots can be viewed and counted in a microscope or, preferably, in an ELISpot reader.

While this procedure is simple and straightforward, it does have certain weaknesses some of which are related to the antigen. To obtain distinct and well focused spots, the antigen needs to be coated at a relatively high concentration and in a way that leaves the relevant epitope/s accessible for binding. Furthermore, the antigen needs to withstand incubation at 37°C in the presence of cells and serum-containing medium without being degraded. In practice, supply of antigen is often limited and the fate of the antigen, when bound to the membrane and exposed to the culturing conditions, is difficult to control. This may result in poor spot quality often accompanied by a loss of sensitivity.

Figure 1. Schematic illustration of the two ways of making antigen-specific B-cell ELISpot using antigen for coating (A) or biotinylated antigen for detection (B). In the example, analysis is restricted to IgG-producing cells.



Antigen-specific IgG ELISpot

To avoid some of these problems, we have developed a novel B-cell ELISpot format based on using the antigen for detection. As shown in Figure 1B, the assay starts by coating the ELISpot plate with capture anti-Ig antibodies. These antibodies can be chosen to bind a specific isotype (e.g. IgG or IgA). During the cultivation with cells, antibodies from the ASC are bound by the capture antibody independent of their antigen specificity. To detect only those cells that secrete antibodies with the appropriate antigen specificity, biotinylated antigen is added followed by incubation with Streptavidin-enzyme and finally the addition of substrate. In our experience with a number of antigen systems, this alternative way of performing the B-cell ELISpot results in more distinct and easily evaluated spots, lower background staining of the membrane and, in many cases, improved sensitivity (see examples in Fig. 2 and 3). In addition to these advantages, approximately 20 to 200 times less antigen is needed and the antigen is not subject to the potentially degrading conditions during cell cultivation [4,5].

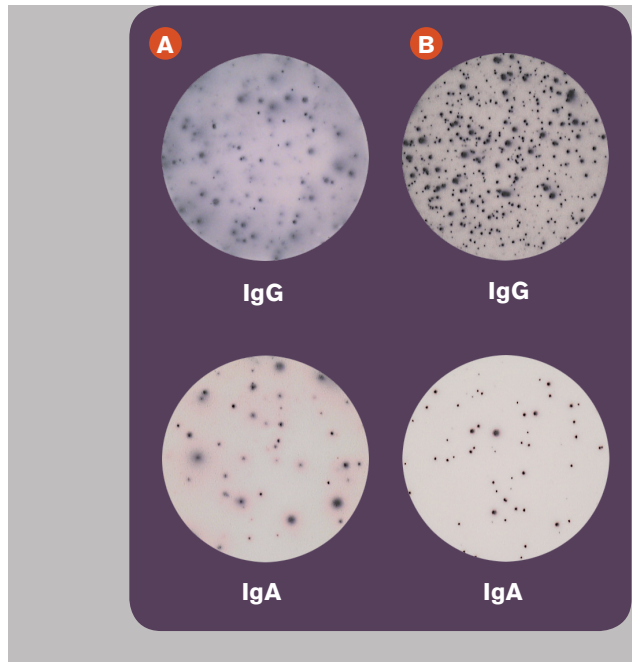


Figure 2. B-cell ELISpot analysis of anti-ovalbumin IgG and IgA responses in immunized mice. Splenocytes (250 000 cells/well) from Balb/c mice immunized three times with ovalbumin were incubated overnight in ELISpot plates coated with ovalbumin (A) or antibodies to mouse IgG or IgA (B). Detection was made by the addition of biotinylated antibodies to mouse IgG or IgA (A) or by biotinylated ovalbumin (B).

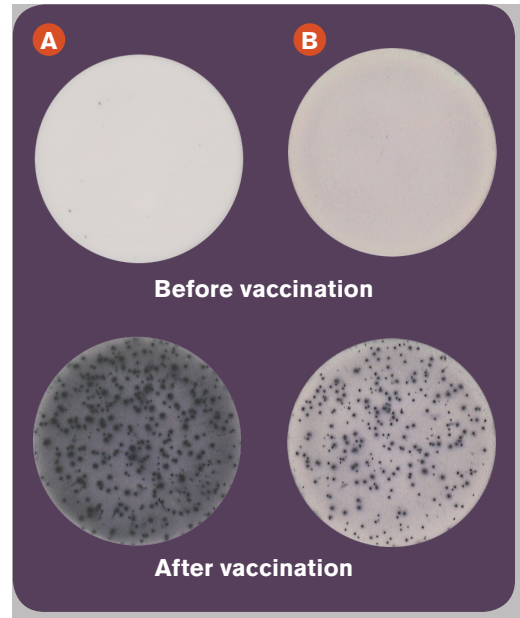


Figure 3. Swine flu-specific IgG ELISpot. Human PBMC were collected before and seven days after vaccination with the intramuscularly administered influenza vaccine Pandemrix®. Ag-specific IgG-producing cells were analyzed using Pandemrix at 1 µg/well for coating (A) or at 0.03µg/well as a biotinylated detection reagent (B).

Sources of B cells

While most B cells can be tested in the ELISpot assay, conditions will differ depending on the source and state of the cells. For instance, when using samples from blood or lymphoid tissue, cells may be in an activated form as a consequence of an acute infection or a recent immunization/vaccination or in the form of memory cells. Whereas activated cells may already be in a state where they secrete antibodies (lymphoblastoid or plasma cells), memory B cells may require several days of stimulation with polyclonal activators

before they start to secrete antibodies. With the conditions normally used, naïve B cells will not be picked up in the assay.

If cells have been activated *in vivo*, they can be added to the plates and incubated in cell culture medium without any further stimulation. However, studies have shown that the time window in which one can detect ASC in blood after e.g. vaccination is narrow [6]. Typically cells need to be collected 5 to 10 days after the administration of antigen. After this, a sharp drop in the number of circulating ASC occurs. Memory B cells, on the other hand, need to be stimulated before they produce detectable amounts of antibody. This is typically done in a pre-incubation step where the cells are cultured in the presence of factors that promote proliferation and maturation of the B cells. There exist many protocols of stimulation often including a combination of different activating signals, like R848 together with IL-2 (included in Mabtech's kits). The period of stimulation varies but is normally 3 to 8 days, where the longer incubation times are used with the purpose of expanding the number of B cells in order to facilitate the detection of low frequency responses. After the pre-incubation, before addition to the ELISpot plates, cells are washed to remove any antibodies present in the culture medium.

Since the *in vitro* expansion of the cells will skew the proportion of B cells in a sample, the number of antigen-specific spots is normally compared to the total number of ASC present. The latter is determined by using biotinylated anti-Ig instead of biotinylated antigen (see Fig. 4 and 5).

Cell samples from humans are usually in the form of peripheral blood mononuclear cells (PBMC) but may also be obtained from lymphoid organs or mucosal sites. As the B cells normally constitute only a minor fraction of the cells (5-12% of PBMC), they may be enriched by positive selection using e.g. anti-CD19 magnetic beads or by depleting non-B cells. Enrichment leads to increased sensitivity simply due to more B cells being analyzed in the individual wells.

Mouse B cells are usually obtained from the spleen or other lymphoid organs but can also come from peripheral blood. In the latter case, it may be difficult to obtain sufficient amounts of cells whereas spleen cells are easily obtained in great numbers and also contain a larger proportion of B cells compared to the blood.

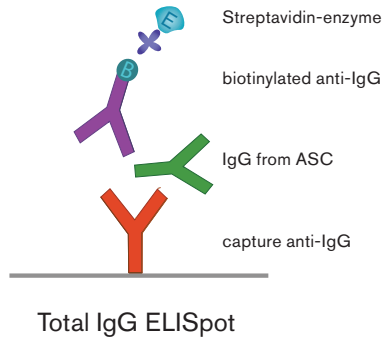


Figure 4. Schematic illustration of total IgG ELISpot.

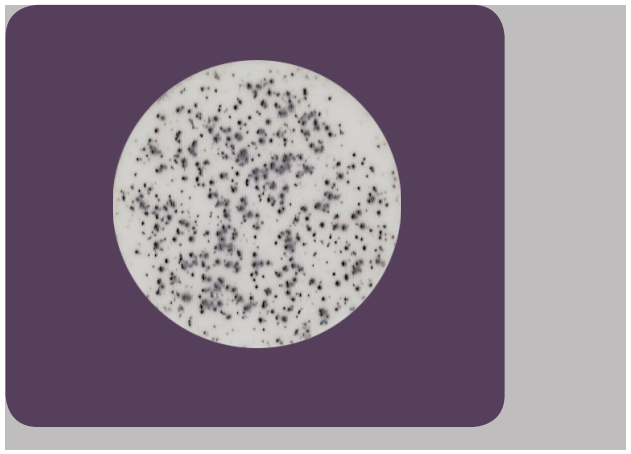


Figure 5. Detection of total number IgG producing cells using in vitro *stimulated ASC. Human PBMC were stimulated with R848 and IL-2 for 3 days. The cells were washed, added to the ELISpot plate (50 000 cells/well) and incubated for 20h without further stimulation.*

Methodological aspects

Requirements for a successful B-cell ELISpot include the use of PVDF membrane plates, pre-activation of the membrane with ethanol and the coating with a relatively high concentration of antibodies or antigen. These measures combine to give well defined spots of high intensity, which will not only facilitate spot counting and evaluation but also increase the sensitivity of the assay.

As in all immunological assays, reagents should be of high quality and validated for optimal performance. For instance, when using anti-IgG antibodies, these should display similar reactivity to all IgG subclasses and isotype-specific reagents should be exclusively reactive with a particular isotype. Apart from the capture antibodies and detection reagents, the medium and serum used for culturing the cells also need to be of high quality. To assure this, multiple serum batches should be pre-tested. Fetal calf serum is typically used for the assay and one should avoid using autologous serum since antibodies present in this serum will interfere with the assay.

As mentioned earlier, the number of cells added to the wells is highly dependent on the expected frequency of positive cells. However, even in cases where antigen-specific cells are expected to be present at very low frequencies, cell numbers over $5x10^5$ cells/well should be avoided as this may result in multiple layers of cells where cells in the upper layer/s tend to produce weaker and less focused spots.

Applications

The B-cell ELISpot represents a powerful tool to analyze various aspects of the antibody immune response. It may be particularly suitable in situations where a high degree of sensitivity is required or when the response is best studied at the cellular level.

Two major application areas have been in the detection of B-cell responses to natural infections and those elicited by vaccination [7-9]. A typical example of such a vaccine-induced response is shown in Figure 6 where the number of antigen-specific IgA-secreting cells to an orally administered cholera vaccine was investigated before and after vaccination.

As previously mentioned, ELISpot can be used for analyzing B cells in peripheral blood as well as B cells present in various lymphoid tissues and other sites harboring immune cells such as mucosal layers and synovial fluid. For instance, it has been used to determine the distribution of antigen-specific B cells in lymphoid tissues after vaccination and to demonstrate the primary confinement of long-lived memory B cells to the spleen [10, 11].

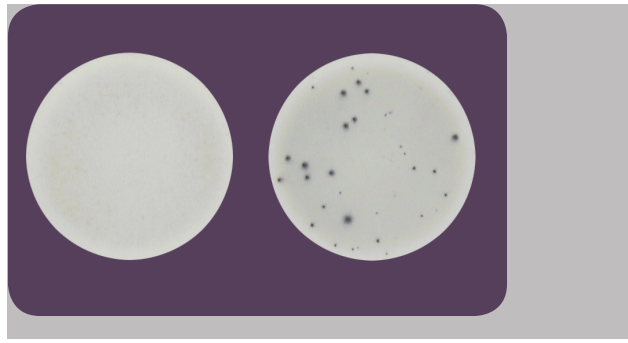


Figure 6. B-cell ELISpot analysis of human IgA response against cholera toxin B. PBMC were collected before (left) and seven days after a second vaccination (right) with the orally administered cholera vaccine, DUKORAL®. Cells (250 000 cells/well) were incubated overnight in ELISpot plates coated with anti-human IgA. ASC specific for the vaccine were detected with biotinylated cholera toxin B.

B-cell FluoroSpot

As an attractive alternative to the ELISpot, antibody-secreting cells may also be analyzed by FluoroSpot using fluorophore-conjugated secondary reagents for detection. This technique is as sensitive as the ELISpot but offers the additional possibility to, in the same well, separately define and enumerate B cells secreting antibodies of different isotypes. This can be done in an antigen-specific manner as depicted in Figure 7 or with the purpose to determine the total number of B cells producing different immunoglobulin isotypes (Fig. 8). Apart from an easy procedure, the fact that more than one type of antibody response can be measured in the same well makes the technique particularly suitable in situations where the source of cells or the amount of antigen is limited.

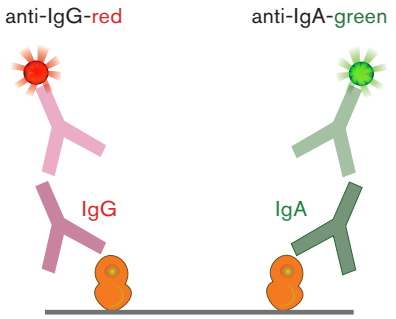


Figure 7. Schematic illustration of antigen specific B-cell FluoroSpot using coated antigen. Antibodies from B cells secreting antigen-specific IgG or IgA are detected using differently labeled, isotype-specific detection antibodies, making spots appear as either red (IgG) or green (IgA).

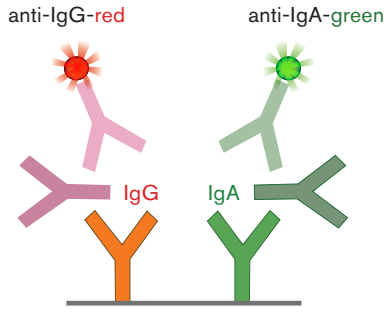


Figure 8. Determination of the total number of B cells secreting IgG or IgA. Wells coated with two different capture mAbs specific for IgG and IgA were incubated with B cells and bound antibodies were detected by the addition of two fluorophore-labeled mAbs specific for IgG and IgA, respectively.

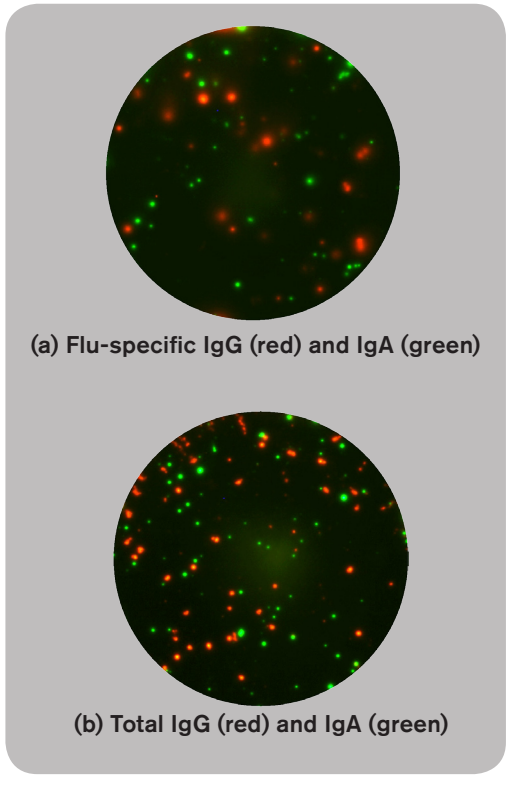


Figure 9. FluoroSpot analysis of swine flu specific IgG and IgA secretion by in vivo *activated human B cells. PBMC were collected before and one week after vaccination with Pandemrix. Wells were coated with 0.75 µg of antigen per well. After overnight culturing of PBMC (200 000 cells/well), spots were detected by adding a mixture of anti-IgG-red and anti-IgA-green (a). Analysis of B cells secreting IgG and IgA is shown as a reference (b). Wells were coated with a mixture of anti-IgG and anti-IgA capture mAbs. After overnight culturing of PBMC (100 000 cells/well), spots were detected by adding a mixture of anti-IgG-red and anti-IgA-green.*

Two examples of vaccine-induced antibody responses are shown in Figures 9 and 10. Here the number of antigen-specific IgG and IgA secreting cells were investigated before and after vaccination with Pandemrix (swine flu) or Dukoral (cholera), respectively. In both examples there were no detectable spots before vaccination.

We believe that the many beneficial properties of the B-cell ELISpot and FluoroSpot make them excellent assays to be used in studies aiming to provide further knowledge about B-cell immunity in general as well as in specific applications where B cells play either a protective role (e.g. infection and cancer) or may be linked to disease (e.g. autoimmunity and allergy).

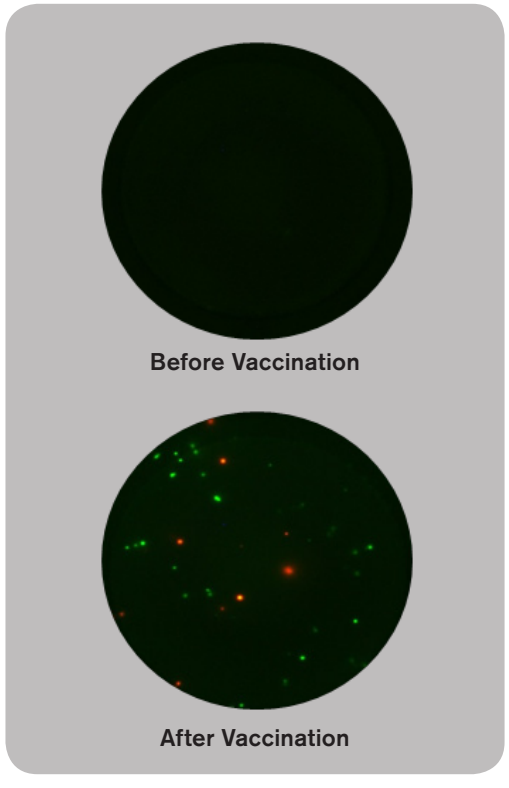


Figure 10. Detection and enumeration of cholera-specific IgG and IgA secreting B cells. Human PBMC were collected before and seven days after vaccination with the oral vaccine Dukoral. Antigen-specific IgG (red) and IgA-(green) secreting cells were analyzed using wells coated with the cholera toxin B subunit (1µg/well).

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