

An antigen microarray immunoassay for multiplex screening of mouse monoclonal antibodies

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The mouse monoclonal antibody (mAb) technology still represents a key source of reagents for research and clinical diagnosis, although it is relatively inefficient and expensive and therefore unsuitable for high-throughput production against a vast repertoire of antigens. In this article, we describe a protocol that combines the immunization of individual mice with complex mixtures of influenza virus strains and a microarray-based immunoassay procedure to perform a parallel screening against the viral antigens. The protocol involves testing the supernatants of somatic cell hybrids against a capture substratum containing an array of different antigens. For each fusion experiment, we carried out more than 25,000 antigen-antibody reactivity tests in less than a week, a throughput that is two orders of magnitude higher than that of traditional antibody detection assays such as enzyme-linked immunosorbent assays and immunofluorescence. Using a limited number of mice, we can develop a vast repertoire of mAbs directed against nuclear and surface proteins of several human and avian influenza virus strains.

INTRODUCTION

As sequencing projects of microbial organisms are being completed, a large number of genes of potential biological and medical relevance are classified as 'unknown' because their unique sequence and structural features elude bioinformatic predictions. The biochemical and functional characterization of these genes is a challenging task that requires the *ad hoc* development of a large number of highly specific antibodies directed against individual components of the microbial proteomes. The somatic cell hybrid technology that is used for generating mouse mAbs¹ has been substantially improved over the past 20 years but still remains a complex and relatively inefficient procedure. The most important limiting factor in the technology is the throughput of the antibody-screening assay that in turn determines the number of distinct antigen-specific mAbs that can be identified at any given time. In a typical experiment, antibody-producing lymphocytes collected from the spleen of antigen-immunized mice are fused with myeloma cells to generate hybrids that produce antigen-specific mAbs and multiply indefinitely². The concomitant growth of thousands of cell hybrids in small wells dictates the need to rapidly identify those cultures producing the relevant antibodies for further expansion and cloning³. Normally, the screening procedure is carried out using immunoassays such as the enzyme-linked immunosorbent assay (ELISA), immunofluorescence and cytofluorimetry^{4–10}. These assays are only suitable for investigating antibody specificity against a limited number of antigens at one time because they are labor intensive, require large volumes of samples and have a low throughput. The production of mAb libraries directed against a vast antigen repertoire in a timely manner requires assay systems capable of performing thousands of immunoassays in parallel. This requirement is far beyond the throughput of conventional immunoassays and the capability of most research laboratories in term of logistics, resources and personnel^{11–15}.

The development and validation of multiplex microarray immunoassays^{16–18} has over the last few years dramatically enhanced the capability of somatic cell hybrid technology to develop mAb libraries with a vast antigen specificity repertoire. Approaches using

microarray technology have also been exploited to expedite the screening of mAb of desired specificity. One approach uses a soft lithographic process called microengraving to screen and retrieve individual antibody-secreting cells¹⁹. Single antibody-secreting cells are distributed onto microscale wells to generate an antibody array substrate that is incubated with different antigens. This strategy allows the isolation of a clonal line of hybridomas that produce the antibody of interest directly without the need for additional cloning. Although this method typically yields large numbers of clones that produce antibodies specific for the target of interest, it is not appropriate for immunization with complex mixtures of antigens, such as whole microorganisms. A method combining immunization with multiple recombinant antigens and an antigen-coated microarray-screening assay (AMA) has been used to generate mAbs directed to different targets²⁰. This method uses chips homogeneously coated with a single antigen and subsequently arrayed with hybridoma supernatants. This approach represents a useful tool to screen mAbs, although is difficult to standardize and quite impractical when hybridomas must be screened against multiple antigens. Moreover, AMA requires a large amount of material to coat the whole surface of a slide (5 µg per slide).

In previous works, we have shown how immunoassays that use an antigen microarray as substratum could be used to detect serum antibodies directed against microbial organisms such as *Toxoplasma gondii*, the rubella virus and the herpes simplex virus^{21–23} to assay allergens^{24,25} and investigate human immune response against a variety of *Plasmodium falciparum* antigens²⁶. The ability to distribute a large number of capture molecules packed in a small area on the same assay substratum allows thousands of distinct antigen-antibody reactions to be performed simultaneously using small quantities of culture supernatants and reagents. This assay format thus incorporates key features such as true parallelism, miniaturization and high throughput, thereby overcoming most of the limitations of traditional immunoassays^{23–25}. The protocol presented here allows the operator to obtain, from a single assay, information not only on the reactivity of a mAb to an antigen or microorganism

but also on whether its specificity is restricted to a single molecule/organism or cross-reacts with others. The array can be designed to include a variety of different antigens or whole microorganisms, resulting in a highly informative assay that drives an early decision on whether or not to select a determined hybridoma, drastically increasing the efficiency of the process and reducing the workload by eliminating unwanted cell cultures at a very early stage. This protocol may be particularly suitable for seeking mAbs recognizing conserved epitopes of highly variable antigens or orthologous proteins from different microorganisms. At the same time, the technology described here is equally appropriate for selecting mAbs reacting exclusively with one specific strain, type, subtype or isolate of one microorganism. Moreover, in our hands, immunization with whole microorganisms, instead of recombinant antigens, has been shown to be a valid strategy to generate hybridomas that produce antibodies directed to glycosyl moieties. The principal limitation of this approach is the number of slides to be processed during the hybridoma screening. More than 100 slides are necessary to complete the screening of individual hybridoma libraries resulting from one fusion procedure. Of course, an instrument able to automatically process slides would overcome the intense work of this phase of the protocol.

The heterogeneous nature of the antigens (protein, sugar residues and lipids) also presents many challenges in all aspects of developing such arrays, from immobilization of the capture molecule to detection of the bound ligand. In addition, there is no simple method of antigen amplification (such as PCR for nucleic acids), and stabilization is yet a further major consideration. Different immobilization protocols have been experimentally validated using a combination of buffers and immobilization substrates (e.g., polylysine, aldehyde, silylated aldehyde or silanated amine). Arrays of proteins covalently bound to aldehyde-coated glass slides were shown to retain their ability to interact specifically with other proteins or with small molecules in solutions²⁷. In the protocol presented here, we have shown that whole microorganisms, such as influenza viruses, can be printed in arrays and used as capture substrates, overcoming difficulties associated with producing recombinant antigens or with purifying molecules from their sources. With regard to array stabilization, we observed that binding of substrates onto aldehyde glass slides required 24 h to

become stable and no significant differences were observed in the assay performance for at least 6 months; thereafter, we experienced a progressive deterioration of the arrays. Notwithstanding these challenges, antigen immunoassays represent suitable tools for translating genomic information originating from sequencing projects of microbial organisms into functional immunological knowledge.

Here, we describe the step-by-step protocol to produce a mAb library directed against distinct influenza virus types and subtypes using multiplex immunization²⁸ with whole inactivated microorganisms (in this example, whole inactivated viruses) as source of antigens and an immunoassay that uses as capture substratum a microarray of influenza antigens. This protocol is particularly useful when the objective is to produce several mAbs, each directed to a distinct component of cellular fractions, microbial organisms and parasites. We illustrate how combining microarray immunoassay and somatic cell hybrid mAb technology is possible to rapidly identify a large number of antibodies displaying a range of different specificities against the microorganisms used in the multiplex immunization regimen.

Experimental design

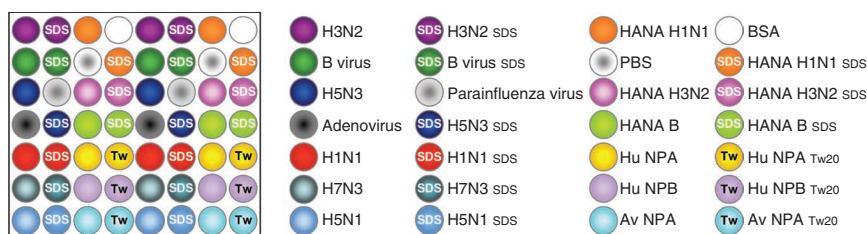
Immunization regimen. The protocol allows the rapid development of mAbs of different specificities starting from animals immunized with whole microorganisms, cell extracts and cell organelles. As an example, we immunized seven groups of mice with six subtypes of whole influenza viruses inactivated by either formaldehyde or β -propiolactone treatment. The first group of animals was injected with a mixture of viruses containing the A subtypes H1N1 and H5N3, and one B strain of influenza virus (Victoria lineage). The second group was immunized with a mixture of H3N2 and H7N3 subtypes of influenza A virus, whereas the other five groups of animals were each injected with individual virus subtypes H5N1, H1N1, H3N2, H7N3 and H5N3 (**Table 1**). Mouse immunization is normally performed by injecting 50–100 μ g of antigen four to five times at 15-d intervals. As we immunized mice with whole influenza viruses, we injected the maximum amount of virus tolerated by the animal to deliver a sufficient amount of each viral protein. The overall amount of virus tolerated by the mice was the equivalent of 21 μ g of total hemagglutinin.

TABLE 1 | Immunization regimen.

Groups of immunized mice	Virus injected	Equivalent μ g of hemagglutinin injected	Immunization regimen		
			Number of injections given to each mouse		
			Complete Freund's adjuvant	Incomplete Freund's adjuvant	PBS
1	B virus + H1N1 + H5N3	7 per virus	1	1	3
2	H3N2 + H7N3	10 per virus	1	1	3
3	H5N1	20	1	1	3
4	H1N1	20	1	1	3
5	H3N2	20	1	1	3
6	H7N3	20	1	1	3
7	H5N3	20	1	1	3

The table shows the seven groups of mice injected with either combinations or single types of influenza virus. The three columns on the right indicate the number of injections administered per mouse in each group to deliver influenza viruses in complete and incomplete Freund's adjuvant and 1 $\times$ PBS. Each injection was performed using the amount of virus equivalent to that of the hemagglutinin indicated in third column.

Figure 1 | Schematic representation of the microarray layout. Inactivated whole viruses, hemagglutinin and neuraminidase subunits (HANA) and recombinant nucleoproteins were arrayed in different buffers to optimize binding and antibody recognition. Inactivated whole influenza viruses (A subtypes: H3N2, H1N1, H5N1, H5N3 and H7N3; B-type virus of Victoria lineage: B virus) and HANA from influenza A subtypes H3N2 (HANA H3N2), H1N1 (HANA H1N1) and B-type virus (HANA B) were printed in either 1× PBS (plain colored circles) or 1× PBS/0.2% SDS (colored circles labeled 'SDS'). Adenovirus, parainfluenza virus and BSA were printed in 1× PBS. The recombinant nucleoproteins have been printed either in 1× PBS (plain colored circles) or 1× PBS/0.01% Tween 20 (colored circles labeled 'Tw'). Av NPA, recombinant nucleoprotein from a representative avian influenza A-type virus; BSA, bovine serum albumin; Hu NPA, recombinant nucleoprotein from a representative human influenza A-type virus; Hu NPB, recombinant nucleoprotein from a representative human influenza B-type virus; PBS, 1× PBS; Tw20, Tween 20.



We mixed 250 µl of virus solution with 250 µl of adjuvants or 1× PBS to be injected into each mouse.

Antigen microarray production. *Antigen selection:* The nature and purity of the arrayed antigens will largely determine the capability of identifying the antibody with the desired reactive profile. As capture substratum, we used an antigen microarray containing a vast combination of influenza virus antigens in the form of inactivated virus strains, purified natural molecules and recombinant proteins (see Array design, below).

Slide choice and antigen binding: Protein heterogeneity dramatically affects the binding efficiency of individual molecular species to chemically modified glass slides (the substrate on which arrays are typically printed). Different glass surfaces must be assessed to optimize protein binding by either adsorption or covalent linkage. In our hands, aldehyde-coated slides showed the best performance in terms of antigen binding and postprocessing background²⁶.

In addition, optimal binding conditions should be experimentally assessed for each antigen preparation at different pH and buffer composition. Formaldehyde-inactivated viruses, such as influenza virus or adenovirus, could be efficiently printed onto aldehyde-coated slides in 1× PBS (pH 7.4) either in the presence or absence of SDS. For most purified proteins, the best array-printing results were obtained when the antigens were resuspended in 1× PBS (pH 7.4) containing 0.01% (vol/vol) Tween 20.

Array design: The array was designed to contain several influenza virus types, subtypes or strains, as well as related (parainfluenza virus) and unrelated microorganisms (adenovirus) as control (Fig. 1). We generated a chip (used across all screening procedures) containing the following components: the B-type of influenza virus (B/Malaysia/2506/2004), five influenza A subtypes (human H1N1 A/Solomon Island/3/2006, H3N2 A/Wisconsin/67/2005, avian H5N1 A/Swan/Italy/2681/2006, H5N3 A/Mollard/Italy/26474/2004, and H7N3 A/Mollard/Italy/33/2001) and purified mixtures of hemagglutinin and neuraminidase from the same clades of human influenza H1N1, H3N2 and B viruses. We also included recombinant bacterial nucleoproteins of A and B types (huNPA from the human clade A/Thailand/CU44/2006(H1N1); AvNPA from the avian clade A/Chicken/HongKong/728/2005(H5N1); and NPB from lineage B/Malaysia/2506/2004), parainfluenza virus and adenovirus. To confirm positive reactions, the antigens should be printed in replicates in different positions of the array. We have experienced a very good reproducibility of the reactivity between replicates of antigens both intra- and interslides. The intra- and interslide coefficients of variation (CV; where CV = (s.d. / mean) × 100) were

~5 and 10%, respectively. Different batches of slides have shown very similar binding efficiency and background (Supplementary Fig. 1). Nevertheless, before printing high numbers of slides, it is advisable to perform a rapid test with known reagents, such as the immune serum or commercial antibodies. We routinely printed each antigen preparation in two replicates within a 7 × 8 array (Fig. 1). Each slide contained four separate arrays, allowing the processing of four supernatants per slide (Supplementary Fig. 2). Array formats larger than 21 × 21 can be easily accommodated on a microscope slide, but at the expense of the number of subarrays that can be printed on each slide, thus increasing the screening cost. To control the immunoreactivity of the arrayed antigens, we included during each screening procedure an assay performed using the immune serum collected from the mouse before starting the fusion procedure. Initially, to monitor the activity of the secondary reagents, we included in the array scalar amounts of IgG and IgM, as previously described^{17,21,22,24,26}. No signal was detected when slides were processed using the secondary conjugated antibody alone. We have not experienced competition between the two secondary reagents when used together. Signals derived from supernatants detected by only one or a mixture of the two anti-isotype secondary antibodies showed no significant difference in intensity. As the Alexa Fluor 647 goat anti-mouse IgM only recognizes epitopes found on the µ heavy chain and does not react to the immunoglobulin light chain, the two secondary antibodies do not interfere with each other.

Microarray printing: Microarray chips were generated through solid pin deposition technology using the Microgrid Compact (Biorobotics). The arrays were printed at 23 °C and 55% humidity and left overnight inside the printing cabinet before being stored with desiccant. Microarray slides can be stored for a maximum of 6 months at room temperature (RT, 20–25 °C); prolonged periods of storage cause a progressive deterioration of the assay performance.

mAb screening microarray immunoassay. As little as 20 µl of culture supernatant can be used to carry out the incubation on the array surface for 1 h after blocking the slides with 5% (wt/vol) BSA. We routinely detected the presence of antibodies bound to the array using, as secondary antibody, a mixture of anti-mouse whole IgG and anti-mouse IgM, conjugated to Alexa Fluor 555 and Alexa Fluor 647, respectively. The combination of two distinct secondary antibodies conjugated to different fluorophores allows for the determination of the immunoglobulin class of the mAb at a very early stage during the screening procedure. Combinations of more than two secondary antibodies can be utilized using an array reader incorporating three to four different

lasers. Processed slides stored at 4 °C retain signal for several weeks and thus can be reanalyzed by laser scanning. The small amount of supernatant required in the procedure represents a great advantage of the system over conventional assays such as ELISA and immunofluorescence. Hybridomas are usually seeded in 96-well plates; thus, the volume of culture medium that can be withdrawn to carry out the screening assays is limited (100–180 µl).

Controls needed for the procedure. Both positive and negative controls should be included to assess the background and the cut-off of positive reactions, as well as to monitor the activity of the secondary reagents. We used the preimmune serum collected from each of the mice selected for the immunization procedure to assess the background signal against the arrayed antigens. Typically, the preimmune sera were tested at a 1/100 dilution. Unrelated anti-

gens such as BSA and different viral extracts (parainfluenza virus and adenovirus) were included to test the specificity of the reaction. Immune sera collected on the day of the fusion were used as positive control to monitor for the presence of the antigen on the microarray and the activity of the secondary reagents. The latter was also assessed in some occasion by including in the array a scalar amount of mouse-purified IgG or IgM^{10,12,13,15,17}. Fusion procedure usually results in 500–600 independent somatic cell hybrids, but only a small number of them (about 5–7%) produce immunoglobulins that react against the antigens used to immunize the mouse. Thus, the high number of negative hybridoma supernatants allows the operator to easily distinguish low signals from genuine positives. Supernatant from P3x63Ag8.653 myeloma cell culture should also be tested to assess the background generated by either the cell growth medium or the secondary conjugated antibody itself.

MATERIALS

REAGENTS

- Polystyrene plates (384 wells, Matrix, Thermo Scientific, cat. no. 4310)
- Aldehyde glass slides (CEL Associates, VALS-25) **▲ CRITICAL** Slides coated with different chemicals (e.g., silylated aldehyde or silanated amine) should be tested to select the best surface to print antigens in the arrays.
- BALB/c female mice (Harlan) **! CAUTION** All animal experiments require the approval of the institutional review board and the animal use and care committees and must be conducted in accordance with institutional and national regulations.
- Bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A7906)
- Complete Freund's adjuvant (Sigma-Aldrich, cat. no. F5881)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D2650)
- EDTA (Sigma-Aldrich, cat. no. E5134)
- Fetal bovine serum (FBS; Invitrogen, cat. no. 16000-044)
- Gene frame (25 µl, 1.0 cm × 1.0 cm; Abgene, cat. no. AB-0576)
- Goat anti-mouse IgG conjugated with fluorophore-Alexa 555 (Invitrogen, cat. no. A-21422)
- Goat anti-mouse IgM conjugated with fluorophore-Alexa 647 (Invitrogen, cat. no. A-21238)
- Hypoxanthine-aminopterin-thymidine medium supplement (50×; Sigma-Aldrich, cat. no. H0262) **! CAUTION** It is toxic by inhalation, in contact with skin and if swallowed; it may cause harm to the unborn child. Manipulate under a fume board; use gloves and wear safety goggles during REAGENT SETUP.
- Hypoxanthine-thymidine (HT) medium supplement (50×; Sigma-Aldrich, cat. no. H0137)
- Hybridoma-enhancing supplement (Sigma-Aldrich, cat. no. H8142) **▲ CRITICAL** We also tested hybridoma-enhancing products from other companies (e.g., Hybridoma fusion and cloning supplement, Roche, cat. no. 11363735001), but in our hands, they were less effective.
- Iscove's modified Dulbecco's medium (IMDM; Invitrogen, cat. no. 21980-065)
- Incomplete Freund's adjuvant (Sigma-Aldrich, cat. no. F5506)
- MEM NEAA (minimal essential medium with nonessential amino acids solution; Invitrogen, cat. no. 11140-050)
- MEM vitamin solution (Invitrogen, cat. no. 11120-037)
- PEG 4000 (Euroclone, cat. no. EMR393001)
- Penicillin-streptomycin (Invitrogen, cat. no. 15070-063)
- Potassium chloride (Sigma-Aldrich, cat. no. P-9541)
- Potassium phosphate (Sigma-Aldrich, cat. no. P-5379)
- P3x63Ag8.653 cells (ATCC, cat. no. CRL-1580)
- Silica gel bags (Sigma-Aldrich, cat. no. S-8394)
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. S8045)
- Sodium pyruvate (Invitrogen, cat. no. 11360-039)
- Sodium chloride (Sigma-Aldrich, cat. no. 71376)
- Sodium phosphate (Sigma-Aldrich, cat. no. S-3264)
- SDS (Sigma-Aldrich, cat. no. L-4390) **! CAUTION** It is harmful if it comes in contact with skin and if swallowed; it is also irritating to eyes and respiratory system. Manipulate under a fume board, use gloves and wear safety goggles during REAGENT SETUP.

- Trypan blue (BioWhittaker, cat. no. MD21793)
- Tween 20 (Sigma-Aldrich, cat. no. P-5927)
- Enzymatic protein deglycosylation kit (Sigma-Aldrich)
- Ethanol
- Liquid nitrogen
- Bidistilled water (H₂O)

EQUIPMENT

- Syringes (10 ml; Terumo, cat. no. SS-1052138)
- Round bottom tubes (13 ml; BD Falcon, cat. no. 352001)
- Tubes (50 ml; BD Falcon, cat. no. 352077)
- Flasks (75 cm² and 25 cm²; Iwaki, cat. no. 3120-25)
- Cell strainer (70 µm; BD, cat. no. 353086)
- Centrifuge (Eppendorf, cat. no. 5810 R)
- Cryoboxes
- Flat bottom 96-well microplates (Iwaki, cat. no. 3860-096)
- Incubator with CO₂
- Microarrayer Microgrid II (Genomic Solutions)
- Microarray Scanner ScanArray Gx (Perkin Elmer)
- Mr. Frosty (Nalgene, cat. no. 5100/0001)
- Orbital shaker (Heidolph Unimax 1010, Heidolph)
- ScanArray Express software
- Scissors
- Sterile hood
- Tas Application Suite software version 2.2.0.6
- Tweezers
- Vertical staining jars
- Filter paper
- Bunsen burner

REAGENT SETUP

Complete IMDM medium with 20% (vol/vol) FBS (myeloma cell medium) To prepare 500 ml of medium, add 100 ml of FBS, 5 ml of MEM NEAA, 5 ml of MEM vitamin solution, 5 ml sodium pyruvate and 5 ml of penicillin-streptomycin to 380 ml of IMDM. The medium can be stored at 4 °C for ~4 weeks.

Complete IMDM medium with 10% (vol/vol) FBS To prepare 500 ml of medium, add 50 ml of FBS, 5 ml of MEM NEAA, 5 ml of MEM vitamin solution, 5 ml of sodium pyruvate and 5 ml of penicillin-streptomycin to 430 ml of IMDM. The medium can be stored at 4 °C for ~4 weeks.

Incomplete IMDM medium (without FBS) To prepare 500 ml of medium, add 5 ml of MEM NEAA, 5 ml of MEM vitamin solution, 5 ml of sodium pyruvate and 5 ml of penicillin-streptomycin to 480 ml of IMDM. The medium can be stored at 4 °C for ~4 weeks.

Ca²⁺ and Mg²⁺-free 1× PBS/EDTA (1 mM) To prepare 500 ml of solution, add EDTA at a final concentration of 1 mM to 1× PBS and sterilize by filtration. The solution can be stored at 4 °C for up to 6 weeks. **▲ CRITICAL** The solution must be used cold.

IMDM/DMSO 2.5% (vol/vol) solution Add 250 µl of DMSO to 10 ml of IMDM. Prepare fresh before use.

PROTOCOL

Peg 4000 Place 2 g of PEG 4000 powder in a glass tube for bacterial growth and sterilize it in autoclave. It can be stored for several weeks at room temperature.

PEG solution Using a Bunsen burner, warm the bottom of the glass tube containing 2 g of autoclaved PEG 4000 powder until it turns liquid. As soon as PEG becomes liquid, gradually add 2 ml of IMDM/DMSO (ratio 1:1). Then add a few drops (~50 μ l) of NaOH (0.5 M) to the PEG solution until the color turns pink. Keep PEG solution at RT and warm it briefly again immediately before use to ensure that it is still completely liquid.

NaOH (0.5 M) To prepare 10 ml of the solution, dissolve 0.2 g of NaOH pellets in 10 ml of bidistilled H_2O and sterilize by filtration. It can be stored at room temperature for several weeks.

PBS (10 $\times$, pH 7.4; Ca^{2+} and Mg^{2+} -free) Dissolve 0.2 grams of KCl, 1.44 grams of Na_2HPO_4 , 0.24 grams of KH_2PO_4 and 8 grams of NaCl in 1 liter of H_2O . The pH should be ~7.4. Sterilize it in autoclave. It can be stored at room temperature for several months.

BSA (10% (wt/vol)) Dissolve 10 g of BSA in 100 ml of H_2O . After filtration through a 0.2- μ m filter, it can be stored at -20 °C for several months.

Rinse buffer PBS (1 $\times$) containing 0.01% (vol/vol) Tween 20. Prepare fresh before use.

Blocking buffer PBS (1 $\times$) containing 2% (wt/vol) BSA. Prepare fresh before use.

Antibody diluent buffer PBS (2 $\times$) containing 0.01% (vol/vol) Tween 20 and 2% (wt/vol) BSA. **▲ CRITICAL** Solution of 2 $\times$ PBS containing 0.01% (vol/vol) Tween 20 can be prepared and stored for several weeks at 4 °C. BSA must be added immediately before use.

Secondary fluorophore-labeled antibody Dilute, together, both goat anti-mouse IgG and IgM conjugated to Alexa 555 and 647, respectively, to 10 μ g ml⁻¹ in antibody diluent buffer. Prepare this solution before use and protect it from light.

Spotting buffer The best spotting buffer should be chosen on the basis of empirical experiments. Most antigens are successfully printed in buffers based on PBS. Adding detergents such as SDS or Tween 20 often results in better deposition of some substances (0.2% (wt/vol) SDS in 1 $\times$ PBS; 0.01% (vol/vol) Tween 20 in 1 $\times$ PBS). Other spotting buffers that can be used are based on borate (pH 9.4) or glycine (pH 2.4).

Antigen preparation for printing The virus and hemagglutinin and neuroaminidase solutions were printed at a concentration equivalent to 50 μ g of hemagglutinin per ml in 1 $\times$ PBS or 1 $\times$ PBS containing 0.2% (wt/vol) SDS. Recombinant nucleoproteins were diluted in 1 $\times$ PBS or 1 $\times$ PBS containing 0.01% (vol/vol) Tween 20 at the spotting concentration of 100 μ g ml⁻¹.

PROCEDURE

Mouse immunization ● TIMING 8 weeks

1| Collect several drops of blood from the tails of each preimmune mouse into separate 1.5-ml tubes by cutting the ends of their tails with scissors (a few millimeters is sufficient).

2| Keep the blood samples at 37 °C for 1.5 h.

3| Centrifuge the blood samples at 1,000g at 4 °C for 10 min.

4| Recover the sera and transfer them into new 1.5-ml tubes; discard the pellet.

■ **PAUSE POINT** The sera can be stored at -80 °C for several years.

5| Immunize at least two mice by mixing an appropriate amount of antigens or whole inactivated microorganism(s) (prepared as described in Experimental design) with an equal volume of complete Freund's adjuvant (vortex or shake to resuspend the mycobacterium before use) in a final volume of 500 μ l.

6| Form an emulsion by vigorously mixing for ~1–2 min. Inject the mice intraperitoneally with no more than 0.5 ml of emulsion.

▲ **CRITICAL STEP** We injected an amount of whole inactivated virus solution per mouse equivalent to a maximum of 21 μ g of total hemagglutinin. A higher amount of virus may cause the death of the animal.

▲ **CRITICAL STEP** The emulsion should be very thick, such that it does not disperse if placed on the surface of a saline solution.

7| After 2 weeks, boost the mice by intraperitoneal injection of the same amount of antigens or whole inactivated microorganism(s) emulsified with an equal volume of incomplete Freund's adjuvant.

8| After a further 2 weeks, boost the mice by intraperitoneal injection of the same amount of antigens or whole inactivated microorganism(s) in PBS without adjuvant.

9| Repeat Step 8 twice.

▲ **CRITICAL STEP** Once the immunization schedule has been completed, the mice can be left untreated until the fusion procedure (Step 44). The fusion procedure should be carried out within 4 d after the last immunization.

Selection of immunized mice for the fusion procedure ● TIMING 6 h

10| At 4 d before fusion, boost mice as in Step 8.

11| At 1 d before fusion, analyze immunized mice by microarray immunoassay to select the animal showing the strongest reactivity toward the arrayed antigens. To do this, collect a few drops of blood (at least 100 μ l) from the tails of mice as in Step 1.

12| Store the blood samples at 37 °C for 1.5 h.

13| Centrifuge the blood samples at 1,000g at 4 °C for 10 min.

14| Recover the sera by pipetting them into new 1.5-ml tubes, and discard the pellet.

■ **PAUSE POINT** The sera can be stored at –80 °C for several years.

15| Test 50 µl of both undiluted and diluted sera (in the ratio 1:100 in 1× PBS) by microarray immunoassay, according to Steps 81–90.

▲ **CRITICAL STEP** Preimmune serum collected in Step 1 above should be used as negative control (undiluted and diluted in the ratio 1:100 in 1× PBS) in the immunoassay to assess the background reactivity of the mouse serum.

16| Select the mouse to be used for the fusion process on the basis of the best signal-to-background reactivity ratio against the immunization and the negative control antigens.

Collection and plating of feeder cells ● **TIMING 3 h**

17| At 1 d before fusion, prepare ten 96-well microplates seeded with mouse peritoneal lavage feeder cells. To isolate enough feeder cells for ten plates (1,000–2,000 feeder cells per well), collect peritoneal washes from one or two BALB/c mice (8 weeks old); first, kill each mouse by cervical dislocation and rinse its abdomen with 70% (vol/vol) ethanol.

18| Under a sterile hood, pinch the skin in the middle of the abdomen and make a small incision.

19| Hold the skin firmly above and below the incision and pull toward the head and tail to expose the abdominal peritoneal membrane.

20| Lift the peritoneal membrane using sterile tweezers and wash the peritoneal cavity with 10 ml of IMDM without FBS by using a 10-ml syringe with a 19-G needle and vigorously pulling the injected solution in and out five or six times.

? **TROUBLESHOOTING**

21| Pool the peritoneal washes in a 50-ml tube if more than one mouse is used.

22| Centrifuge cell suspension at 800g for 10 min at 4 °C.

23| Discard the supernatant and resuspend the cell pellet in 5 ml of IMDM without FBS.

24| Take 20 µl of cells and mix with 4 µl of trypan blue.

25| Determine the yield and viability of cells by trypan blue exclusion and count cells on a hemocytometer²⁹.

26| Dilute feeder cells in IMDM/20% FBS at 1,000–2,000 cells per 100 µl.

27| Seed 100 µl of feeder cells to each well of ten 96-well microplates.

28| Incubate plates at 37 °C in a humidified, 5% CO₂ incubator.

Preparation of P3x63Ag8.653 myeloma cells for fusion ● **TIMING 1.5 h**

29| Collect medium from three 75-cm² flasks with semiconfluent cultures of P3x63Ag8.653 cells and transfer into three 50-ml tubes and store on ice (most cells are nonadherent). Cell cultures are prepared and maintained as described in **Box 1**.

▲ **CRITICAL STEP** Myeloma cell viability is the most critical point for optimal fusion efficiency. A percentage of dead cells above 5% strongly correlates with a poor outcome of the fusion in terms of growing hybridomas. We prepare ten 75-cm² flasks (**Box 1**) in order to select at least three cell culture flasks with the best live-to-dead ratios. Do not continue with the procedure until you obtain a minimum of three 75-cm² flasks showing the desired viability.

30| Wash the remaining adherent cells in the flasks once with 10 ml of ice-cold Ca²⁺ and Mg²⁺-free PBS/1 mM EDTA.

31| Discard the washing medium.

BOX 1 | CELL CULTURE PROCEDURES

Myeloma cell culture

1. Grow myeloma cells (P3x63Ag8.653) in complete IMDM/20% FBS at 37 °C and 5% CO₂.
2. Subculture myeloma cell line every 2 d by splitting in a ratio of 1:2 or 1:3.

▲ **CRITICAL STEP** Before adding the cells, it is best to put the fresh medium in a sterile flask and then place the flask/medium in a CO₂ incubator to warm the medium and allow the CO₂ to 'dissolve' in the medium. The cells should be kept in log-phase growth.

Cell-splitting procedure

3. Once cells are semiconfluent, collect the medium (containing floating cells) from the flask and store on ice in 50-ml tubes.
4. Wash adherent cells remaining in the flasks with ice-cold Ca²⁺ and Mg²⁺-free PBS/1 mM EDTA. Rinse and then discard the washing medium.
5. Detach adherent cells from the bottom of the flask by adding 10 ml of ice-cold Ca²⁺ and Mg²⁺-free PBS/1 mM EDTA solution.
6. Incubate the flasks for 4–5 min on ice, sharply tapping the flask periodically with the palm of your hand; collect the supernatant and add it to the Falcon tube.
7. Centrifuge cells for 10 min at 800g at 4 °C.
8. Discard the supernatant and resuspend the pellet by tapping gently with fingertips on the bottom tube and adding fresh incomplete IMDM medium.
9. Distribute the cell suspension into new flasks containing complete IMDM/20% FBS.

Freezing procedure

10. Collect the cells as described in the 'cell-splitting procedure' above and resuspend the pellet with 3 or 4 ml of ice-cold 90% FBS/10% DMSO. Divide the cell suspension into cold cryovials. Use a freezing container to allow a slow freezing of cells at –80 °C overnight and then move samples into liquid nitrogen.

32| Incubate adherent cells in 10 ml of fresh ice-cold Ca²⁺ and Mg²⁺-free PBS/1 mM EDTA for 1–2 min on ice until cells start floating on the medium.

33| Gently pipette up and down to harvest cells, collect the medium and pool it with the cell culture medium from Step 29.

34| Centrifuge cell suspensions at 800g for 10 min at 4 °C.

35| Decant supernatants and resuspend the dry cell pellets by gently tapping the bottom of the tube several times.

36| Add 10 ml of ice-cold IMDM without FBS to each tube.

37| Pool the three cell suspensions in one 50-ml tube and gently mix to make the solution homogeneous.

38| Split the cell suspension equally in two 50-ml tubes and add 25 ml of ice-cold IMDM without FBS to each.

39| Repeat Steps 34 and 35.

40| Add 2.5 ml of ice-cold IMDM without FBS to each tube and pool the two cell suspensions together.

▲ **CRITICAL STEP** Always keep the cell suspension on ice.

41| Mix 10 µl of cell suspension with 190 µl of IMDM and 40 µl of trypan blue.

42| Determine the yield and viability of cells by trypan blue exclusion and by counting on a hemocytometer²⁹.

43| Transfer 4 × 10⁷ cells into a fresh tube and store it on ice.

Fusion procedure ● **TIMING 2.5 h**

44| Clean dissection tools with 70% (vol/vol) ethanol.

45| To isolate spleen, kill an immunized mouse from Step 10 by cervical dislocation and rinse its abdomen with 70% (vol/vol) ethanol.

46| Under a sterile hood, pinch the skin just above the area where the spleen is localized, make a small incision, remove the skin and, with a sterile scissors, cut the peritoneal membrane to expose the spleen.

47| Using sterile tweezers and scissors, dissect the spleen by cutting the connection from the mouse body and place it in a Petri dish containing 5 ml of IMDM without FBS.

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48| Cut the spleen into small pieces of 2–3 mm using scissors; remove fat tissue if still present.

49| Collect the cell suspension from the Petri dish (from Step 48) with a 25-ml pipette and filter it through a 70- μ m mesh filter (cell strainer) into a 50-ml tube to remove any cell clumps and fat tissue. Smash the remains of spleen fragments using the rubber end of a syringe plunger while filtering.

50| Wash the 70- μ m filter mesh with another 5 ml of IMDM without FBS.

51| Transfer all 10 ml of the splenocytes in IMDM into a 13-ml round-bottomed tube and centrifuge at 800*g* for 10 min at 4 °C.

52| Decant the supernatant and disaggregate the dry spleen cell pellet by gently tapping the end of the tube with the finger several times.

▲ CRITICAL STEP Always keep the cell suspension on ice.

53| Add the suspension of 4×10^7 P3x63Ag8.653 cells from Step 43 to the splenocyte solution.

54| Centrifuge the splenocyte/myeloma cell mix at 800*g* for 10 min at 4 °C.

55| While centrifuging cells, prepare one 50-ml tube with 20 ml of IMDM without FBS and one 50-ml tube with 30 ml of IMDM without FBS (keep them both at RT).

56| Decant supernatant from the centrifuge tube from Step 54 by inverting the tube and, while still keeping the tube facing downward, remove any drops remaining on the tube rim with a micropipette.

▲ CRITICAL STEP The presence of liquid in the cell pellet will negatively affect the efficiency of the fusion.

57| Disaggregate the dry cell pellet by gently tapping the end of the tube with the finger several times so that it coats the bottom of the tube.

58| Add, dropwise, 1 ml of PEG solution over a period of 1 min to the cell suspension. While adding the PEG solution, shake every few drops to ensure homogenization.

59| Add, dropwise (use a 2-ml pipette and shake the tube after every few drops), 10 ml of IMDM from the tube containing 20 ml of the solution (prepared in Step 55) as follows: 1 ml over a period of 1 min; 6.5 ml over a period of 3 min; and 2.5 ml over a period of 1 min.

60| Leave the cell solution for 1 min at RT.

61| Transfer the cell suspension into the 50-ml tube containing the remaining 10 ml of IMDM without FBS (which was used in Step 59).

62| Leave the cell solution for 1 min at RT.

63| Pour the 20-ml cell suspension into the second 50-ml tube from Step 55 containing 30 ml of IMDM without FBS, obtaining a total volume of 50 ml.

64| Leave the cell solution for 5 min at RT.

65| Centrifuge the cell suspension at 800*g* for 10 min at 4 °C. Meanwhile, place 99 ml of IMDM without FBS into a 250-ml cell culture flask and store it at 37 °C and 5% CO₂.

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- 66| Decant the supernatant from the centrifuged cells from Step 65 and disaggregate the dry cell pellet by gently tapping the end of the tube with the finger several times.
- 67| Add 6 ml of IMDM (kept at room temperature) with 20% FBS to the cells.
- 68| Pour resuspended cells into the 250-ml flask containing 99 ml of IMDM (prepared in Step 65).
- 69| Wash the tube from Step 67 with 5 ml of IMDM with 20% FBS to ensure that all cells are transferred into the flask.
- 70| Store the flask containing fusion cells for 10–30 min at 37 °C and 5% CO₂.
- 71| Plate 100 µl per well of fusion cell suspension onto the ten 96-well microplates containing feeder cells (from Step 28).
- 72| Incubate the 96-well microplates at 37 °C and 5% CO₂ overnight.
- 73| The next day, add 20 µl of 5× hypoxanthine-aminopterin-thymidine medium into each well.
- 74| Incubate the 96-well microplates at 37 °C and 5% CO₂ for a week.
- 75| Replace 100 µl of supernatant from each microplate well with 100 µl of fresh IMDM/10% FBS/1× HT and incubate the plates for a further 1 week or until the cell plaques are visible to the eye.
- 76| As soon as hybridoma cell clusters (1–2 mm) appear in the wells and the color of supernatants turns yellow, withdraw 50 µl of supernatant and start the screening procedure (Step 81).
- ▲ **CRITICAL STEP** Myeloma cell hybrids grow at different rates. Two weeks after fusion, ~100 cultures per day usually reach the stage to be screened (~100 cultures can be screened in the course of 1 working day). At that point, testing all hybridomas from one fusion takes about 5–6 d, resulting in ~25 slides to be processed per day.

Microarray sample preparation ● TIMING 1–1.5 h

- 77| Prepare 100 µl of the antigen preparations to be printed in the optimal concentrations and solution buffers.
- 78| Load a minimum of 25 µl—up to a maximum of 100 µl—of each antigen sample into a 384-well microtiter plate (see **Supplementary Fig. 3** for plate and array layout details).
- ▲ **CRITICAL STEP** Spotting buffers and antigen concentrations must be evaluated before using the array as capture substratum of the mAb screening assay using commercial mAbs and sera from immunized mice. Antigens have always been printed at least in duplicate, along with sample buffers and 2% BSA as negative controls. Our spotting concentration ranges from 20 to 200 µg ml⁻¹ for each antigen.

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Array printing ● TIMING 1–72 h (depending on the number of slides printed)

- 79| The robot should be set up to print as many array replicates as possible in a single slide. The number of chips that can be printed in a single slide depends, of course, on the array size. Four replicates of 8 × 7 arrays can be accommodated in a single slide. We normally use aldehyde-coated slides, which have showed the best performance in our hands, but other types of coating can be evaluated. Samples for the printing are transferred from a 384-well microtiter plate to defined positions on glass slides using solid steel pins (**Supplementary Fig. 3**).
- 80| Once printed, store the slides for at least 24 h inside the robot cabinet. A stable and controlled environment (~55% humidity, 23 °C), favors the binding of proteins to the glass surface. Store slides in the dark in boxes containing desiccant silica gel bags to avoid moisture during storage.
- ▲ **CRITICAL STEP** When using aldehyde-coated slides, the performance of the microarray assay increases progressively during the days after printing. Although slides can be used at 24 h after printing, better performances can be observed after 1 week.

Hybridoma screening procedure ● TIMING 2–3 h for 12–15 slides; 1 week for all hybridomas from one fusion procedure

- 81| *Slide preparation:* Stick a gene frame (1.0 cm × 1.0 cm) on the slide, ensuring that the array is in the center, and place the slides in a plastic box (humid chamber) filled with wet filter paper to avoid evaporation of reagents during incubation.

82| Blocking step: To block nonspecific binding, pipette 100 μl of blocking solution (1 $\times$ PBS/2% BSA) in each array area (within the gene frame) and incubate the slides for 60 min at RT inside the humid chamber.

83| Washing step: Tap the slide to discard the liquid from the area contained in the gene frame. Place the slide into a staining jar containing 50 ml of rinse buffer. Wash the slide for 3 min while shaking constantly (160 r.p.m.). Take the slides out one by one (if more than one is processed at the same time) and tap the slide to get rid of the remaining rinse solution.

84| Sample incubation: Incubate the slide with 30–40 μl of cell hybrid supernatant from Step 76 per chip for 30 min at RT.

85| Repeat Step 83.

86| Secondary antibody incubation: Prepare a solution containing 10 $\mu\text{g ml}^{-1}$ of each secondary antibody (goat anti-mouse IgG and IgM conjugated with Alexa 555 and 647, respectively) in antibody diluent buffer. Prepare this solution just a few minutes before use. Incubate the slide with 30 μl of the secondary antibody solution for 15 min at RT.

87| Gene-frame removing step: Repeat Step 83 without shaking the slide. Carefully remove the gene frames using tweezers.

88| Repeat Step 83.

89| Drying step: Dry the slide by centrifugation in a 50-ml tube for 1 min at 2,000g.

Data acquisition ● TIMING 5 min per slide

90| Analyze the slides with a microarray laser scanner set to gather the signal emitted by the two fluorophores. Each should be under constant instrument settings, i.e., 90% of laser power, 60% of photomultiplier gain and 10 μm of scan resolution.

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91| Select positive hybridomas by choosing those for which the signal collected onto individual spots is 2 s.d. above the mean of negative controls in all replicates.

▲ **CRITICAL STEP** Supernatant containing an unrelated mAb should be included in the immunoassay to evaluate the background reading value of each spot. Preimmune and postimmune sera should always be included as negative and positive samples in the immunoassay during the screening step to determine the background against the arrayed antigens and controls.

Hybridoma cloning procedure ● TIMING 30 min per clone

92| Harvest cells from the selected well by pipetting up and down.

93| Dilute cells in complete medium to a final volume of 200 μl .

94| Mix 20 μl of cells with 4 μl of trypan blue.

95| Determine the number of cells on a hemocytometer.

96| Seed two 96-well microplates with 1 and 5 cells per well, respectively, in complete IMDM medium with 10% (vol/vol) FBS, 1 $\times$ HT and 10% (vol/vol) hybridoma-enhancing supplement.

97| After 2 weeks, cell clusters will be evident in some wells. The number of cell-containing wells in one 96-well microplate should be less than 30–40% to consider the cloning procedure successful.

98| Screen ten clones using microarray procedure as in Steps 81–91. Screen more clones if no positive clones have been detected.

99| Harvest cells by pipetting up and down from two positive wells and expand clones by sequential growth in a six-well plate and then in several 25- cm^2 flasks in order to freeze several stocks of the clones. Hybridoma clones are grown and split in the same way as myeloma cells (**Box 1**). For optimal growth, some hybridoma clones also require medium supplemented with 10% hybridoma-enhancing supplement after culture expansion.

▲ **CRITICAL STEP** Some hybridomas lose the ability to produce mAbs because of loss of chromosomes during their duplication. The nonproducing population of hybridoma cells in the cultures has a growth advantage over the producing population. The cloning procedure must be performed as early as possible to prevent nonproducing cells from overgrowing mAb-producing cells.



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Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
20 and 47	Contamination of the hybridomas	Contamination can occur during the feeder cell collection and spleen dissection	Avoid notching or cutting the gut Avoid touching the fur or any external mouse part with scissors or tweezers
78	Shape and size of the spots	Various spotting solutions or surfactant concentrations can affect size, shape and surface binding properties of the spots	Optimize array printing conditions by trying different spotting buffers at different pH and by adding different concentrations of surfactants
	“Comet tail” shape is seen in the printed array	High concentration of proteins in the printing buffer can increase the risk of comet tails caused by an excess of unbound material in the spots	Print the antigen under different concentrations
90	High background	High background caused by high antibody concentration or prolonged incubation of secondary antibody	Decrease secondary antibody concentration Decrease the incubation time of secondary antibodies Carry out a third washing step after removing the gene frames

● TIMING

A schematic stepwise representation of the time required to carry out the entire procedure from the last immunization to the storage of positive clones is shown in **Figure 2**.

Steps 1–9, Mouse immunization: 8 weeks

Steps 10–16, Selection of immunized mice for the fusion procedure: ~6 h

Steps 17–28, Collection and plating of peritoneal feeder cells: 3 h

Steps 29–43, Preparation of P3x63Ag8.653 myeloma cells: 1 week to obtain three 75-cm² flasks with semiconfluent cultures starting from a frozen aliquot of cells, plus 1.5 h to harvest and wash cells for fusion procedure.

Steps 44–76, Fusion procedure: ~2.5 h

Steps 77 and 78, Microarray sample preparation: 1–1.5 h

Steps 79 and 80, Variable. The time to produce arrays depends on the number and complexity of the arrays as well as the throughput of the printing equipment. Typically, using the Microarrayer Microgrid II arrayer, we print 100 slides containing four arrays composed of 56 spots in a period of ~72 h.

Steps 81–89, Screening of the culture supernatants: 5–7 d for fusion (~25 slides per day, which allows the analysis of 100 cell hybrid supernatants)

Steps 90 and 91, Data acquisition: 5 min per slide

Steps 92–99, Cloning by limiting dilution of one positive cell hybrid: ~30 min per clone

ANTICIPATED RESULTS

We have used the protocol described here to develop a library of mAbs directed against a range of human and avian influenza viruses, with the objective to generate reagents for immunoassays that discriminate among a wide range of influenza virus types and subtypes. We obtained an average of ~500 independent hybridomas from

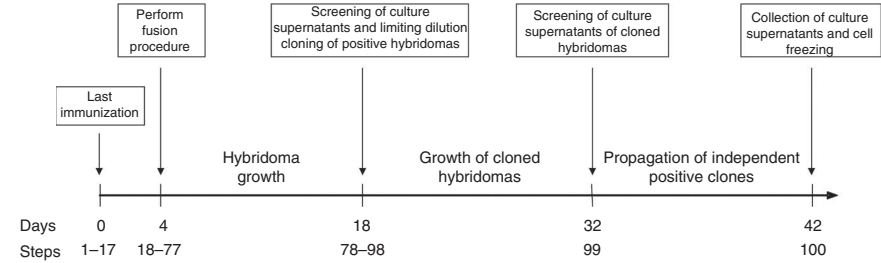


Figure 2 | Timeline of the experimental procedure to generate and screen somatic cell hybrids producing antibodies with desired specificity.

TABLE 3 | Repertoire of isolated mAbs.

MAb reactivity profile against arrayed antigens	Number of mAbs
NPA (human and avian)	24
NPB	3
NPA-NPB	3
H1N1	3
H3N2	2
H5N1	1
H7N3	2
B virus	2
H3N2–H1N1	10
H5N1–H7N3	3
H5N3–H7N3	13
H5N1–H5N3–H7N3	22
H3N2–H1N1–B virus	15
H3N2–H5N1–H5N3–H7N3	1
H3N2–H1N1–B virus–H5N1	3
H3N2–H1N1–B virus–H5N1–H5N3–H7N3	1
Total mAbs	108

The table shows the reactivity profiles of different mAbs against the arrayed antigens, including the nucleoproteins A and B (NPA and NPB) and the subtypes of influenza virus used to immunize the mice, and the number of mAbs that share the same profile.

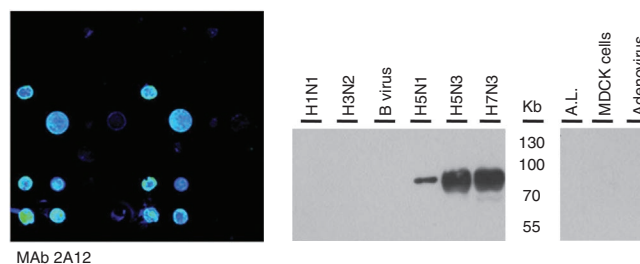


Figure 3 | Reactivity analysis of mAb 2A12 (see **Supplementary Table 1**). The specificity of the mAb against an epitope common to all avian viruses was deduced on the basis of the microarray profile (left panel); using the array layout shown in **Fig. 1** and confirmed in immunoblot experiments (right panel), for which influenza virus extracts were run on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with mAb 2A12. The amount of virus loaded in each gel lane corresponded to 1.5 µg of total hemagglutinin. Allantoid liquid from uninfected chicken eggs (A.L.) and Madin-Darby canine kidney cell extract (MDCK cells) were used as negative control samples as influenza viruses were grown in either chicken eggs or MDCK cell cultures. Adenovirus extract was used to assess potential cross-reactivity of anti-influenza mAbs to other viruses.

each fusion procedure. By using the antigen microarray immunoassay procedure, we could carry out a total of ~25,000 antigen-antibody determinations per fusion (~125,000 in total) within the time frame of single-antigen ELISA screening. This approach not only allowed us to rapidly identify mAbs reacting against different antigens but also to assess their fine specificity in terms of cross-reactivity against different virus types and subtypes (**Table 3**). The knowledge of the fine specificity of the individual mAbs at this early stage of screening provided invaluable information for guiding the selection of hybridomas for further analysis, cloning and storage, with obvious benefits in terms of resource optimization. We obtained a total of 108 mAbs covering a wide spectrum of reactivity against individual virus antigens (**Supplementary Table 1**). Immunizations with virus mixtures yielded a number of mAbs directed toward epitopes shared among distantly related influenza virus types that reacted against a broad range of avian and human viruses (**Fig. 3**). Biochemical analysis showed that most of the mAbs displaying the highest broad-range reactivity were directed against glycosyl residues (**Supplementary Fig. 4**). We also obtained a number of highly specific mAbs that selectively recognized type- and subtype-specific virus proteins, including nucleoprotein (**Fig. 4**), hemagglutinin and neuroaminidase (**Fig. 5**). These findings show that the protocol described here, which combines somatic hybrid

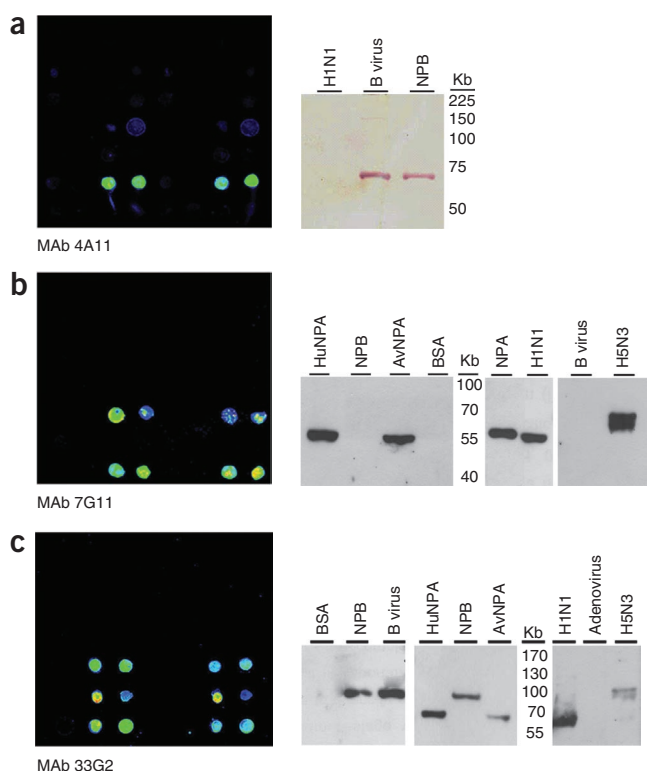
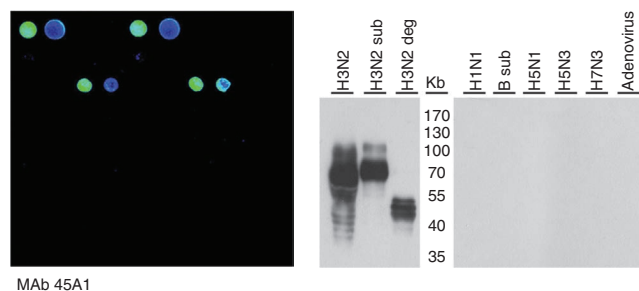


Figure 4 | Reactivity analysis of mAbs 4A11, 7G11 and 33G2 (see **Supplementary Table 1**). The specificity of the antibodies against different epitopes of avian and human influenza A and B viruses was deduced on the basis of their unique microarray profiles and confirmed in immunoblot. The mAbs 4A11 and 7G11 selectively recognize (a) NPB and (b) NPA, respectively, whereas (c) 33G2 is able to selectively recognize both NPB and NPA. Spots containing influenza viruses in the microarray assays were not detected by anti-NP mAbs, as viruses were printed as nonpermeabilized whole-virus solution, making NPs not accessible to antibodies. Recombinant NPs and BSA were loaded in a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with mAbs; NP, nucleoprotein.

Figure 5 | Identification of virus-specific antibodies. The microarray (left panel) and immunoblot (right panel) analysis demonstrate that mAb 45A1 (see **Supplementary Table 1**) selectively recognizes only the A-subtype virus H3N2. The immunoblot reactivity with the purified hemagglutinin (HA) and neuroaminidase (NA) fraction of the H3N2 virus (H3N2 sub) and the molecular weight shift observed after deglycosylation of the H3N2 virus suggest that this mAb reacts with either HA or NA. H3N2 or B sub, purified HA and NA fraction of the virus; H3N2 deg, influenza virus deglycosylated using the Enzymatic Protein Deglycosylation Kit (Sigma-Aldrich).



fusion for mAb production and microarray immunoassay for antibody screening, has the throughput and ease of use to be successfully used in the development of mAb libraries directed against a vast repertoire of antigens.

Note: Supplementary information is available via the HTML version of this article.

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