Fluorescence correlation spectroscopy as a sensitive and useful tool for revealing potential overlaps between the epitopes of monoclonal antibodies on viral particles

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ABSTRACT
Although the enzyme-linked immunosorbent assay (ELISA) is well established for quantitating epitopes on inactivated virions used as vaccines, it is less suited for detecting potential overlaps between the epitopes recognized by different antibodies raised against the virions. We used fluorescence correlation spectroscopy (FCS) to detect the potential overlaps between 3 monoclonal antibodies (mAbs 4B7-1H8-2E10, 1E3-3G4, 4H8-3A12-2D3) selected for their ability to specifically recognize poliovirus type 3. Competition of the Alexa488-labeled mAbs with non-labeled mAbs revealed that mAbs 4B7-1H8-2E10 and 4H8-3A12-2D3 compete strongly for their binding sites on the virions, suggesting an important overlap of their epitopes. This was confirmed by the cryo-electron microscopy (cryo EM) structure of the poliovirus type 3 complexed with the corresponding antigen-binding fragments (Fabs) of the mAbs, which revealed that Fabs 4B7-1H8-2E10 and 4H8-3A12-2D3 epitopes share common amino acids. In contrast, a less efficient competition between mAb 1E3-3G4 and mAb 4H8-3A12-2D3 was observed by FCS, and there was no competition between mAbs 1E3-3G4 and 4B7-1H8-2E10. The Fab 1E3-3G4 epitope was found by cryoEM to be close to but distinct from the epitopes of both Fabs 4H8-3A12-2D3 and 4B7-1H8-2E10. Therefore, the FCS data additionally suggest that mAbs 4H8-3A12-2D3 and 4B7-1H8-2E10 bind in a different orientation to their epitopes, so that only the former sterically clashes with the mAb 1E3-3G4 bound to its epitope. Our results demonstrate that FCS can be a highly sensitive and useful tool for assessing the potential overlap of mAbs on viral particles.

Introduction
Despite worldwide vaccination since the 1950s, poliovirus is still not eradicated. The use of 2 different vaccines, inactivated poliovirus vaccine (IPV) and oral poliovirus vaccine (OPV), developed by Salk et al. and Sabin, respectively, has dramatically decreased the number of paralytic poliomyelitis cases. As a result of its safety profile, IPV has been in use in many countries to control polio, while OPV has been the vaccine of choice in many others during the last years. Polioviruses are composed of a single-strand RNA genome encapsulated in an icosahedral protein capsid. These small non-enveloped and spherical (300 Å in diameter) viruses can be divided into 3 distinct serotypes (1 to 3). Polioviruses, like all enteroviruses, are pH stable, remain active after crossing the stomach, and reach the intestine where they replicate in the mucosal cells. In some cases following viremia polioviruses are able to destroy lower motor neurons, leading to paralysis without permanent sensory loss. The poliovirus capsid encapsidates a 7-kb RNA genome of positive strand polarity, and is characterized by 60 copies of 4 viral polypeptides named VP1, VP2, VP3, and VP4, assembled in an icosahedrally symmetric geometry. The crystallographic structures of all serotypes have been characterized since 1985 and structural differences among them have been highlighted, especially in the loop regions of the viral capsid proteins. Overall antigenicity is quantified by the D-antigen assay, which induces protective antibodies. The antigenic structure of polioviruses consists of at least 4 different antigenic sites. Overall antigenicity is quantified by the D-antigen assay, which is used to assess the potency of IPV vaccines. Several ELISA-based methods have been developed to measure the D-antigen content using polyclonal or monoclonal (mAbs) antibodies, but these methods cannot reliably detect epitope overlaps. To address this question, fluorescence correlation spectroscopy (FCS) could be used. This technique is based on the analysis of fluorescence intensity fluctuations in the small observation (femtoliter) volume of a confocal or a 2-photon microscope. Analysis of these fluctuations determines the diffusion coefficients of the different fluorescent species in solution. In the case of freely diffusing species, diffusion rates are inversely proportional to the size of the tagged species. Because antibodies are 5-8 times smaller than the polivirus, the
diffusion of antibodies bound to polioviruses is expected to be significantly slower than that of free antibodies, enabling bound and free antibodies to be easily discriminated. FCS also quantifies the average number, and thus the concentration of each fluorescent species in the observation volume. By detecting changes in the relative concentrations of bound and free species, FCS should therefore be suited to detect competition between different mAbs for overlapping epitopes.

We used FCS to determine through competition experiments the potential overlap of epitopes recognized by 3 specific mAbs against poliovirus type 3. Moreover, as recently performed with polioviruses type 1 and 2, cryo-electron microscopy (CryoEM) and 3-dimentional (3D) reconstruction techniques were used to map the epitopes of poliovirus type 3 that are recognized by the antigen-binding fragments (Fabs) of the 3 mAbs, in order to validate the overlap between epitopes suggested by FCS.

Results

This study was performed with 3 mAbs (1E3-3G4, 4B7-1H8-2E10 and 4H8-3A12-2D3) directed against wild-type poliovirus type 3 (Saukett strain). These mAbs were shown by surface plasmon resonance (SPR) to exhibit high functional affinity (kd < 200 μM), as well as a high neutralizing power. The mAb 1050, an anti-type 2 poliovirus (MEF-I), showing no specific affinity for type 3 poliovirus, was used as a negative control. In a first attempt, we have tried to evidence possible overlap between mAb epitopes by SPR technology. The results were not convincing because it was difficult, under SPR conditions, to saturate the virus surface with one mAb before the injection of the second mAb.

Binding of the monoclonal antibodies to type 3 poliovirus, as monitored by fluorescence correlation spectroscopy

To evidence possible overlaps between the virus surface epitopes of different mAbs, fluorescence correlation spectroscopy (FCS) measurements were performed to monitor the competition between the different mAbs for their epitopes. The FCS technique is based on the analysis of the fluorescence intensity fluctuations within the small focal volume (femtoliter) defined by a confocal or a 2-photon microscope. These fluctuations are mainly due to the diffusion of the fluorescent species through the focal volume. Since mAbs and polioviruses are 150 kDa and 8 MDa, respectively, we labeled the smallest partner, i.e., the mAb, with Alexa488 to monitor its binding to the poliovirus. The three Alexa488-labeled mAbs specific to poliovirus type 3 and the Alexa488-labeled mAb 1050 control in their free form showed similar autocorrelation curves (Fig. 1). The maximum entropy method (MEM) fitting, which makes no a priori assumption of the number of species in solution, suggested the presence of one major species in solution with less than 10% of residual free dyes (Fig. S1), but only with freshly labeled and purified mAbs. Noticeably, after 2 days of storage, the percentage of free dyes increased above 20%, suggesting that dyes were likely released from the mAbs to which they were adsorbed. Thus, experiments were done only with mAbs labeled and purified on the day of the experiment.

Similar values of diffusion coefficients were obtained for the different mAbs using equation 1 of the Materials and Methods section (Table 1). Assuming that mAbs had a spherical shape, a hydrodynamic radius ( Rh) of 3.5 ±/− 0.3 nm could be inferred for the labeled mAbs, in line with the literature and the theoretical Rh value (3.5 nm) calculated from its molecular weight (~150 kDa) according to equation 5. Moreover, the average number of fluorescent particles in the focal volume (N) deduced from the autocorrelation curves fitted by equation 1 was found to be in excellent agreement (less than 10% difference) with the theoretical value calculated from the concentration of mAbs determined by absorbance measurements.

After adding poliovirus type 3 particles at a molar ratio of 10:1 (mAbvirus) to the labeled mAbs, the MEM analysis of the autocorrelation curves indicated the presence of a single species in all cases (Fig. S1). However, while a significant increase in the diffusion time compared to free mAbs was observed with mAbs 1E3-3G4, 4B7-1H8-2E10 and 4H8-3A12-2D3, no change in diffusion time was observed for mAb 1050, which was used as a negative control. By fitting the autocorrelation curves (Fig. 1a and b) with equation 1, diffusion coefficients of 12–16 μm/s were calculated for the 3 mAbs (mAb 1E3-3G4, 4B7-1H8-2E10 and 4H8-3A12-2D3) specific to poliovirus type 3 (Table 1). These diffusion constants correspond to a hydrodynamic radius of 16+/−3 nm, in good agreement with the 17.5 nm hydrodynamic radius of the poliovirus type 3 (Saukett strain) measured by dynamic light scattering and the 14 nm value calculated for a globular protein of 9.5 MDa (8 MDa for the viral particle and 10 times 150 kDa for mAbs). The change in the diffusion time was accompanied by a nearly 10-fold increase in the G(0) value (Fig. 1a) and brightness (data not shown), indicating a 10-fold decrease in the number of diffusing species. The changes in the diffusion constants together with the changes in the number of diffusing species and their brightness indicate that all mAbs are bound to the viral particles in these conditions, in line with the high affinity of the mAbs for the viral particles and the sub-saturating concentrations of mAbs used. In contrast, the absence of change in the diffusion time and number of diffusing particles for mAb 1050 indicates that, as expected, this antibody does not interact with the poliovirus type 3. Thus, our data show that the FCS approach is a valid tool to monitor interactions between the poliovirus type 3 and mAbs targeting it in solution.

In a next step, our objective was to check whether the antibodies can bind simultaneously to their epitopes or compete with each other. To this aim, we monitored by FCS the binding of one of the labeled mAbs to the poliovirus type 3 particles pre-incubated with one of the other antibodies in its non-labeled form. To perform this competition, we first attempted to determine appropriate experimental conditions. One major concern in FCS measurements is the presence of fluorescent aggregates that result in large fluorescent spikes that distort the autocorrelation curves. To see whether aggregates could form, we incubated the mAbs with the polioviruses at ratios varying from 1:1 to 400:1. As the poliovirus is characterized by 60 copies of 4 viral polypeptides, a fluorescent spike 60 times brighter than a single free labeled mAb necessarily comes from an aggregate. For the different ratios, the percentage of photon counting traces with aggregates was determined (Table S1). At
low ratios (≤ 20:1 mAb:virus), no aggregate was detected. Aggregates could be perceived in a few curves at a mAb:virus ratio of 40. At mAb:virus ratios > 100, numerous aggregates appeared, which prevented the analysis of the autocorrelation curves. Therefore, competition experiments were performed, taking care that the total amount of antibodies added to the
Table 1. Diffusion coefficients of free mAbs and their complexes with the poliovirus.

| mAbs          | Diffusion coefficients (μm² s⁻¹) |
|---------------|---------------------------------|
|               | Free mAbs | mAb: Poliovirus (ratio 1:1) |
| 1E3-3G4       | 57 ± 3    | 12 ± 2                     |
| 4H8-3A12-2D3  | 58 ± 8    | 16 ± 4                     |
| 4B7-1H8-2E10  | 60 ± 3    | 12 ± 4                     |
| 1050          | 61 ± 3    | 50 ± 20                    |

*The diffusion constants were obtained from the autocorrelation curves in Fig. 1. The values are expressed as mean ± standard error of the mean for a triplicate of 200 autocorrelation curves.

virus particles should not exceed a ratio of 30:1. Accordingly, the poliovirus (2.5 nM) was pre-incubated with a 20-fold excess of non-labeled mAbs 1E3-3G4/4H8-3A12-2D3/4B7-1H8-2E10 or mAb 1050 antibodies and challenged with an Alexa488-labeled antibody added at a 10:1 (mAb: virus) ratio.

As expected, pre-incubation of the poliovirus particles with non-labeled mAb 1050 was found to only marginally alter the autocorrelation curves recorded with the Alexa488-labeled mAb 4H8-3A12-2D3 (Fig. 2a and b, compare pink and red curves). This further confirms that the mAb 1050 antibody does not bind to the poliovirus particle, and thus does not compete with mAb 4H8-3A12-2D3. In sharp contrast, pre-incubation of the particles with non-labeled mAb 4H8-3A12-2D3 was found to strongly reduce the binding of the same antibody in its labeled form, as could be seen by the strong decrease in the diffusion time and the G(0) value (Fig. 2a and b, compare green and red curves). In fact, the autocorrelation curve in the presence of non-labeled mAb 4H8-3A12-2D3 became comparable to that of the free labeled mAb 4H8-3A12-2D3 in the absence of viral particles (Fig. 2a and b, black dotted curves). As the G(0) value was somewhat higher than the autocorrelation curve of the free mAb (Fig. 2a, inset), a fraction of the labeled mAb is still bound to the viral particles. To estimate this fraction, we fitted the autocorrelation curve with equation 2 of the Materials and Methods section. This fraction was found to be of 22 +/− 15% (with a value of 1.2 for Q, the relative brightness of the complex compared to the free mAb), compared to 95 +/− 5% (with Q = 10) for the autocorrelation curves of the same labeled mAbs in the absence of competing mAbs or in the presence of mAb 1050 fitted by equation 2. This strong competition between non-labeled and labeled mAb 4H8-3A12-2D3 antibodies for their epitopes on the poliovirus type 3 particles suggests that the number of antibodies that can bind to the virus may be significantly less than the theoretical number (60) of epitopes on the virus. A comparable effect was observed when the binding of mAb 4H8-3A12-2D3 was monitored in the presence of non-labeled mAb 4B7-1H8-2E10 added at a 20:1 mAb: virus ratio to the poliovirus. Indeed, we observed a strong decrease in both the diffusion times and the G(0) values (Fig. 2a and b, compare yellow and red curves), so that the autocorrelation curve became very similar to that obtained with the free mAb 4H8-3A12-2D3 in the absence of virus (Fig. 2a and b, black curves). In this case, the final fraction of bound mAb 4H8-3A12-2D3 (18 +/− 12% with Q = 2) is similar to that obtained in the presence of non-labeled mAb 4H8-3A12-2D3, clearly indicating that the epitopes of the 2 mAbs strongly overlap. A significant effect on the autocorrelation was also observed when the virus particles were pre-incubated with a 20:1 mAb virus ratio of non-labeled mAb 1E3-3G4 antibody (Fig. 2a and b, compare blue and red curves). Though the effect was less pronounced than with mAb 4B7-1H8-2E10, the significant decrease in the G(0) value clearly indicated a decrease in the number of bound mAb 4H8-3A12-2D3 in the presence of mAb 1E3-3G4. Using equation 2, the fraction of bound mAb 4H8-3A12-2D3 was found to drop to 73 +/− 10% (with Q = 4.2), suggesting that the binding of mAb 1E3-3G4 to its epitope does not prevent but reduces the access of the mAb 4H8-3A12-2D3 to its epitope.

To confirm our conclusions on the overlap between the epitopes, the FCS experiments were repeated by replacing the labeled mAb 4H8-3A12-2D3 by the Alexa488-labeled mAb 4B7-1H8-2E10 (Fig. S2 in Supplementary data). As expected, addition of an excess of non-labeled mAb 4H8-3A12-2D3 or 4B7-1H8-2E10 resulted in autocorrelation curves that were similar to that of the labeled mAb 4B7-1H8-2E10 in its free form, clearly confirming an overlap between the epitopes of the 2 mAbs. Using equation 2, the fraction of bound labeled mAb 4B7-1H8-2E10 was below 10% (with Q = 1). In contrast, the autocorrelation curve of the labeled mAb 4B7-1H8-2E10 added to viral particles incubated with non-labeled mAb 1E3-3G4 showed a shift toward longer correlation times together with a limited change in the G(0) value, indicating that the 2 mAbs can bind together to the same viral particles, and thus that the overlap between their epitopes is marginal.

To substantiate the observations by FCS on the overlap between the mAbs epitopes, CryoEM structures of the complexes of the poliovirus with the corresponding Fab fragments of the 3 monoclonal antibodies were solved to clearly identify the binding sites of the 3 mAbs.

CryoEM structure of poliovirus type 3 complexed to Fabs

The 3D reconstruction volume was obtained by refining an initial 3D model calculated from the atomic structure of the type 3 Polio virus (1pvc code) provided in the Protein Data Bank (PDB). No protein modeling was required because the virus vaccine sequence presents an identity level on VP1, VP2 and VP3 higher than 98.3% with the sequence of the virus used in the PDB. The structure was first rotated so that its axis of symmetry of order 5 is aligned with the z axis. The structure was then filtered with a low-pass filter of 20 Å resolution. The density range volumes of the reference Type 3 polio virus and the immune complex of the virus + Fabs were normalized, and a reduced resolution low-pass filtering corresponding to a resolution of 15 Å was applied to the reference Type 3 polio virus. Subtraction volume was performed on both virus and virus + Fab corrected volumes. The resulting 3D density map corresponds to the contribution of the Fab immune complex. Noise treatment (band pass filter) was applied to the resulting volume, which was then colorized and superimposed on the reference volume of the Type 3 polio virus. An isosurface or transparent mode (mesh) visualization using the Chimera software clearly shows the position of the Fabs 4H8-3A12-2D3, 1E3-3G4 and 4B7-1H8-2E10 on the surface of the type 3 poliovirus capsid (Fig. 3).
The resolutions obtained for each reconstruction either for the free poliovirus or the poliovirus complexed to Fab are better than those obtained for the type 1 poliovirus\textsuperscript{21} and the type 2 poliovirus.\textsuperscript{20} Noticeably, the estimated radius of the viral particle alone is 12.5 nm, so that the radius of the virus coated by mAbs should be 19.5 nm (12.5 nm +2 × 3.5 nm),

**Figure 2.** Competition of mAbs 1E3-3G4, 4H8-3A12-2D3, 4B7-1H8-2E10 and 1050 for the binding of Alexa488-labeled mAb 4H8-3A12-2D3 to poliovirus particles, as monitored by Fluorescence Correlation Spectroscopy. Raw (a) and normalized (b) autocorrelation curves of Alexa488-labeled mAb 4H8-3A12-2D3 (25 nM) added to 2.5 nM poliovirus particles in the absence (red) or in the presence of 50 nM non-labeled mAb 1050 (pink), 1E3-3G4 (blue), 4H8-3A12-2D3 (green) and 4B7-1H8-2E10 (yellow). The FCS curve of free Alexa488-labeled mAb 4H8-3A12-2D3 (50 nM) is given for comparison (black curve). The raw data were fitted using equation 1 or 2 (solid lines) of the Materials and Methods section, as described in the text. The inset in Fig. 2a is a zoom of Fig. 2a to highlight the autocorrelation curves having a large fraction of free mAbs. Experiments were performed in PBS buffer 10 mM, NaCl 150 mM, pH 7.4 at 20°C.
in good agreement with the 16+/−3 nm hydrodynamic radius measured by FCS. The reconstructions of the complexes with the 3 Fabs (Fig. 3b–d) revealed 5 extra densities around each 5-fold axis compared with the virus particles alone (Fig. 3a). These extra densities represent the Fabs. As five Fabs are facing each other across the icosahedral 5-fold axis, a total of 60 Fabs are bound to the virus particles, indicating a much lower degree of steric hindrance between bound Fabs, compared to mAbs.

To further map the epitopes, we zoomed on the Fab/virus junctions from the superimposition images shown in Fig. 3, which allowed us identifying the core amino acids probably involved in the Fab epitopes on the virus. The residues exhibiting the highest probability of contact with the Fabs were selected as described previously20,21 and zoomed in Fig. 4.

For all selected epitopes, the sequences of the vaccine strain and the strain used for the 3D structure in the PDB are identical. From Fig. 4, it is clearly seen that the Fabs 4H8-3A12-2D3 and 4B7-1H8-2E10 recognize the same loop on VP1, so that the epitopes for these 2 mAbs are largely overlapping. This fully rationalizes the mutual competition between the 2 mAbs observed by FCS. In contrast, the 1E3-3G4 epitope appears independent from the 2 other ones, but nevertheless in close proximity to them as further indicated in Fig. 5. As the mAbs are much bulkier than their corresponding Fabs, the close proximity of the epitopes of mAbs 4H8-3A12-2D3 and 1E3-3G4 can easily rationalize the competition between the 2 mAbs observed by FCS. Moreover, in contrast to the strong overlap of the mAbs 4H8-3A12-2D3 and 4B7-1H8-2E10 epitopes, which clearly prevents any simultaneous binding of the 2 mAbs on their overlapping epitopes, there may be only a steric hindrance between mAbs 4H8-3A12-2D3 and 1E3-3G4 with a mutual decrease of their binding constants on 2 proximal sites, but not a full exclusion. Finally, the absence of competition observed by FCS between mAbs 4B7-1H8-2E10 and 1E3-3G4 suggests that the 2 mAbs can bind simultaneously to their proximal epitopes with limited steric hindrance. This further suggests that mAbs 4H8-3A12-2D3 and 4B7-1H8-2E10 likely bind with different orientations on their respective epitopes, so that a steric clash with mAb 1E3-3G4 appears only with the former one. The difference in orientation between mAbs 4H8-3A12-2D3 and 4B7-1H8-2E10 on the viral particle is in line with the nearly perpendicular orientation of their respective epitopes (Fig. 5).

Figure 3. CryoEM structures of Type 3 Polio Virus in the free form (a) or complexed to Fab 4H8-3A12-2D3 (b), Fab 1E3-3G4 (c) or Fab 4B7-1H8-2E10 (d). The final resolutions were 13.2 Å, 14.6 Å, 14.1 Å and 13.9 Å for a–d, respectively.

Figure 4. Epitope mapping. The 4H8-3A12-2D3, 1E3-3G4, and 4B7-1H8-2E10 Fabs are in orange, pink and cyan, respectively. Otherwise, VP1 is shown in red, VP2 in blue and VP3 in yellow. Amino acid residues of the epitopes are highlighted.
In this study, FCS was used to monitor the binding of 3 specific monoclonal antibodies (mAbs 1E3-3G4, 4B7-1H8-2E10 and 4H8-3A12-2D3) selected by SPR for their ability to specifically recognize poliovirus type 3 with high affinity. FCS clearly demonstrated the mAbs’ ability to bind to poliovirus in diluted solution, but, due to mAb-induced aggregation of viruses at mAb/virus ratios >30, it was unable to unambiguously quantify the total number of binding sites on the virus. This aggregation is likely connected both to the cross-link of virions as a result of the presence of 2 binding sites per mAb and the probable solubility loss of the viral particles when they are coated by multiple mAbs. Furthermore, competition experiments showed strong competition between mAbs 4B7-1H8-2E10 and 4H8-3A12-2D3, which is consistent with the strong overlap of the epitopes of their Fabbs. In particular, CryoEM showed that they notably share the same VP1 loop. FCS also showed less competition between mAbs 4H8-3A12-2D3 and 1E3-3G4, and marginal competition between mAbs 4B7-1H8-2E10 and 1E3-3G4. These observations were less easy to predict from the CryoEM data showing that the Fab 1E3-3G4 epitope was close to, but distinct from, the epitopes of both Fabbs 4H8-3A12-2D3 and 4B7-1H8-2E10 (Fig. 5). Therefore, the FCS data suggest that mAbs 4H8-3A12-2D3 and 4B7-1H8-2E10 bind in a different orientation to their epitopes, so that only the former sterically clashes with the mAb 1E3-3G4 bound to its epitope.

Taken together, our data show that FCS constitutes a unique tool for assessing epitope overlap between mAbs raised against a viral particle. This technique exhibits several distinctive and unique advantages. Indeed, FCS measurements are much faster (they require a few hours) and less tedious than structural techniques such as CryoEM. Additionally, in contrast to ELISA and SPR techniques, FCS does not require one of the binding partners to be immobilized on a surface, which can perturb binding. One limitation is that FCS requires fluorescent labeling of at least one mAb, but this can be easily achieved with commercial kits. Moreover, FCS will probably not be able to discriminate a true overlap of epitopes with the case where epitopes are within distances comparable with the dimensions of the mAb molecules. Another limitation is the sensitivity of the technique to aggregation, which necessitates preliminary experiments to determine the mAb:virion ratio not to be exceeded in competition experiments.

**Discussion**

FCS measurements were performed on a home-built multichannel setup based on an Olympus IX70 inverted microscope thermostated at 25°C. The excitation light at 780 nm was provided by a mode-locked femtosecond laser (Insight, Spectra Physics) delivering 100 fs pulses at 80 MHz frequency. The laser beam was focused by a 60× (NA = 1.2) water immersion objective (Olympus). The excitation power was about 2 mW at the sample. This condition was found to provide an optimal signal/noise ratio and minimize the probe photobleaching.

Emitted fluorescence was filtered using a short-pass filter with a cutoff wavelength of 680 nm (F75–680; AHF, Germany). A second dichroic (LP 585, Chroma) was used to split the emission in green (BP 525–39, Semrock) and red channels (BP 660–52, Semrock). For both channels, fluorescence was directed to a fiber-coupled APD (SPCM-AQR-14-FC; Perkin Elmer), and the normalized autocorrelation curve was calculated on-line with a hardware correlator (ALV5000, ALV GmbH, Germany). Multiple FCS runs of short duration (5 s) were performed on solutions of mAbs tagged with AlexaFluor 488 in the absence and in the presence of poliovirus particles. For each condition, 100 acquisitions of 5 seconds duration were performed after 15 min equilibration in 96-well plates. Using the QuickFit 3.0 software, a first analysis was done on the mean curve of all autocorrelation curves by MEM to determine without a priori

**Materials and methods**

**Materials**

Wild-type inactivated poliovirus type 3 (Saukett strain) (IPV3) was provided by the Production Department of Sanofi Pasteur. Purified anti-type 3 Poliovirus (Saukett strain) mAbs 4B7-1H8-2E10, 1E3-3G4, 4H8-3A12-2D3 were obtained from Biotem (France). These IgG mAbs have been generated by mice immunization with inactivated poliovirus type 3 (Saukett strain). All three mAbs neutralize the Saukett type 3 strain (data not shown). As a negative control, one anti-type 2 wild-type poliovirus (MEF-I strain) mAb 1050 obtained from the National Institute for Biological Standards and Control (NIBSC) was used. The generation and characterization of this mAb have been reported by Minor et al. Corresponding Fabbs were produced as previously described. The mAbs were labeled with AlexaFluor 488 using Antibody Labeling Kits (Life technologies). The concentration and the degree of labeling of the labeled mAbs were determined by UV-vis absorption using a Cary 4000 (Varian) spectrophotometer according to the labeling kits manufacturer’s instructions.
the number of diffusing species in solution. Then, all autocorrelation curves were analyzed independently on a home-made Matlab program, using the standard 3D diffusion model with one (Eq. 1) or 2 (Eq. 2) diffusing species depending on the MEM results:

\[
G(t) = \frac{1}{N} \times \left[ \frac{1}{1 + \frac{t}{\tau_{D}}} \times \frac{1}{\sqrt{1 + \frac{t}{s^2 \times \tau_{D}}}} \right]
\]

(1)

\[
G(t) = \left( \frac{1}{N_1 + Q \times N_2} \right)^2 \times \left( N_1 \frac{1}{1 + \frac{t}{\tau_{D}}} \times \frac{1}{\sqrt{1 + \frac{t}{s^2 \times \tau_{D}}}} \right)
+ \left( N_2 \times Q^2 \frac{1}{1 + \frac{t}{\tau_{D}}} \times \frac{1}{\sqrt{1 + \frac{t}{s^2 \times \tau_{D}}}} \right)
\]

(2)

where \( N, N_1 \) and \( N_2 \) are the average numbers of fluorescent species diffusing through the excitation volume, \( Q \) is the relative brightness of species 2 versus species 1 (the estimation of \( Q \) value is detailed in SI), \( \tau \) is the lag time, \( \tau_{D1}, \tau_{D2} \) and \( \tau_{D} \) are the average diffusion times of the fluorescent species in the focal volume and \( s \) is the ratio of the axial to lateral radii of the excitation volume. The experimental curves were analyzed by using the Global-analysis approach. The fit parameters were obtained using the Marquardt-Levenberg nonlinear least squares method. The diffusion coefficients \( D \) were calculated from the diffusion time (\( \tau_{D} \)) and the lateral radius (\( \omega_{XY} \)) of the focal volume:

\[
D = \frac{\omega_{XY}^2}{4 \tau_{D}}
\]

(3)

Assuming a spherical shape, the hydrodynamic radius (\( R_h \)) of a diffusing species was calculated by:

\[
R_h = \frac{kT}{6\pi\eta D}
\]

(4)

where \( k \) is the Boltzmann constant, \( T \) is the temperature and \( \eta \) is the viscosity of the solvent. This value can be compared to the theoretical \( R_h \) value calculated from the expected molecular weight by the relation:

\[
R_h = \sqrt{\frac{3 \times MW \times \varphi}{4\pi}}
\]

(5)

with a specific gravity \( \varphi \) of 0.74 cm\(^3\)g\(^{-1}\).

The focal volume (\( \omega_{XY} \) and \( s \)) was calibrated each day prior to experiments using a 50 nM solution of tetramethylrhodamine (\( D = 421 \mu m^2/s\)). Values of 300 nm and 3.8 were typically obtained for \( \omega_{XY} \) and \( s \), respectively. Using the focal volume determined as described above and the obtained average number of fluorescent species (\( N \) with eq. 1 or \( N_1+N_2 \) with eq. 2), the concentration of fluorescent species can thus be deduced. This parameter was obtained by dividing the average fluorescence intensity by the average number (\( N \)) of fluorescent species in the focal volume for each individual curve (5 s acquisition) or through a fit of the autocorrelation curves to eq. 2.

**Cryo-electron microscopy**

All observations were carried out on a JEOL JEM-2100F electron microscope at 200 kV with a magnification of x50,000 and following a dose of approximately 10 electrons per Angström square per second. The digitization of selected negative films was performed using a Nikon Coolscan 9000ED film scanner, using a density of 2880 DPI (100 dots per mm), which corresponds to a pixel size of 2.4 Å.

**Particles selection**

Best micrographs were used for selection of individual particles using the EMAN software. The coordinates of each particle (the upper left corner of the window size of 300 pixels x 300 pixels used for the extraction of particles) were saved in EMAN format and then converted to readable SPIDER format. In total, this selection produced images of individual particles. Each individual image has been normalized so that the average of the dynamic range of the image is zero and the standard deviation is 1.

**Optical characterization of selected images**

CTFit package was used to estimate the maximum and minimum focal values for each individual particle. For a fast analysis, we reduce the size of micrographs by 2 in each dimension; the size of the window for calculating the power spectrum was 256 × 256 pixels. The difference between the maximum and minimum values of focal images was calculated for each particle, and images whose astigmatism was greater than 1000 Å were rejected from analysis. Remaining particles (having astigmatism lower than 1000 Å) were classified into sub-focal groups of 800 Å and used for 3D reconstruction method that corrects the contrast transfer function of the electron microscope by collecting a series of volumes calculated from a focal series of images. As the volumes of the series should have similar resolutions, focal groups with less than 60 particles were not taken into account for the reconstruction.

**Three dimensional reconstruction of single particle specimens using reference projections with defocus groups**

Once a set of particle images has been obtained, an initial 3D reconstruction was calculated using coarse projection angles. This was followed by refinement, which iteratively adjusted the angles for finer resolution. Reference 3D reconstruction volume was obtained by refining an initial 3D model calculated from the atomic structure of the Poliovirus type 3 provided in the PDB. The PDB (1pvc code) structure was used in the present study as the 3D structure for the vaccine poliovirus type 3. No protein modeling was required as the vaccine sequence presents
The angular refinement was done (SPIDER image processing package) using the method of experimental images alignment with the projections of the 3D model of the particle calculated at the previous iteration (3D reference). The directions of projections are uniformly distributed on a sphere, but we reduced the space between the directions (i.e., the discretization of guidance parameters) iteratively. For the first 4 iterations, the step size was 3°. Four subsequent iterations were made with a step of 2.5°. From iteration 9 to 12, the step size was 2°, and from iteration 13, the step was 1.5°. At each iteration, the computed 3D model has been masked in order to serve as a new 3D reference in the next iteration. The mask was calculated by binarization of the 3D reference used in the first iteration followed by a low-pass filter to smooth the edges. For rapid refinement, each iteration reconstruction was made using interpolation in the Fourier space. The icosahedral symmetry was imposed during the reconstruction. In this way, the resolution was first improved by 20 Å (first iteration) to 15 Å (14th iteration). Then, a band pass filter was applied to the 3D reference from iteration 16 to give more importance to the alignment details. The band pass filter was composed of 2 filters: low pass 11.5 Å and high pass 25.5 Å. With this filter, the resolution was refined below 14–15 Å at iteration 18. The resolution could not be improved in subsequent iterations.

**References**

1. Cochi SL, Hegg L, Kaur A, Pandak C, Jafari H. The Global Polio Eradication Initiative: Progress, Lessons Learned, And Polio Legacy Transition Planning. Health Aff (Millwood) 2016; 35:277-83; PMID:26858381; http://dx.doi.org/10.1377/hlthaff.2015.1104
2. Salk JE, Krech U, Youngner JS, Bennett BL, Lewis LJ, Bazeley PL. Formaldehyde treatment and safety testing of experimental poliomylitis vaccines. Am J Public Health Nations Health 1954; 44:563-70; PMID:13148396; http://dx.doi.org/10.2105/AJPH.44.5.563
3. Sabin AB. Properties and behavior of orally administered attenuated poliovirus vaccine. J Am Med Assoc 1957; 164:1216-23; PMID:13438685; http://dx.doi.org/10.1001/jama.1957.62980110080008
4. Chumakov K, Ehrenfeld E. New generation of inactivated poliovirus vaccines for universal immunization after eradication of poliomyelitis. Clin Infect Dis 2008; 47:1587-92; PMID:18990066; http://dx.doi.org/10.1086/593310
5. Racaniello VR. Picornaviroidae: The Viruses and Their Replication. In: Lippincott-Raven, ed. Fields virology, 2013:685-722
6. Minor PD, Ferguson M, Evans DM, Almond JW, Icenogle JP. Antigenic structure of polioviruses of serotypes 1, 2 and 3. J Gen Virol 1986; 67:1283-91; PMID:2425046; http://dx.doi.org/10.1099/0022-1317-67-7-1283
7. Melnick JL. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Lippincott-Raven, ed. Lippincott-Raven, 1996:655-712
8. Nathanson N. The pathogenesis of poliomyelitis: what we don’t know. Adv Virus Res 2008; 71:1-50; PMID:18585526; http://dx.doi.org/10.1016/S0065-3527(08)00001-8
9. Filman DJ, Syed R, Chow M, Macadam AJ, Minor PD, Hogle JM. Structural factors that control conformational transitions and serotype specificity in type 3 poliovirus. EMBO J 1989; 8:1567-79; PMID:2548847
10. Hogle JM, Chow M, Filman DJ. Three-dimensional structure of poliovirus at 2.9 A resolution. Science 1985; 229:1358-65; PMID:2994218; http://dx.doi.org/10.1126/science.2994218
11. Ferguson M, Wood DJ, Minor PD. Antigenic structure of poliovirus in inactivated vaccines. J Gen Virol 1993; 74:685-90; PMID:7682250; http://dx.doi.org/10.1099/0022-1317-74-4-685
12. Minor PD. Antigenic structure of picornaviruses. Curr Top Microbiol Immunol 1990; 161:121-54; PMID:2169382; http://dx.doi.org/10.1007/978-3-642-75602-3_5
13. Rezapkin G, Martin J, Chumakov K. Analysis of antigenic profiles of inactivated poliovirus vaccine and vaccine-derived polioviruses by block-ELISA method. Biologicals 2005; 33:29-39; PMID:15713554; http://dx.doi.org/10.1016/j.biologicals.2004.11.001
14. Sawyer LA, McInnis J, Albrecht P. Quantitation of D-Antigen Content in Inactivated Poliovirus Vaccine Derived from Wild-Type or Sabin Strains. Biologicals 1993; 21:169-77; PMID:8297601; http://dx.doi.org/10.1006/biol.1993.1070
15. Sawyer LA, Wood D, Ferguson M, Crainic R, Beuvery EC, McInnis J, Albrecht P. Potency of wild-type or sabin trivalent inactivated poliovirus vaccine, by enzyme-linked immunosorbent assay using monoclonal antibodies specific for each antigenic site. Biologicals 1997; 25:299-306; PMID:9324998; http://dx.doi.org/10.1006/biol.1997.0100
16. van der Marel P, Hazendonk AG, van Wezel AL. D-antigen determination in polio vaccine production: comparison of gel diffusion and ELISA methods. Dev Biol Stand 1981; 47:101-8; PMID:6262140
17. Magde D, Elson E, Webb WW. Thermodynamic Fluctuations in a Reacting System: Measurement by Fluorescence Correlation Spectroscopy. Physical Rev Letters 1972; 29:705-8; http://dx.doi.org/10.1103/PhysRevLett.29.705

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.
18. Berland KM, So PT, Gratton E. Two-photon fluorescence correlation spectroscopy: method and application to the intracellular environment. Biophys J 1995; 68:694-701; PMID:7696520; http://dx.doi.org/10.1016/S0006-3495(95)80230-4
19. Haustein E, Schwille P. Fluorescence correlation spectroscopy: novel variations of an established technique. Annu Rev Biophys Biomol Struct 2007; 36:151-69; PMID:17477838; http://dx.doi.org/10.1146/annurev.biophys.36.040306.132612
20. Bannwarth L, Girerd-Chambaz Y, Arteni A, Guigner JM, Ronzon F, Manin C, Venien-Bryan C. Mapping of the epitopes of poliovirus type 2 in complex with antibodies. Mol Immunol 2015; 67:233-9; PMID:26059753; http://dx.doi.org/10.1016/j.molimm.2015.05.013
21. Bannwarth L, Girerd-Chambaz Y, Arteni AA, Guigner JM, Lemains J, Ronzon F, Manin C, Venien-Bryan C. Structural studies of virus-antibody immune complexes (poliovirus type I): Characterization of the epitopes in 3D. Mol Immunol 2015; 63:279-86; PMID:25146483; http://dx.doi.org/10.1016/j.molimm.2014.07.014
22. Campbell MG, Veesler D, Cheng A, Potter CS, Carragher B. 2.8 Å resolution reconstruction of the Thermoplasma acidophilum 20S proteasome using cryo-electron microscopy. eLife 2015; 4:e06380; http://dx.doi.org/10.7554/eLife.06380
23. Azoulay J, Clamme J, Darlix J, Roques B, Mely Y. Destabilization of the HIV-1 complementary sequence of TAR by the nucleocapsid protein through activation of conformational fluctuations. J Mol Biol 2003; 326:691-700; PMID:12581633; http://dx.doi.org/10.1016/S0022-2836(02)01430-4
24. Clamme JP, Azoulay J, Mely Y. Monitoring of the formation and dissociation of polyethyleneimine/DNA complexes by two photon fluorescence correlation spectroscopy. Biophys J 2003; 84:1960-8; PMID:12609898; http://dx.doi.org/10.1016/S0006-3495(03)75004-8
25. Kim SA, Heinze KG, Schwille P. Fluorescence correlation spectroscopy in living cells. Nat Methods 2007; 4:963-73; PMID:17971781; http://dx.doi.org/10.1038/nmeth1104
26. Ries J, Bayer M, Csucs G, Dirix R, Solimena M, Ewers H, Schwille P. Automated suppression of sample-related artifacts in Fluorescence Correlation Spectroscopy. Opt Express 2010; 18:11073-82; PMID:20588964; http://dx.doi.org/10.1364/OE.18.011073
27. Sengupta P, Garai K, Balaji J, Periasamy N, Matti S. Measuring size distribution in highly heterogeneous systems with fluorescence correlation spectroscopy. Biophys J 2003; 84:1977-84; PMID:12609900; http://dx.doi.org/10.1016/S0006-3495(03)75006-1
28. Beecham J, Gratton E, Ameloot M, Kruitson J, Brand L. The Global Analysis of Fluorescence Intensity and Anisotropy Decay Data: Second-Generation Theory and Programs. In: Lakowicz J, ed. Topics in Fluorescence Spectroscopy Springer, US:, 2002:241-305; http://dx.doi.org/10.1007/0-306-47058-6_5
29. Marquardt DW. An algorithm for Least-Squares estimation of nonlinear parameters. SIAM J Appl Math 1963; 11:431-41; http://dx.doi.org/10.1137/0111030
30. Fischer H, Polikarpov I, Craievich AF. Average protein density is a molecular-weight-dependent function. Protein Sci 2004; 13:2825-8; PMID:15388866; http://dx.doi.org/10.1110/ps.04688204
31. Didier P, Godet J, Mely Y. Two-photon two-focus fluorescence correlation spectroscopy with a tunable distance between the excitation volumes. J Fluoresc 2009; 19:561-5; PMID:18923813; http://dx.doi.org/10.1007/s10895-008-0424-0
32. Manin C, Naville S, Gueugnon M, Dupuy M, de Alba YB, Adam O. Method for the simultaneous assay of the different poliovirus types using surface plasmon resonance technology. Vaccine 2013; 31:1034-9; PMID:23277095; http://dx.doi.org/10.1016/j.vaccine.2012.12.046
33. Tetin SY, Stroupe SD. Antibodies in diagnostic applications. Curr Pharm Biotechno 2004; 5:9-16; PMID:14965206; http://dx.doi.org/10.2174/1389201043489602
34. Jossang T, Feder J, Rosenqvist E. Photon correlation spectroscopy of human IgG. Journal of protein chemistry 1988; 7:165-71; PMID:3255367; http://dx.doi.org/10.1007/BF01025246
35. Dietrich J, Andreason L, Andersen P, Agger E. Inducing Dose Sparing with Inactivated Polio Virus Formulated in Adjuvant CAF01. Plos One 2014; 9:e:100879; PMID:24956110; http://dx.doi.org/10.1371/journal.pone.0100879