Author's response to reviews

Title: Automated image-based assay for evaluation of HIV neutralization and cell-to-cell fusion inhibition

Authors:

Enas Sheik-khalil (enas.sheik-khalil@med.lu.se)
Mark-Anthony Bray (mbray@broadinstitute.org)
Gülsen Özkaya Sahin (gulsen.ozkaya_sahin@med.lu.se)
Gabriella Scarlatti (scarlatti.gabriella@hsr.it)
Marianne Jansson (marianne.jansson@med.lu.se)
Anne E carpenter (anne@broadinstitute.org)
Eva M Fenyö (eva_maria.fenko@med.lu.se)

Version: 3
Date: 27 July 2014

Author's response to reviews: see over
Dear Dr. Nazarenko

Thank you for your looking into my submission to BMC Infectious Diseases. I have made the changes to my manuscript asked by the journal.

We thank both reviewers for their thorough review of our manuscript. We believe that it has helped us to improve.

Reviewer #1

Enas Sheik-Khalil et al. described an automation of existing manual HIV neutralization assay. This study lacks novelty and is a simple automation of existing manual assay. In addition, conclusion made based on plaque area as a major parameter of cell-cell fusion is not convincing.

To evaluate virus neutralization and cell-to-cell fusion within the same assay format has never been presented for HIV before. This is spelled out in the Abstract and discussed in the Discussion. To further emphasize the novelty we modified the first sentence of the Conclusions: “Here we report on a novel image-based automated plaque-reduction assay where both HIV neutralization and inhibition of cell-cell fusion can be analyzed, for the first time within the boundaries of the same assay format.”

Additional Comments:

1. In Figure 1E, 1F and 1G, it is unclear whether the giant cell formed is due to merging of multiple cells and clear bright field and DAPI staining picture showing multiple nuclei in a giant cell should be provided.

   We include a new picture, FIGURE 3, to show this.

2. In Figure 1G, whether the highlighted small areas are based on DAPI stained nuclei or possible location of nuclei detected by the Imaging software in a giant cell is not clear.

   The small areas in blue are based on DAPI stained nuclei inside a syncytia detected by the imaging software. Figures 1E-G have been replaced by a new figure to more clearly show both nuclei and syncytia detected by the imaging software. Please see FIGURE 2 and FIGURE 3.
3. In Figure 2, the DAPI staining could not show the various nuclei present in a giant cell though it appears as a smear. Clear picture showing either DAPI nuclei staining or bright field images showing a giant cell with nuclei may be presented.

In previous figure 2 (now renumbered to figure 3), the main purpose is to show the detection of syncytia and not nuclei. We here aim to show how the imaging software was manually and carefully tuned to detect and define syncytia borders. DAPI nuclei staining showing a giant cell with nuclei is instead presented in a new figure, FIGURE 2 and FIGURE 3.

4. In Figure 3, the manual and automated assay results have higher variability. When a manual assay to be automated for high-throughput analysis purpose, more than 10-15% variance is a major concern. Particularly in neutralization assays, if high similarity is questionable when automating existing manual assay that may leads to errors and increase in false positives etc. In addition, no statistical analysis was performed on the graph.

The manual and the automatic readout are separate experiments, this has now been better clarified in the text: Page 4 and page 16 and in Figure legend 5. Thus an inter-assay variation analysis is more applicable in this case. The variation of neutralization assays performed on different days (inter-assay variation) is higher than those performed on the same day (intra-assay variation) (Shi et al. 2002).

In the present experiments we tested the MN(P) isolate with TriMab in three assays performed on three different days and compared IC50 results running a t-test. No significant differences were found in IC50 values.

Our automatic readout was performed after fine tuning the threshold correction factor (TCF) on plaque detection in GHOST(3)- CCR5/CXCR4 cells for HIV-1-infected and uninfected cases (as explained in figure 4). This was made manually and after carefully reviewing many plates and pictures. The IdentifyPrimaryObjects module in the APR image analysis pipeline has thus been adjusted to read foreground/background to match the manual reading. The results of the automatic readout and what would be the corresponding manual readout within the same experiment are thus closely similar, if not identical.

In order to compare the overall performance of repeat assays for the two readouts, we chose an inter-assay comparison and present the mean performance of the two readouts in Figure 5.

5. The authors claim from Figure 3, that the established automated assay’s sensitivity is higher than manual assay. It appears that at multiple data points, automated assay readout was lesser as compared to the manual assay data points.

Indeed, in three cases the sensitivity of the automatic reading appeared to be lesser than the manual reading. In 11 cases, however, automatic reading appears to be more sensitive. This is discussed in the text.

6. Figure 4 is highly convincing and plaque area is correlated with viral infectivity inhibition by an inhibitory agent. However, whether it is true for decrease in syncytia formation or inhibition of
cell-cell fusion is unclear as infectivity itself will decrease once virus is neutralized.

The number of plaques and mean plaque area has been measured in parallel for every virus-inhibitory reagent combination.

Infectivity in GHOST(3) cells are measured by fluorescence, Syncytia formation is a result of cell-cell fusion and the GHOST(3) cells trigger fluorescence upon HIV-infection by the LTR promotor. The definition of IC90 means that the number of plaques is decreased with 90%. Plaques in our system are composed of fluorescent cells; either single cells or multinucleated cells (syncytia). Both single-cell and multinucleated cells fluoresce with the same intensity and are detected by the APR image analysis program in the same manner and have the same overall intensity. At IC90 we found a decrease in mean plaque area. This is thus a result of decreased cell-cell fusion as fluorescence itself does not “decrease” once virus is neutralized. A cell is either infected or not.

It may be important to quantify syncytia and check whether syncytia number is also decreased accordingly or unfused single infected cell numbers are decreased should be presented.

We thank the reviewer for this interesting thought; our system is indeed suitable to address such a question. We consider this to be a different project, outside the scope of the present work (note that the reviewer labeled this comment “discretional”).

7. The major conclusion drawn from Figure 5, plaque area is a measure of cell- cell fusion or syncytia formation. This will be convincing if the authors quantitate syncytia by other assay such as Fluorescence based assay (Sabina winschman et al., J. Clin. Microbiol., 2000; Fluorescence based quantitative assay for detecting HIV-1 induced syncytia) and correlate with the total cells merged is indeed equivalent to the plaque area that is a direct measure of number of fused cells.

Very interesting work, thank you for calling our attention to it. Winschman et al. demonstrate that the reduced cell number measured in HIV-infected cultures is predominantly due to formation of multinucleated giant cells and not to the cell lysis frequently observed in such cultures. The two-color fusion assay allows microscopic detection of double-stained fusion cells by color.

Our assay differs in several aspects. Our assay can detect a single cell being HIV-infected; something not possible with Winschman’s method. Our assay is automated and, furthermore, can provide information on neutralization in parallel with the information of plaque area. The specific information the reviewer asks for can, however, be extracted from our assay by cropping the mosaic picture and focus on single syncytia. The new figure 3 illustrates this as a possibility. However, these studies are outside the focus of the present paper.

8. In Figure 5B, the steep decline in neutralizing activity and an immediate increase is an artifact or real can be checked by changing the dilution factor from 1:20 to 1:40 to smaller dilutions such as 1:5/10/15/20/25/30/40 etc.
Dilutions have already been made. However, the steep decline in neutralization gives a very strong proof that the decline in plaque area is correlated to neutralization only and not due to an artifact of any kind.

9. In Figure 5D, it is unclear why TriMab neutralization activity and plaque area are not reversibly correlative and should be explained.

IC90 has not been reached with the virus-inhibitory reagent combination depicted in Figure 5D (now Figure 8D).
Reviewer #2

Major compulsory revisions:

1. The comparison of neutralization sensitivity by the three presented techniques should not be relegated to supplemental material. This is a very interesting set of data, and the authors should be upfront about the similarities and discrepancies between their new assay and the results generated by the more established neutralization sensitivity assays.

The comparison of the three techniques has been extended in the results section, page 8, as suggested. Supplementary figure 1 and 2 are now figure 6A and 6B.

2. The authors make some very qualitative statements regarding the within-virus and within-plasma comparisons shown in Figures S1 and S2. In both cases, a geometric mean is calculated for the IC50 generated. Clearly in many cases the three assays report similar IC50s as illustrated by 92BR025 and MN(P) in figure S2. However, in several cases there is a large amount of heterogeneity in the IC50 values reported by each assay, for example 92UG024 and SF162 in figure S2. The authors should run ANOVAs (or the non-parametric equivalents) with post tests to determine whether there are significant differences between the outcomes of the different assays. This may not reflect inadequacy of the authors’ novel assay to calculate accurate and reproducible IC50s, but should absolutely be considered by readers when evaluating the assay for their own needs.

We have now performed statistical analysis and extended the results section “Comparison of APR assay with other HIV-1 neutralization assays”, page 9.

3. Furthermore, since the materials and methods section make no mention of the protocols used to calculate IC50s in the pseudovirus and PBMC neutralization assays, it is impossible to critically compare the results presented in Figures S1 and S2. The authors should provide descriptions of these assays and indicate whether they were run in parallel with the new assay presented here or if they are historical values that have been published elsewhere.

The assays were run in parallel with the same inhibitory reagents and viruses within the NeutNet network. Results have been published in a different format as referred to in the text (reference 3). We have now added brief descriptions of all three assays and referred to a detailed protocol on the website Europrise.org.

4. On line 237 of the manuscript, the authors state that there is low intra-well variation. Since they are presenting a new assay for evaluation by readers, it is of the utmost importance to quantify not only the intra-well variation, but also the repeatability of the results gathered in the assay. In order to convince readers that this assay will give consistent results the authors should present an analysis that scientifically and statistically demonstrates it’s consistency in capable hands.

The variation of neutralization assays performed on different days (inter-assay variation) is higher than those performed on the same day (intra-assay variation) (Shi et al. 2002).
In the present experiments we tested the MN(P) isolate with TriMab in three assays performed on three different days and compared IC50 results running a t-test. No significant differences were found in IC50 values.

5. Does Figure 4 represent a separate experiment from the screen using the previously determined IC50, IC75, and IC90 concentrations of the plasmas, or is this data mined from the large-scale screen? If the latter is true, please explain how the plasma concentrations were chosen to represent IC50, IC75, and IC90.

The data that appear in Figure 7 (old Figure 4) was mined from the large-scale screen. This has now been clarified (page 11). The Figure has been re-drawn as suggested in point 11.

Minor essential revisions:

6. line 162-163: sentence needs to be rephrased.

Sentence has been rephrased.

7. lines 248-251: This sentence needs restructuring.

Sentence has been restructured.

8. Figure 1: do panels e-g represent an inset of the larger field composite images in a-d? if so, please show it.

Figure 1 has been changed and now comprises panel a-d. Two new figures have been added, Figures 2 and 3, comprising 6 panels each. Figure 3 is an magnified inset of Figure 2. Figure legends have been modified accordingly.

9. Figure 1: state what the colors in panels d, e, f, and g represent in the figure legend.

This has now been clarified in figure legends 1, 2 and 3.

10. Figure 3: the labels on the axes should clearly indicate that IC50 is being presented here, i.e. "IC50 by automated readout"

Labels have been changed, as suggested.

11. Figure 4: to more clearly show that the values in this graph are from different plasma concentrations of the same virus-plasma pairs, the graph should be changed from the box-and-whisker format, to a column format where individual values representing repeated measures are connected by a line and summary statistics are superimposed.
This has now been changed. Figure is now called figure 7.

12. Figure 5: Enlarge the representative lines in the figure legends of this graph so that readers can distinguish the lines appropriately. An alternative could be to have a single legend for the whole figure showing the type of line for % neutralization and mean plaque area.

Figure legend has been restructured. Figure is now called figure 8.