Revealing retroviral integration complex assembly with mass photometry and MassFluidix™ HC

Retroviral integration into the host genome by HIV-1 is a complex process that is important for therapeutics. Assembly of the integration complex, the intasome, could serve as a therapeutic target as preventing its formation would block viral integration. Here, we demonstrate that mass photometry can overcome existing analytical challenges to enable the study of HIV-1 intasome assembly.

In HIV-1, a nucleoprotein complex known as the intasome plays a key role in retroviral DNA integration, integrating a copy of the viral genome DNA into host DNA.[1] The intasome is an important therapeutic target,[2,3] as integrase strand transfer inhibitors (INSTIs) that block its catalytic function are front-line HIV treatments.[4] However, rapidly emerging resistance to existing treatments necessitates further research and drug development. Additionally the mechanism for HIV-1 intasome assembly remains poorly understood. The assembly's large size, inherent flexibility and heterogeneity have made it challenging to study.

The intasome is formed through interaction of HIV-1 integrase (IN) monomers with the viral DNA genomic ends, centered around a core tetramer of integrase.[5] The assembled intasome can then capture host target DNA and mediates the integration. In doing so, it works with cellular cofactors, including the protein LEDGF/p75, which tethers the IN assembly to chromatin.

The complete intasome is a 16-mer of IN bound to viral DNA and at least two LEDGF/p75 subunits – with a total mass of ~670 kDa. Prepared samples of the intasome tend to be highly heterogeneous, as, in theory, 33 different assembly stoichiometries are possible, ranging in mass from 15.5 kDa to 907 kDa (Table 1).

Box 1: Refeyn's MassFluidix HC system

- A rapid-dilution microfluidics system that can be coupled to a mass photometer
- Dilutes samples up to 10,000x in <37 ms before measurement begins
- Enables the low concentrations required for standard mass photometry, giving access to
  - Low-affinity binding interactions
  - Dilution-sensitive, higher-order complexes

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Several challenges make the intasome assembly difficult to study using traditional techniques:

- The complete intasome with full-length proteins is too large for the entire structure to be solved at once using crystallography or analyzed by gel electrophoresis.
- The assembly is not spherical – resulting in heterogeneous measurements by dynamic light scattering (DLS), which measures the hydrodynamic radius.
- The structure of the intasome is flexible, which makes it challenging to analyze by cryo-EM or crystallography. This is especially true of full length LEDGF/p75.
- LEDGF/p75 has multiple potential binding sites on the complex, so LEDGF/75 binding is lost in cryo-EM analysis, which averages the structure from a large dataset.
- Samples are highly heterogeneous.

In light of these challenges, mass photometry, which measures the mass of individual proteins in solution,[5] offers a promising solution.

Mass photometry's advantages in this context include:

- It can measure proteins in the range 30 kDa to 5 MDa.
- It is insensitive to shape.
- As a single-molecule technique, it captures sample heterogeneity.

Standard mass photometry requires samples to be at low (nanomolar) concentrations for measurements, which can

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affect complex formation. Initial experiments indicated that intasome complexes disassembled when samples were diluted manually to the concentration required (not shown). Mass photometry's low-concentration requirement can be overcome, however, with a rapid-dilution microfluidic system (MassFluidix™ HC, Box 1).

In this application note, we apply mass photometry with MassFluidix HC (MP-MFx) to investigate the assembly of HIV-1 intasomes. We confirm that intasome assembly requires LEDGF/p75 and that mass photometry results are consistent with those from size-exclusion chromatography (SEC). Then, mass photometry reveals the dependence of intasome assembly on integrase concentration. Overall, the results demonstrate that MP-MFx form a powerful tool for studying large, heterogeneous complexes that are intractable with other methods.

MP-MFx and SEC confirm that intasome assembly requires LEDGF/p75

First, we analyzed samples containing the HIV-1 WT integrase and LEDGF/p75 by mass photometry with MassFluidix HC and also by SEC (Fig. 1). The results showed that peaks corresponding to populations of assembled intasome (~670 kDa for mass photometry; ~8.7 mL for SEC) appeared. For both techniques, when the measurements were repeated for samples of HIV-1 WT integrase only (no LEDGF/p75) or the HIV-1 integrase with a mutation resulting in only tetramer formation, the population of fully assembled intasome complex (consisting of 16 integrase subunits) was negligible.

Overall, the results showed that intasome complex assembly only occurred when both HIV-1 WT and LEDGF/p75 were both present. They also showed agreement between SEC and mass photometry with MassFluidix HC.

Table 1 Sample species with corresponding mass. Mass values for individual species (integrase, LEDGF/p75 and viral DNA) are listed. Also given are the mass range for the various assemblies that could theoretically form involving either dimers, tetramers, octamers or 16-mers of HIV-1 integrase – alone or together with viral DNA and multiple LEDGF/p75 molecules.

Species	Mass of single molecules or mass range of theoretical assemblies
Viral DNA oligos	15.5 kDa
Monomer of integrase	32.2 kDa
LEDGF/p75	60.3 kDa
Dimer of integrase – alone or in assembly with DNA and/or up to 1 LEDGF/p75	64.4 – 124.7 kDa
Tetramer of integrase – alone or in assembly with DNA and/or up to 1 LEDGF/p75	128.8 – 400.8 kDa
Octamer of integrase – alone or in assembly with DNA and/or up to 1 LEDGF/p75	257.6 – 529.6 kDa
16-mer of integrase – alone or in assembly with DNA and/or up to 1 LEDGF/p75	515.2 – 907.7 kDa

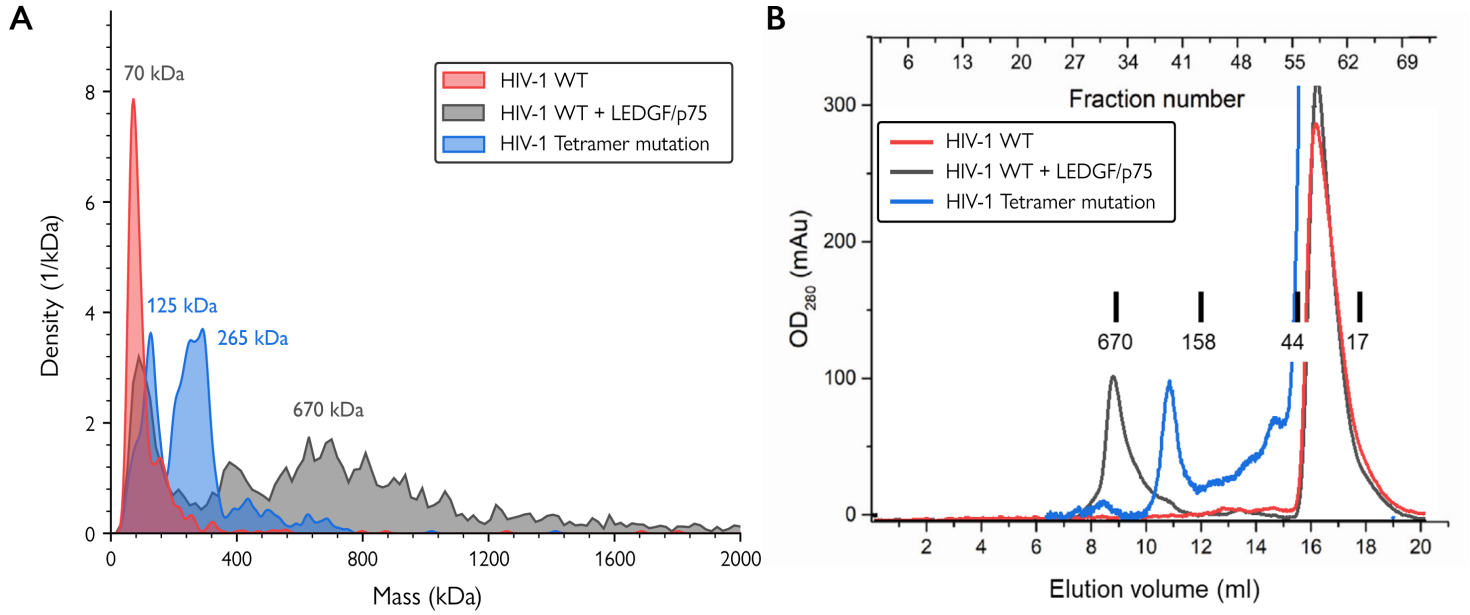

Fig. 1 Intasome complex assembly requires the presence of LEDGF/p75, according to mass photometry and SEC. Samples containing either i) HIV-1 WT only (red) ii) HIV-1 WT and LEDGF/p75 (black/grey) or iii) HIV-1 with a mutation resulting in only tetramer formation and LEDGF/p75 (blue) were analyzed by A) mass photometry and B) SEC. For mass photometry, samples started at 500 nM concentration and underwent 50x rapid dilution via MassFluidix HC to 10 nM for the measurement. For SEC, the black vertical lines indicate the elution of protein standards with the masses of 670 kDa, 158 kDa, 44 kDa and 17 kDa.

Intasome assembly is highly sensitive to integrase concentration

Next, we assessed how integrase concentration affects intasome assembly. To do this, we used mass photometry and MassFluidix HC to analyze samples containing a ratio of integrase:LEDGF/p75:viral DNA at 2:1:1 from 1 nM to 500 nM. The results showed that, overall, the size of the dominant oligomer population increased with increasing integrase concentration (Fig. 2).

At 1 nM integrase, dimers (~100 kDa, corresponding to integrase dimer complexes with viral DNA) were the most prominent population. At 100 nM, there was a mix of dimers and tetramers (~220 kDa, corresponding to integrase tetramer complexes, viral DNA and 2 LEDGF/p75). At 200 nM, tetramers dominated; at 300 nM, there was a mix of octamers (~400 kDa, corresponding to integrase octamer complexes, viral DNA and 2 LEDGF/p75) and some dimers. Finally, once the integrase concentration reached 500 nM, the complete 16-mer intasome assembly (~670 kDa, corresponding to 16 integrase, viral DNA and 2 LEDGF/p75) was observed. For this last sample (500 nM), the counts corresponding to populations of smaller oligomers were low, indicating that most of the integrase protein was stabilized in the instasome assemblies at this concentration. The histogram at 500 nM was also broad, displaying the heterogeneity of the complexes formed – consistent with expectations.

To confirm the observation that intasome assembly occurs in samples analyzed at 500 nM intasome concentration, we analyzed four biological replicates. The results were consistent, with each showing lower-mass populations (~100 kDa) that correspond to integrase dimer species with viral DNA, and additional populations in the mass ranges corresponding to assemblies involving integrase octamers and 16-mers (Fig. 3).

Quantification of the percentages of counts in the octamer and 16-mer assembly mass ranges confirmed the replicability of the result, with standard deviation (SD) <1% for octamer and <5% for 16-mer assemblies (Table 2). Given the evident heterogeneity of the samples, the high degree of replicability is striking.

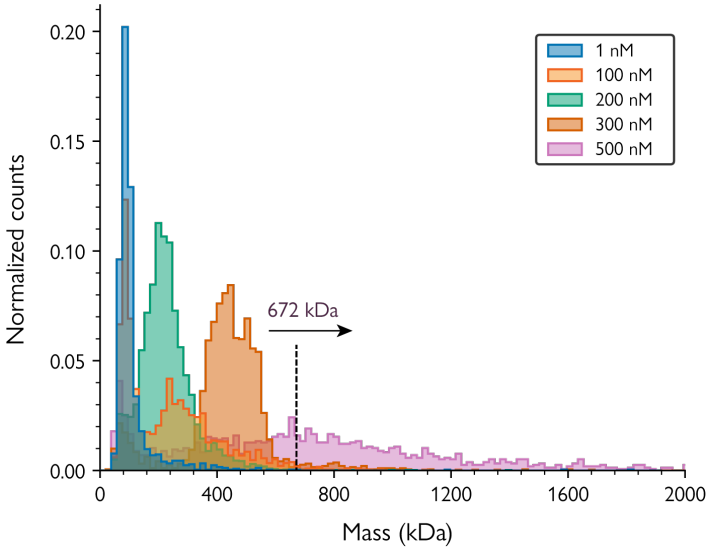

Fig. 2 MP-MFx reveals dependence of intasome assembly on integrase concentration. Data shown for analysis of samples containing varying concentrations of intasome complex assemble with a ratio of integrase:LEDGF/p75:viral DNA at 2:1:1, as indicated. The samples underwent 50x rapid dilution via MassFluidix HC for the measurement.

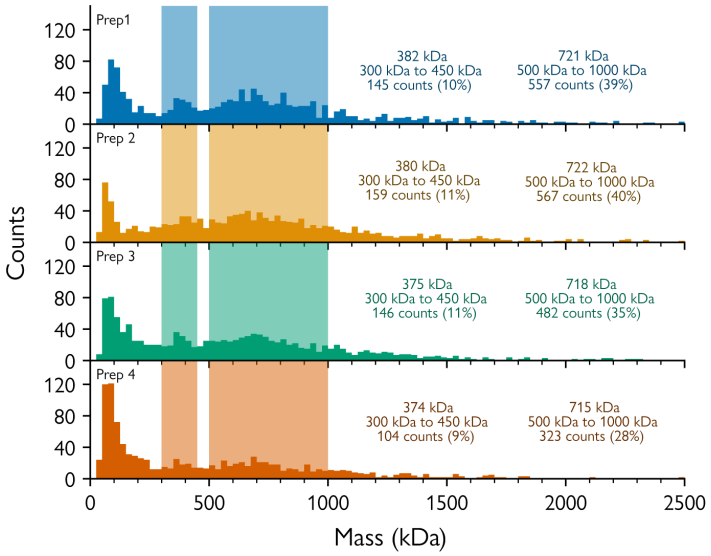

Fig. 3 Reproducibility of intasome assembly across biological replicates, as observed by mass photometry with MassFluidix HC. Mass histograms for the analysis of each replicate are shown, with two mass range windows indicated to identify counts corresponding to octamer complexes (300 – 450 kDa) and 16-mer complexes (500 – 1000 kDa). These ranges were selected based on the expected mass ranges for the species as well as observations of where peaks mainly appeared for octamers and 16-mers (e.g. in Fig. 2).

Table 2 Reproducibility of 8-mer and 16-mer counts. The number of counts (and percentage of total counts) in the mass ranges corresponding to integrase octamer complexes (300 – 450 kDa) and 16-mer complexes (500 – 1000 kDa) are given for the four replicates (Prep 1–4) shown in Fig. 3. Mean ± standard deviation (SD) is also given for the % of total counts.

	% of total counts in mass range	
	300 – 450 kDa	500 – 1000 kDa
Prep 1	10%	39%
Prep 2	11%	40%
Prep 3	11%	35%
Prep 4	9%	28%
Mean ± SD	10.3 ± 0.83%	35.5 ± 4.7%

Discussion

A better understanding of HIV-1 intasome assembly is critical for ongoing development of HIV-1 therapeutics. However, the assembly's large size and heterogeneity have made it difficult to study with traditional methods, including crystallography, gel electrophoresis, cryo-EM and DLS. Here, we have shown that mass photometry combined with MassFluidix HC is a powerful technique in this context.

Mass photometry can measure proteins in an appropriate mass range (30 kDa to 5 MDa), it is insensitive to shape and, as a single-molecule technique, it is straightforward to analyze heterogeneous samples and reveal the heterogeneity present. Meanwhile, the addition of rapid dilution via the MassFluidix HC system overcomes the limitation that samples must be very dilute to enable mass photometry analysis. This approach made it possible to reveal the sensitivity of intasome assembly to integrase concentration, paving the way for further investigation of the assembly mechanism.

References

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Materials and methods

Samples

All samples were provided by Ross Larue. Full-length purified HIV-1 integrase and LEDGF/p75 were purified as described.[6] Short viral DNA oligos consisting of the first 25/27 nucleotides of the viral DNA ends were purchased from IDT. Intasome assemblies were performed while maintaining a ratio of 2:1:1 (typically integrase (40 µM), viral DNA (20 µM) and LEDGF/p75 (20 µM)). Proteins and oligos were mixed in a buffer containing 1 M NaCl, 25 mM Na-Phosphate pH 6.0, 500 mM NaCl, 2 mM DTT, 10% glycerol, 50 mM NDSB 256 and 20 µM ZnCl2, then they were incubated in a serial dialysis of at least 8 h in varying NaCl concentrations with the same buffer components (125 and then 500 mM) and then analyzed.

Mass photometry

All mass photometry measurements were performed by Ross Larue's research group with a OneMP mass photometer (Refeyn, Ltd.). Each movie was recorded for 60 seconds.

Where indicated, a Refeyn MassFluidix HC system was used with the mass photometer. MassFluidix HC Gen 2 (5-channel) chips were used for all MassFluidix HC experiments except those shown in Fig. 2, where Gen 1 (3-channel) chips were used.

Data was acquired with AcquireMP and analyzed with DiscoverMP software (Refeyn, Ltd).

Buffer used for mass photometry measurements: 500 mM NaCl, 25 mM Na-Phosphate pH 6.0, 20 µM ZnCl2, 10% glycerol and 2 mM DTT.

Size-exclusion chromatography (SEC)

Experiments were performed with a Superdex 200 10/300 GL column (GE Healthcare) at 0.5 mL/min in elution buffer containing 25 mM Na-Phosphate pH 6.0, 500 mM NaCl, 2 mM DTT, 10% glycerol and 20 µM ZnCl2. IN (40 µm), viral DNA (20 µM) and LEDGF/p75 (20 µM) were mixed and incubated in a serial dialysis of at least 8 hrs in varying NaCl concentrations (125 and then 500 mM) and then subjected to SEC.

The column was calibrated with the following proteins: Bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B12 (1,350 Da).

Proteins were detected by absorbance at 280 nm, viral DNA by absorbance at 260 nm. All procedures were performed at 4°C.

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