Isolation of *Leishmania* promastigote flagella

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Running Head

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Abstract

Eukaryotic flagella are conserved multifunctional organelles with roles in motility, intercellular interactions and signal transduction. *Leishmania* possess a single flagellum at all stages of their life cycle. Flagella of promastigote forms in the fly are long and motile, with a canonical 9+2 microtubule axoneme and an extra-axonemal paraflagellar rod (PFR). This protocol describes a simple method for the isolation of *Leishmania mexicana* promastigote flagella, optimised to yield intact flagella that retain both the cytoskeletal elements (9+2 axoneme and PFR) and the surrounding membrane. The isolated flagella and deflagellated cell bodies are suitable for analysis by electron microscopy, protein mass spectrometry and lipidomics.

Keywords

Leishmania, kinetoplastids, flagella, cell fractionation

1. Introduction

Eukaryotic flagella and cilia are highly conserved structures. Motile flagella typically have a 9+2 microtubule (MT) structure, where a nine-fold symmetric cylinder of doublet microtubules surrounds a central pair of singlet MTs (CP). The coordinated activity of dynein motor proteins anchored on the doublet MTs generates a flagellar waveform, thought to be regulated by the CP and associated protein complexes. Cilia with a simpler 9+0 MT architecture typically perform sensory functions, which can be mediated through the interactions of external stimuli with receptors localised to the ciliary membrane [1]. Proteomic studies of diverse ciliated cell types [2-5], including trypanosomatids [6-9], have shown that flagella and cilia are composed of several hundred proteins, with a core set of evolutionarily conserved proteins and lineage-specific elaborations and losses [10]. Genetic studies and biochemical analyses of wild type and mutant flagella enabled the development of models for dynein-driven motility [11,12] and advances in cryo-electron microscopy now help to test and refine these models [13,14]. Recent technological advances in gene editing have accelerated the rate at which knockout mutants can be produced [15] and offer opportunities for precision editing to alter just a few residues in a protein, for example. Further biochemical studies on wild type and mutant *Leishmania* flagella thus have the potential to yield new insights into the mechanisms underpinning the diverse functions of this fascinating organelle, which includes motility, cell morphogenesis, attachment to the insect vector and possibly sensory functions [16,17].

Here we developed a protocol for the isolation of *Leishmania mexicana* promastigote flagella (**Figure 1**), optimised to yield intact flagella that retain both the cytoskeletal elements (9+2 axoneme and paraflagellar rod (PFR)) and the surrounding membrane, leaving the basal body and transition zone (TZ) in the deflagellated cell body (**Figure 2A**). This will enable studies both on the cytoskeletal apparatus driving motility as well as interrogation of surface and membrane-associated molecules and their potential role in adhesion and signalling.

The widespread interest in flagella and cilia produced numerous deciliation / deflagellation protocols for different cells, starting with the first reports of isolating cilia from microorganisms such as *Tetrahymena* in the 1950s and 60s [18,19], culminating in an optimised procedure that combined a calcium shock with 2-4 shearings using a 10 ml glass syringe fitted with an 18-G needle [20]. Subsequent work, on the green alga *Chlamydomonas* and other ciliated cells, produced deflagellation methods where separation of the flagella from the cell bodies typically requires application of mechanical force in conjunction with specific chemical treatments [21]. A number of protocols have been used over the years to isolate flagella or flagellar sub-fractions from trypanosomatids [22-27,6,9]. Whilst different flagellar isolation protocols follow broadly similar principles, obtaining pure fractions of the flagellar substructures of interest requires optimisation for each cell type and species. For development of the protocol described here, a number of methods based on chemical approaches such as pH shock, ethanol treatment or drugs like dibucaine were tried and found to yield only small quantities of free *L. mexicana* flagella. Ethanol treatment combined with mechanical shearing resulted in cell fixation, while treatment with different dibucaine concentrations, incubation times or mechanical assistance permeabilised the membrane and deflagellated only some cells in the population. Shearing in a Dounce type homogeniser did result in some deflagellated cells but yielded low numbers. Passaging *L. mexicana* promastigotes through a syringe proved the most efficient at detaching the majority of flagella but this stripped the axonemes of their membranes. A combination of syringe disruption and addition of Ca^{2+} finally enabled detachment of the external flagella complete with membrane. The flagella can then be isolated away from the deflagellated cell bodies by gradient centrifugation. Analysis of the cell fractions is facilitated by using an *L. mexicana* cell line expressing a fluorescently tagged flagellar membrane protein. The resulting flagella are suitable for downstream analysis by transmission electron microscopy, protein analysis by SDS-PAGE, western blotting and mass spectrometry [28], and lipidomics.

2. Materials

Follow local rules for safe handling and containment of *Leishmania* spp. and handling and disposal of hazardous chemicals.

Deflagellation and Density Gradient Centrifugation

1. Phosphate buffered saline (PBS): 137 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2. To make a 5x concentrated solution, weigh out 40 g NaCl, 1 g KCl, 7.1 g Na_2HPO_4 and 1.35 g KH_2PO_4 and dissolve in 1 L water. Dilute 1:5 in water to make 1x PBS.
2. 10 mM PIPES [*see* **Note 1**]: 10 mM NaCl, 10 mM piperazine-N,N′-bis(2-ethanesulfonic acid), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.32 M sucrose, adjusted to pH 7.2. Add 500 ml dH_2O to a 1 L glass bottle and add 10 solid NaOH pellets while stirring. When the pellets have dissolved add 3 g PIPES, 0.22 g $CaCl_2 \cdot 6H_2O$, 0.2 g $MgCl_2 \cdot 6H_2O$ and 0.58 g NaCl. Titrate to pH 7.2 while mixing, using 1 M NaOH solution. Add 109.44 g sucrose to this solution. Keep mixing until completely dissolved and fill up to 1 L with dH_2O.
3. 166 mM PIPES: 166 mM NaCl, 166 mM piperazine-N,N′-bis(2-ethanesulfonic acid), 16.6 mM $CaCl_2$, 16.6 mM $MgCl_2$, adjusted to pH 7.2. Prepare as indicated for 10 mM PIPES, but omitting the sucrose.
4. 1 M Ca^{2+} solution
5. 2 M sucrose solution
6. 5 mM E-64: dissolve 5 mg in 2920 µl DMSO.
7. 20 mM Leupeptin hydrochloride: dissolve 5 mg in 525 µl ddH_2O.
8. 3 mM Pepstatin A: dissolve 5 mg in 2425 µl 100% ethanol and incubate for 1 h at 60°C.
9. 200 mM Phenylmethylsulfonyl fluoride (PMSF): dissolve 100 mg in 2850 µl 100% methanol.
10. 1000x Hoechst: Dissolve 100 mg Hoechst 33342 in 10 ml dH_2O (10 mg/ml stock). Protect tube from light and keep under agitation overnight until completely dissolved.

11. Equipment and consumables: 200 µl plastic gel loading pipette tip (Starlab) [*see* **Note 2**], 10 ml disposable syringe, haemocytometer [*see* **Note 3**], microcentrifuge, benchtop centrifuge, ultracentrifuge and ultra-centrifugation tubes, phase contrast microscope, fluorescence microscope.

3. Methods

An overview of the workflow is shown in **Figure 1**.

3.1 Preparing Cells

1. Use an exponentially growing *Leishmania* promastigote culture [*see* **Notes 4** and **5**] to set up a 200 ml culture starting with $1 \cdot 10^6$ cells/ml, then grow for ~24 h until there are $1 \cdot 10^7$ cells/ml.
2. Decant culture into four 50 ml Falcon centrifuge tubes and collect $2 \cdot 10^9$ cells by centrifugation at 800 x *g* for 15 min at 4°C.
3. For all following steps, keep samples at 4°C and cool buffers on ice before adding to samples.
4. Discard supernatant and combine the four cell pellets by resuspending in a total of 20 ml PBS and centrifuge as above.
5. Discard the supernatant and resuspended the cells in 5 ml 10 mM PIPES.
6. Place 8 *µ*l cell suspension on a microscope slide and add a small volume of 1000x Hoechst stock [*see* **Note 6**]. Examine sample on a fluorescence microscope (**Figure 2B**).

3.2 Deflagellation

1. Add 0.375 ml of 1 M Ca^{2+} solution (final conc. 0.075 M) and protease inhibitors (12.5 *µ*l Leupeptin, 12.5 *µ*l PMSF, 12.5 *µ*l Pepstatin A and 5 *µ*l E-64 to the cell suspension.
2. Attach a 200 µl gel loading pipette tip to a 10 ml disposable syringe.

3. Deflagellate cells by repeatedly drawing cell suspension through the gel loading tip into the syringe and pushing out again. Perform this a total of 100 times until flagella are sheared from the *Leishmania* cell bodies [*see* **Note 7**].
4. Make a 1:2 dilution of the sample by mixing 10 *µ*l sample with 10 *µ*l 10 mM PIPES and then dilute again 1:25 (1:50 dilution of the sample). Add 10 *µ*l of the 1:50 dilution of the sample onto a haemocytometer and quantify the number of flagella and cell bodies and the percentage of deflagellated cells in a 0.1 µl square with a phase contrast microscope (**Figure 2B**).
5. If tagged cells are used, place 7 *µ*l of the 1:2 sample dilution on a glass microscope slide, add a small volume of 1000x Hoechst [*see* **Note 6**] and examine sample on a fluorescence microscope to quantify the percentage of flagella retaining their membrane (**Figure 2B**).

3.3 Separation of Flagella and Deflagellated Cell Bodies

Flagella and cell bodies were separated through density gradient centrifugation, using a modified version of the protocol in [9].

1. Prepare one 9 ml sucrose-bed containing three layers of 10 mM PIPES with 33% (upper), 53% (middle) and 63% (bottom) (w/v) sucrose: Mix the ingredients listed in **Table 1** for each sucrose concentration. Place the 63% sucrose solution in a 15 ml Falcon tube, then carefully layer the 53% and the 33% sucrose solutions on top.

   Table 1

Sucrose (%)	V [ml] 2 M sucrose	V [ml] ddH_2O	V [ml] 0.166 M PIPES pH 7.2
33	1.446	1.374	0.18
53	2.323	0.497	0.18
63	2.761	0.059	0.18

2. Carefully place the sample (from **Section 3.2** above) on top of the sucrose-bed. Take care not to mix the layers.
3. Centrifuge at 800 x *g* for 15 min at 4°C.

4. Prepare another 33% sucrose layer (as specified in **Table 1**) and pipette in a 15 ml Falcon tube.
5. Collect the top layer of the first sucrose-bed (~4.5 ml, flagella fraction) and place it on top of the 33% sucrose layer (from Step 4 above).
6. Discard the other sucrose layers, which contain a mixture of flagella and cell bodies [*see* **Note 8**].
7. Resuspend the pellet in the 63% sucrose layer in 10 ml 10 mM PIPES (contains the deflagellated cell bodies [*see* **Note 9**]) and keep on ice.
8. Centrifuge both the second sucrose-bed with the flagella fraction and the resuspended pellet (cell body fraction) at 800 x *g* for 10 min at 4°C.
9. Discard the supernatant from the cell body fraction and add 20 µl 10 mM PIPES to the pellet. With remnants from the supernatant the final volume should be ~40 µl.
10. Transfer 36.5 µl of this fraction to a 0.5 ml Eppendorf tube and keep on ice.
11. Transfer the top layer of the flagella fraction to a 3.5 ml ultra-centrifugation tube.
12. Discard the remainder of the second sucrose bed.
13. Centrifuge the flagella fraction at 100,000 x *g* for 1 h at 4°C.
14. Discard the supernatant and resuspend the pellet containing the isolated flagella in 10 mM PIPES, adding PIPES in small volumes so that the final volume is exactly 36.5 µl.
15. Remove 0.5 µl from the flagella fraction and dilute with 10 mM PIPES (first 1:40 and then 1:25, to a final dilution of 1:1000). Use the 1:000 dilution to determine the yield and purity [*see* **Note 10**] by counting on a haemocytometer and if a tagged cell line was used, use the 1:40 dilution to determine the proportion of flagella with retained membrane using a fluorescence microscope (**Figure 2B**).
16. Remove 0.5 µl from the final cell body fraction and analyse as for the flagella fraction (Step 15 above; **Figure 2B**).

17. The isolated flagella and cell bodies can then be used for protein mass spectrometry, further biochemical fractionation (**Figure 2C**) [*see* **Note 11**] or light and transmission electron microscopy [*see* **Note 12**].

4. Notes

1. PIPES buffer was used to prepare samples compatible with glutaraldehyde fixation for transmission electron microscopy analysis. An alternative buffer that works for the deflagellation protocol (but is not compatible with glutaraldehyde fixation because it contains Tris) is STC isotonic buffer (0.32 M sucrose containing 0.03 M Tris pH 7.2, 0.001 M $CaCl_2$). The addition of Ca^{2+} to the deflagellation buffer precludes resuspending samples in PBS as we noted a phosphate precipitation.
2. Plastic gel loading tips are used instead of syringe needles for safety reasons to avoid the possibility of needle stick-injuries. The tips used in this protocol had an inner diameter of ~0.35 mm, which corresponds to a 23 gauge needle.
3. For counting of live *Leishmania*, we recommend using disposable haemocytometers such as “C-Chip” Digital Bio, Neubauer Improved DMC-N01 for safety reasons.
4. The density and health of the *Leishmania* culture at the start before homogenization are crucial to ensure a high yield.
5. This protocol can be used with *Leishmania* wild-type promastigotes or with a cell line that expresses fluorescently tagged proteins marking subcellular structures of interest. Expression of the small myristoylated protein 1 (SMP1 [29]; LmxM.20.1310) fused to a fluorescent protein at the C-terminus (e.g. SMP1::GFP) allows monitoring of the presence of the flagellar membrane throughout the isolation procedure. Tagged cell lines can be generated by transgene expression from an episome [30], using CRISPR-Cas9 tagging [15] or Fusion PCR tagging [31].

6. To achieve this, use a P10 pipette with tip and set pipette to 1 µl. Pipette Hoechst 1000x stock solution in and out. Using the same pipette tip, pipette 8 µl cell suspension on the microscope slide up and down.
7. The number of passages was optimized to yield a sample where >99% of all cells were deflagellated.
8. Attempts to purify this layer further to increase the flagellar yield resulted in flagella without attached membrane.
9. The sucrose bed exhibited different gradient properties when using PIPES or a Tris-based buffer (STC). While a clear a pellet is visible in the 63% layer of the PIPES sucrose-bed, this is not obvious for Tris sucrose-beds. Therefore, when using STC (*see* **Note 1**), take both the 53% and 63% layer and dilute to 15 ml with STC to recover cell bodies from the Tris sucrose-bed.
10. This protocol is optimized for high purity of isolated flagella. Expect a yield of ca. $2 \cdot 10^8$ flagella (about 10% of the starting material).
11. For protein analysis (e.g. mass spectrometry or Western blots) supplement cell body and flagellar fractions with 4 µl pre-diluted protease inhibitor cocktail (1 µl Leupeptin (1:10 dilution of stock), 1 µl PMSF (1:10 dilution of stock), 1 µl Pepstatin A (1:10 dilution of stock) and 1 µl E-64 (1:25 dilution of stock)). Samples can then be subjected to further fractionation (e.g. separation into detergent-soluble and insoluble components by extraction with 1% octylglucoside [28]). Samples should be resuspended in suitable sample buffer (e.g. Laemmli buffer) and these samples can be stored at -80°C.
12. For transmission electron microscopy, collect cell fractions by centrifugation (flagellar fraction: 30 min at 18,500 x *g*; cell body fraction: 15 min, 800 x *g*) and fix by overlaying pellets with 500 µl 10 mM PIPES containing 2.5% (v/v) glutaraldehyde overnight at 4°C. Prepare samples for transmission electron microscopy using the chemical fixation protocol described in [32].

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Figure Captions

Figure 1. Workflow of deflagellation procedure

Schematic overview of the workflow. Red asterisk (*) indicate samples that should be examined microscopically to quantify yield and purity. Adapted from [28].

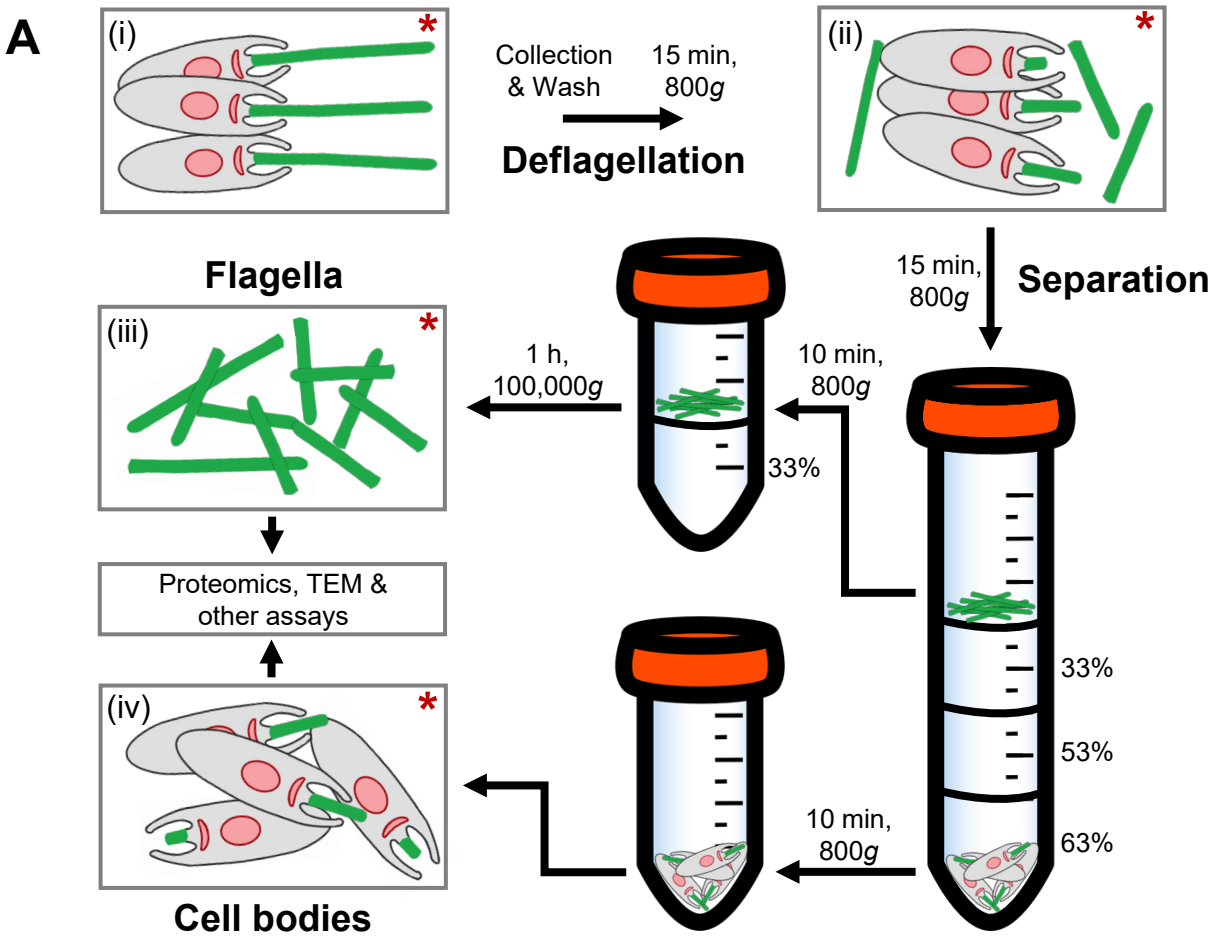
Figure 2. Expected results

(A) Cartoon showing the point where flagella are severed from the cell body. The basal body and proximal part of the flagellum remain attached to the cell body, releasing the external flagellum (axoneme, paraflagellar rod and membrane). **(B)** Composite of phase contrast images and fluorescence signal from Hoechst DNA stain (red) and SMP1::GFP (green) marking the flagellar membrane, taken at different steps of the protocol: (i) whole *L. mexicana* cells before deflagellation, (ii) after deflagellation, (iii) flagellar fraction, (iv) cell body fraction. Scale bar is 20 μm. Image adapted from [28]. **(C)** The flagellar and cell body fractions were separated into 1% octylglucoside soluble (Fs, Cs) and insoluble (Fi, Ci) fractions, separated alongside whole cell lysates (*L. mex* SMP1::GFP) on a 10%

polyacrylamide gel and stained with SYPRO Ruby Protein Gel Stain (Molecular Probes). Distinct banding patterns are observed for each fraction. The numbers below the gel picture indicate the cell equivalent for the amount of protein loaded.

Funding statement

TB was supported by a Medical Research Council PhD studentship (15/16_MSD_836338), FD was supported by an Erasmus grant, RW is a Sir Henry Dale Fellow, supported by Wellcome Trust grant 211075/Z/18/Z, EG is a Royal Society University Research Fellow.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Cell bodies

Figure 1

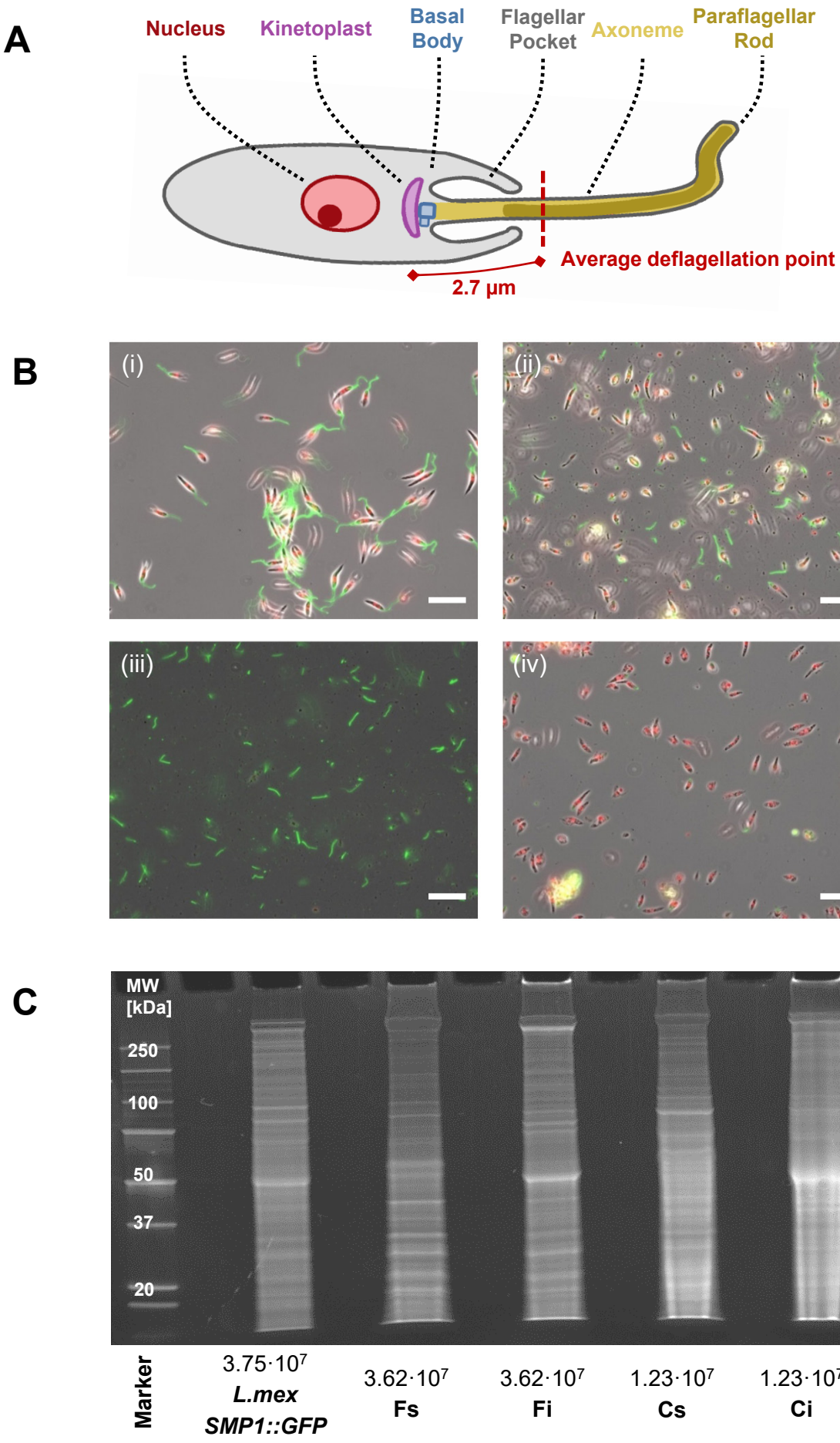
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Figure 2