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Running title: Purification of tubulin from cultured cells

Abstract

A method was designed to purify tubulin from limited volumes of cultured cells, which can be performed in less than 4 hours. The method is based on the preservation of intact microtubule arrays during cell lysis in a large volume of buffer, followed by disassembly of microtubules in a small volume of cold buffer. This allows a good enrichment in tubulin, which is then purified by one cycle of polymerisation/ depolymerisation and a cation exchange chromatography. Such a procedure has been employed successfully on suspension-cultured and on adherent HeLa cells. Tubulin obtained was ≥90% pure, assembly-competent and composed of alpha/ beta I and alpha/ beta IV isotypes. Microtubules made with this tubulin displayed specific properties such as resistance to dilution, maybe related to their specific dynamic behaviour.

Keywords: tubulin/ microtubule/ purification/ HeLa

Abbreviations: MAPs, microtubule-associated proteins; HTub, tubulin purified from HeLa cells; PCTub, tubulin purified from bovine brain; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenyl-methyl-sulfonyl-fluoride.

Introduction

In all eukaryotic cells, the heterodimeric alpha/beta tubulin is the subunit of the microtubules and therefore plays a key role in intracellular positioning of organelles, in trafficking of vesicles, and in mitosis. According to its central role in cell functions, tubulin is tightly regulated by a variety of effectors associated either with microtubules or with the free tubulin pool [1]. Tubulin is also the target of many anti tumour drugs [2]. Therefore, purification of tubulin is required to study tubulin properties as well as its interactions with effectors and drugs.

Historically, most experiments have been carried out with tubulin purified from mammalian brain tissue [3-6]. The main reason was that tubulin is particularly abundant in brain tissue, where it represents about 20% of total proteins. Attempts to purify tubulin from other sources than brain tissue have faced two difficulties: the low abundance (~3-4%) of tubulin in many cell types, and the low amount of the starting material, comparing with the hundreds of grams common in brain preparations [7].

Three main methods to purify cultured cell tubulin have been described. The first one consisted in four successive cycles of polymerisation/ depolymerisation of cell tubulin followed by DEAE column chromatography [8, 9]. However, the yield of these preparations was relatively low because of the loss of tubulin at each polymerisation and depolymerisation step. The second method to purify cultured cell tubulin included the microtubule stabilising drug Taxol, in order to enhance tubulin polymerisation and yields of purified tubulin [10, 11]. Because of the presence of Taxol, this kind of purification rendered tubulin inadequate for experiments concerning tubulin dynamics and their regulations. A third method has been described, exhibiting better yields than the first method, without the use of Taxol. This method included as a first step of purification a rapid ion exchange fractionation, followed by an elution with sodium glutamate which preserves the tubulin molecule [7]. Using such a

procedure, it has been shown recently that intrinsic properties of tubulin purified from cultured HeLa cells markedly differed from those of brain tissue tubulin [12]. HeLa cell tubulin was found to be different in its beta tubulin isotype composition and to display slower dynamics than brain tubulin.

However, these last observations have been made possible while starting from large volumes (40-70 litres) of suspension-cultured HeLa cells. I propose here an alternative way to purify tubulin from cultured cells. This procedure allows recovering workable amounts of tubulin from smaller volumes (2-5 litres) of suspension-cultured HeLa cells. This method has also been tested successfully on adherent HeLa cells. It is based on the pre-purification of the microtubule polymer fraction of cells, with discarding of the free cytosolic proteins. The tubulin obtained at the end of the purification procedure is MAP-free and competent for polymerisation, with or without nucleation on centrosomes. Interestingly, microtubules made from this HeLa cell tubulin (called HTub) were more resistant to depolymerising conditions than microtubules made from brain tubulin (PCTub). HTub was also more efficiently phosphorylated by some kinases than brain tubulin (Fourest-Lieuvin et al., in preparation).

Materials and methods

Cell culture and total cell extract

HeLa S3 cells were grown at 37°C in suspension cultures of 2-3 litres in MEM Eagle Joklik's formulation medium (Cambrex Bio Science), supplemented with 10 % horse serum (Invitrogen) and 1 % penicillin/streptomycin (Invitrogen). HeLa adherent cells were grown in RPMI medium complemented with 10 % foetal calf serum and 1 % penicillin/streptomycin. For total cell extract, HeLa cells grown at confluence in a 100 mm petri dish were scraped in 500 µl of 1% SDS, sonicated for 1 min and then supplemented with Laemmli buffer.

Purification of HeLa cell tubulin (HTub)

Purification of tubulin was performed either from suspension cultured cells (HeLa S3 cells) or from adherent cells (HeLa). During the first steps of the purification, cell microtubule networks in cells have to be preserved. Therefore, centrifugations were performed at 37°C. If the centrifuge that is used cannot raise the temperature, its nacelles must be warmed at 37°C before the first centrifugation.

Two litres of exponentially growing HeLa S3 cells at a density of about 10^6 cells/ml (a total of ~2.10^9 cells) were centrifuged at 320g for 3 min at 37°C. When starting from adherent HeLa cell cultures, ~2.10^9 cells were rinsed with warm PBS, trypsinized at 37°C and trypsin was neutralised in warm culture medium. Cells were then collected by centrifugation as above. In both cases, the cell pellets were pooled in 40 ml of PEM (100 mM Pipes pH 6.7, 1 mM EGTA, 1 mM $MgCl_2$) at 37°C and centrifuged as above. The cell pellet was resuspended for cell lysis in 40 ml of OPT buffer at 37°C (OPT for "OPTimum for microtubule preservation"; 80 mM Pipes pH 6.7, 1 mM EGTA, 1 mM $MgCl_2$, 0.5% Triton-X100, 10% Glycerol, 1 µM pepstatine, 400 µM PMSF). Lysed cells were centrifuged at 320g for 3 min at 37°C and the supernatant was discarded carefully. The pellet was resuspended with shearing

in 2 ml of OPT at 4°C and incubated for 15 min on ice. At the end of this incubation, the pellet was sheared thoroughly once again, and ultracentrifuged at 200,000g for 10 min at 4°C. The supernatant, called HOPT extract, was collected. I obtained routinely 3.8-4 ml of HOPT extract. This extract could be frozen at –80°C or processed directly as described below.

Nine ml of HOPT extracts were supplemented with 5 mM $MgCl_2$, 1 mM GTP, and 5% DMSO (final concentrations). The solution was then incubated for 30 min at 35°C to allow microtubule polymerisation. The polymerised sample was laid on a cushion of PEM/ 60% glycerol/ 400 µM PMSF at 35°C, and ultracentrifuged at 200,000g for 20 min at 35°C. The microtubule pellet was washed carefully, without resuspension, with 3 ml of warm (35°C) PEM50 (50 mM Pipes pH 6.7, 1 mM EGTA, 1 mM $MgCl_2$, 1 µM pepstatine and 400 µM PMSF). Cold PEM50 (600 µl) was then added on the pellet, which was depolymerised on ice for 15 min before resuspension. The resulting tubulin suspension was cleared by ultracentrifugation at 200,000g for 10 min at 4°C and called HTub1x. A cation exchange chromatography was then performed using 300 µl of packed Fractogel® EMD SO_3^- (Merck) pre-equilibrated with 20 volumes of PEM50/ 400 µM PMSF. HTub1x was batch-absorbed to the 300 µl of Fractogel into a disposable Poly-Prep® column (Biorad). The HTub1x-Fractogel slurry in the column was mixed gently on a tabletop rocker for 15 min at 4°C before spinning at 430g for 1 min at 4°C. The filtrate was composed most exclusively of HeLa cell tubulin and was called HTub. The concentrations of Pipes and GTP were then raised to 100 mM and 1 mM, respectively, before storage at –80°C. The concentration and purity of HTub was evaluated by SDS-PAGE, comparing with known concentrations of bovine brain tubulin. Tubulin purification from bovine brain was performed as described [13]. Relative quantities of tubulins on a gel were evaluated using the Quantity one® software (Biorad).

Turbidimetry assay

A 50 µl solution of 20 µM HTub in PEM/ 10 % glycerol plus 1mM GTP and 5 mM $MgCl_2$ was incubated at 37°C in a polystyrene 96-well plate with flat bottom wells (Costar, Corning). Tubulin assembly was monitored by measuring optical density at 350 nm (OD_{350}) every 30 sec in a Spectra Max Plus spectrophotometer (Molecular Devices).

Aster assembly on isolated centrosomes

Centrosomes purified as described [14] from KE37 human lymphoid cell line were a gift from Dr A.V. Popov. A solution of 0.5 µl centrosomes (~ 10,000 centrosomes), 5 mM $MgCl_2$, 1 mM GTP was added to HTub (20 µM) in a total volume of 20 µl. Asters of microtubules were allowed to grow for 20 min at 37°C and cross-linked in 1 ml of 1% glutaraldehyde/ PEM before centrifugation onto coverslips as described [15]. Asters on coverslips were then processed for immunofluorescence.

Test of microtubule resistance to dilution

The capacity of HeLa cell microtubules to resist to depolymerising conditions such as dilution was tested. HeLa cell tubulin and brain tubulin were assembled and then centrifuged onto coverslips before or after dilution as follows.

HTub or PCTub at 25 µM in PEM/ 10 % glycerol were supplemented with 1 mM GTP and 5 mM $MgCl_2$. Aliquots (20 µl) of each solution were incubated for 35 min at 37°C. One aliquot of HTub and one of PCTub were crosslinked in 2 ml of 1% glutaraldehyde/ 60% glycerol/ PEM for control of polymerisation. The other aliquots were diluted to 1:25 (final volume 500 µl) in OPT buffer pre-warmed at 37°C, gently mixed by inverting the tubes, and incubated again for 15 min at 37°C. These aliquots were then crosslinked by adding 1.5 ml of 1.3% glutaraldehyde/ 60% glycerol/ PEM. Samples were centrifuged through a PEM-10%

glycerol cushion on glass coverslips at 65,000g for 30 min at room temperature. Glass coverslips were then retrieved and fixed for immunofluorescence as described below.

Immunofluorescence

Microtubules and asters of microtubules on coverslips were fixed in cold (-20°C) methanol, and stained with anti beta tubulin TUB2.1 monoclonal antibody (Sigma) and Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories). Immunofluorescence staining was visualised on a Zeiss Axioscop conventional microscope.

Western blotting

After SDS PAGE and transfer, blots were incubated with anti-beta I or anti-beta IV tubulin monoclonal antibodies (Abcam, Cambridge, UK) as primary antibodies, and then with a peroxidase-conjugated goat anti-mouse IgG antibody (Sigma).

Results and discussion

Purification of HeLa cell tubulin

The tubulin purification procedure described here was based on two important points. Firstly, the microtubule arrays were kept as intact as possible by performing the first steps of the purification at physiological temperature (Fig. 1 steps 1 to 3, see also Materials and Methods). The cells were maintained at 37°C during centrifugations, during washing in PEM and during cell lysis. Secondly, cell lysis was performed in a large volume of lysis (OPT) buffer (Fig. 1, step 3), in order to dilute soluble proteins that were not associated with the cytoskeleton. Lysing cells in a large volume of buffer led to the loss of the cellular free tubulin pool, which represents about half of total cell tubulin [16]. Nonetheless, this loss was the price to pay for an easy and fast pre-purification of tubulin.

After this cell lysis, insoluble material, comprising nuclei and the whole cytoskeleton, was pelleted and resuspended in a small volume of cold OPT buffer (Fig. 1, step 4). The cold temperature (4°C) depolymerised microtubules. Nuclei, and other cold-resisting components, were then pelleted by ultracentrifugation. The supernatant, called HOPT extract, was largely enriched in tubulin (Fig. 2, lane 2), as compared to a whole cell extract (Fig. 2, lane1).

To further eliminate proteins that were not directly associated with microtubules or tubulin, a cycle of polymerisation/ depolymerisation of the tubulin present in HOPT extract was performed (Fig. 1, steps 5 and 6). After this polymerisation/ depolymerisation cycle and an ultracentrifugation, the resulting suspension contained mainly tubulin plus MAPs (HTub1x; Fig 2, lane 3). For example, the non-neuronal MAPs TOGp and E-MAP115 could still be detected by Western blotting at this step (data not shown).

Tubulin was separated from MAPs by performing a cation exchange chromatography (step 7), which binds positive-charged MAPs. HTub1x was batch-absorbed to the cation exchange gel in a disposable column, before spinning the column. The spinning step allowed

the recovery of the column filtrate while avoiding diluting it too much. The filtrate contained MAP-free tubulin, HTub (Fig 2, lane 4). However, sometimes, a faint band at 70 kDa was visible on gels. This band has been analysed by mass spectrometry and has revealed to be Hsc 70 (data not shown). Hsc 70 is often found associated with HeLa cell tubulin [9, 17], although it has no direct effect on microtubule polymerisation (V. Gache, unpublished data).

As our purification method comprised a cell lysis step in a Triton X100-containing buffer (OPT buffer), Triton X100 might be still present in the final solution of HTub tubulin. For this reason, I always evaluated tubulin concentration by SDS PAGE instead of by measuring OD_{280}.

The entire purification procedure lasted about 4 hours. Nevertheless, it was more convenient to split the procedure: as HOPT extracts could be frozen, it was possible to accumulate some of these extracts before continuing the purification.

Estimation of the tubulin purification yield

From 3 litres of exponentially growing cells in suspension cultures (~3.10^9cells), it was possible to recover 5.5 to 6 ml of HOPT at ~2 mg/ml of tubulin, i.e. a total of 10-12 mg of tubulin. From this amount of HOPT extract, 1 mg of purified HTub was routinely obtained. This means a recovery of about 10% of the tubulin contained in HOPT extracts. Therefore, using this purification method, 1 mg of tubulin was typically obtained from 3.10^9 cells. As HeLa tubulin could polymerise at a lower critical concentration than brain tubulin [12], it was possible to perform more experiments with 1 mg of HeLa tubulin than with the same amount of brain tubulin.

Polymerisation of HTub

To assess that HTub was competent for microtubule assembly, a turbidimetry assay was performed (Fig 3A). This assay showed that 20 μM HTub could assemble following a sigmoid curve as expected [18]. When the sample was exposed to cold temperature for 10 min, OD_{350} nearly returned to its basal level, showing that HTub assembled into cold-sensitive genuine microtubules. This last point was confirmed by experiments of centrifugation of microtubules onto glass coverslips after polymerisation, and immunostaining of these microtubules (Fig 4B).

Finally, I also showed that HTub could be nucleated on centrosomes, forming asters of microtubules with a length of about 40 μm in my conditions (Fig 3B).

HTub properties

In a previous work, we showed that HeLa and NIH3T3 cell microtubules were intrinsically stable after cell lysis, resisting to depolymerising conditions like dilution or cold temperature [16]. These properties contrasted with those of purified bovine brain microtubules polymerised in vitro, which are labile when exposed to cold temperature or to dilution. Stability of lysed cell microtubules has been attributed to their association with MAPs, as it was the case for cold stability induced by association of microtubules with STOP proteins [19, 20]. However, many cell types, like HeLa cells, did not contain any STOP protein. Their microtubules did not resist to cold temperature but still resist to dilution [16]. A MAP specifically responsible for resistance to dilution has not been found yet. Therefore, I tested whether HeLa cell tubulin, without any MAP, could form microtubules resisting dilution.

Solutions of HTub or PCTub were allowed to polymerise, and then diluted to 1:25 in warm OPT buffer during 15 min. Microtubules were crosslinked before (Fig 4 A, B) or after

dilution (Fig 4 C, D), then centrifuged onto coverslips and stained with an anti-beta tubulin antibody. PCTub microtubules entirely disassembled after dilution (Fig 4C), while an appreciable amount of HTub microtubules remained in same conditions (Fig 4D). When quantified using a quantitative filter assay as in ref. [21], the amount of remaining HTub microtubules represented 15 ± 1% of the total amount of polymerised HTub (data from 4 independent experiments). Consequently, purified HeLa cell microtubules are intrinsically more resistant to dilution than purified bovine brain microtubules.

This difference between bovine brain and HeLa cell microtubules could be explained by differences in their dynamic properties. Microtubules made of brain tubulin exhibit in vitro a well-described dynamic behaviour called dynamic instability [22-24]. In this process, microtubule ends switch between periods of growth, rapid shortening and pause. The transition between growth and shortening is called “catastrophe”, and the transition shortening-growth is called “rescue”. Newton et al. [12] showed that purified HeLa cell microtubules exhibited minimal dynamic instability in vitro. These microtubules grew very slowly, they rarely transitioned to rapid shortening, and spent a majority of the time in a paused state. In addition, the catastrophe frequency of HeLa cell microtubules was very low (13% of that for bovine brain microtubules). Accordingly, it seems possible that HeLa cell microtubules were less affected by dilution because of their reduced intrinsic catastrophe frequency.

Newton at al. [12] also showed that beta tubulin isotype composition of HeLa tubulin differed from that of brain tubulin. HeLa beta tubulin is composed of ~80% beta I and 20% beta IV tubulin, while brain beta tubulin is composed mainly of beta II and beta III tubulins, with only 14% beta IV and 3 % beta I tubulins. As the method for tubulin purification described here was different of that of Newton and collaborators, the presence of beta I and beta IV tubulin isotypes in HTub was tested. Both beta I and beta IV isotypes were detected in

HTub, the beta I isotype being predominant (Fig 5). Therefore, this method, including the pre-purification of microtubule networks, does not seem to select one peculiar beta isotype.

This isotype composition of HTub may explain its relative stability to dilution. Previous evidence has shown that microtubules made from different beta tubulin isotypes have different polymerisation properties as well as different dynamic instability behaviours [25-28]. For instance, microtubules composed of alpha/ beta III tubulin are more dynamic than microtubules composed of only alpha/ beta II or alpha/ beta IV tubulins [25]. If stability to dilution is relied to dynamic properties, it seems possible that stability of HTub microtubules is a consequence of their alpha/ beta I and alpha/ beta IV tubulin content.

Conclusion

Here is described a cell tubulin purification method that allows the obtention of purified tubulin starting from reasonable amounts of cultured cells. This method could be performed both on suspension cultured or adherent HeLa cells. Although it was not tested in this study, this protocol should work as well on other cell types. I found that tubulin purified according to this protocol was comparable with tubulin purified by Newton et al. [12]. Moreover, we show in another work that HeLa cell tubulin is a better substrate than brain tubulin for some protein kinases, like Cdk1 (Fourest-Lieuvin et al., manuscript in preparation). Therefore, this purified cell tubulin has revealed to be very useful for studies of tubulin regulations by cellular effectors such as protein kinases or phosphatases.

Acknowledgments

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Figure legends

Figure 1. Diagram illustrating the HTub tubulin preparation. See details in Materials and Methods.

Figure 2. Purification of HTub tubulin from HeLa cells. Equal volumes of each sample were loaded on a 7.5% acrylamide gel for SDS PAGE. Samples were: (1) Total extract of HeLa cells; (2) HOPT extract; (3) HTub1x; (4) HTub. Molecular weights are indicated in kDa.

Figure 3. HTub tubulin is competent for microtubule assembly. (A) The polymerisation of HTub (20 μM) was monitored by turbidimetry at 350 nm during 15 min at 37°C. The sample was then incubated on ice for 10 min, and the OD_{350} was checked again at the end of the incubation on ice. (B) HTub (20 μM) was mixed with centrosomes and incubated for 20 min at 37°C before fixation. Asters were centrifuged onto coverslips and immunostained with an anti beta tubulin antibody. Bar 40 μm.

Figure 4. HTub microtubules are resistant to dilution. PCTub (A, C) or HTub (B, D) at 25 μM were polymerised at 37°C for 35 min, then diluted to 1:25 in buffer at 37°C and incubated again for 15 min before crosslinking. Microtubules were centrifuged onto coverslips before immunostaining with an anti beta tubulin antibody. (A, B) Controls of polymerisation. (C, D) Diluted samples. Bar 20 μM.

Figure 5. HTub is composed of alpha/ beta I and alpha/ beta IV tubulin isotypes. HTub (10 μg) and PCTub (15 μg) were loaded on a gel and processed for SDS PAGE and Western blotting (WB) with anti-beta I tubulin monoclonal antibody (left panel) and with anti-beta IV tubulin monoclonal antibody (right panel). Ponceau staining of blots is also shown, as indicated.

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Fig 1 purification of tubulin

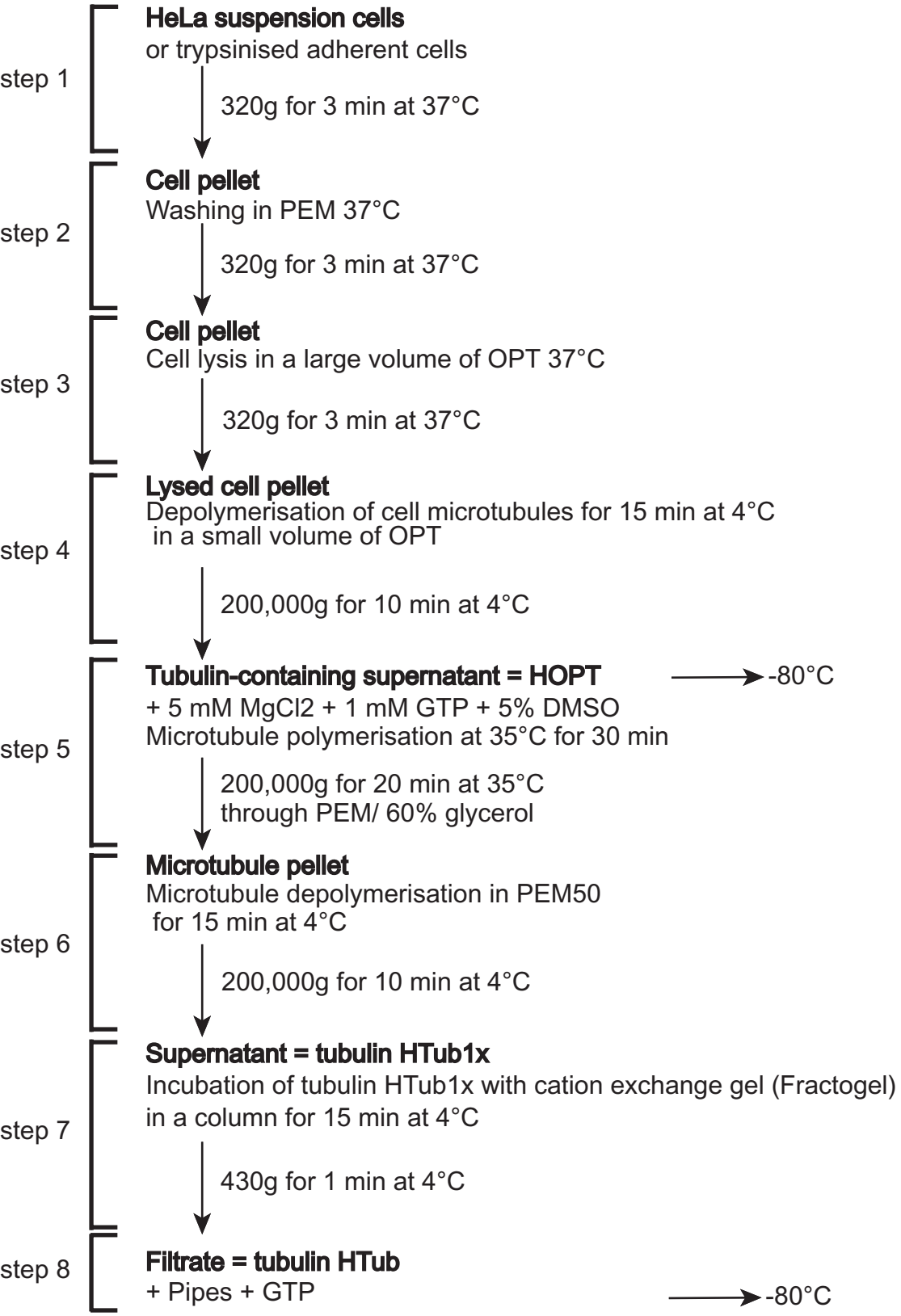


Fig 1, Fourest-Lieuvin

Fig 2 purification of tubulin

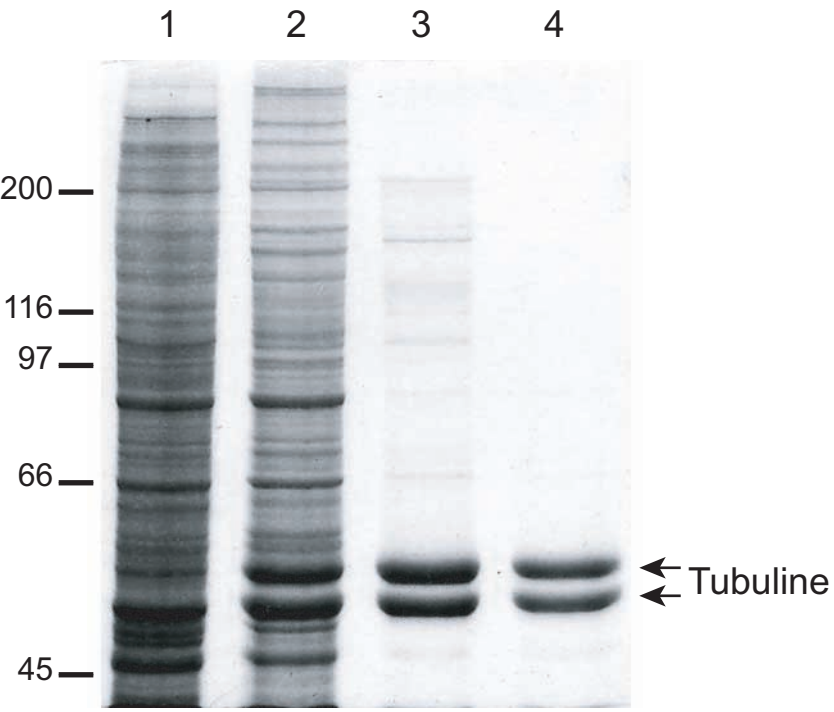
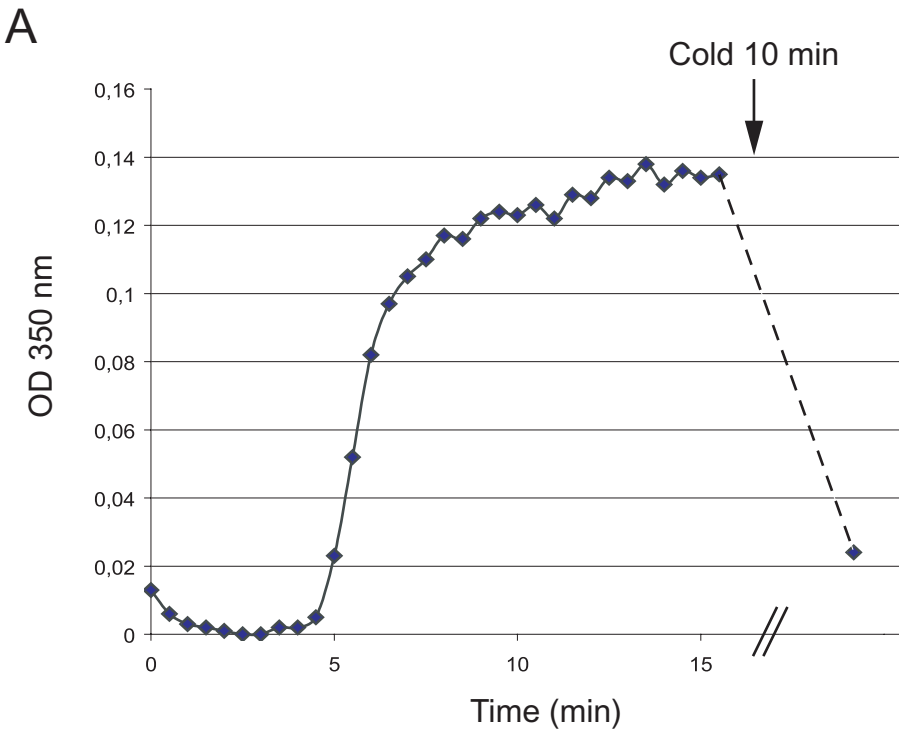


Fig 2, Fourest-Lieuvin

Fig 3 purification of tubulin



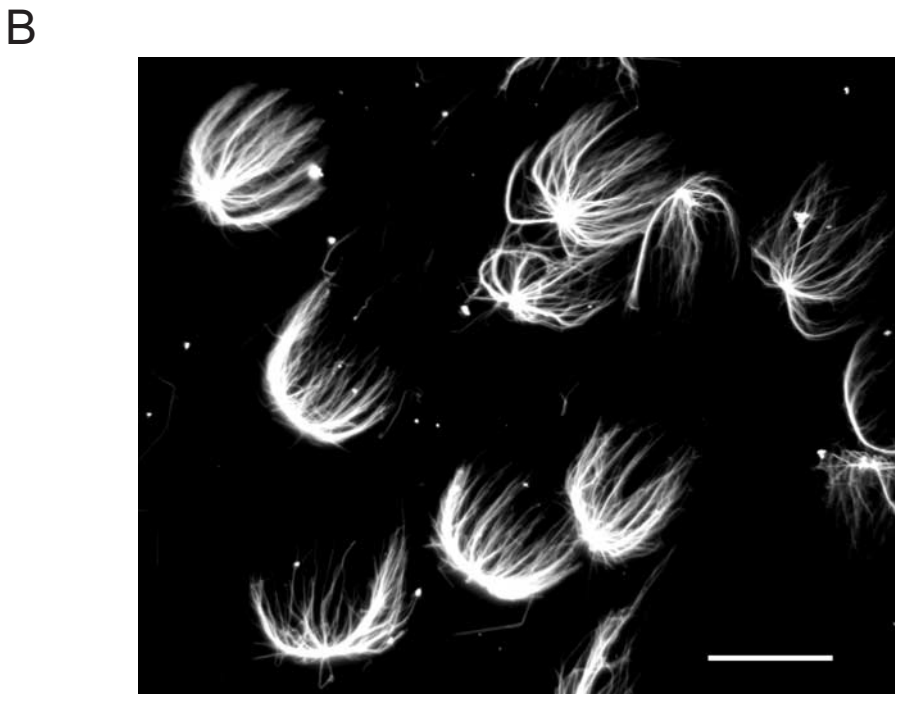


Fig 3, Fourest-Lieuvin

Fig 4 purification of tubulin

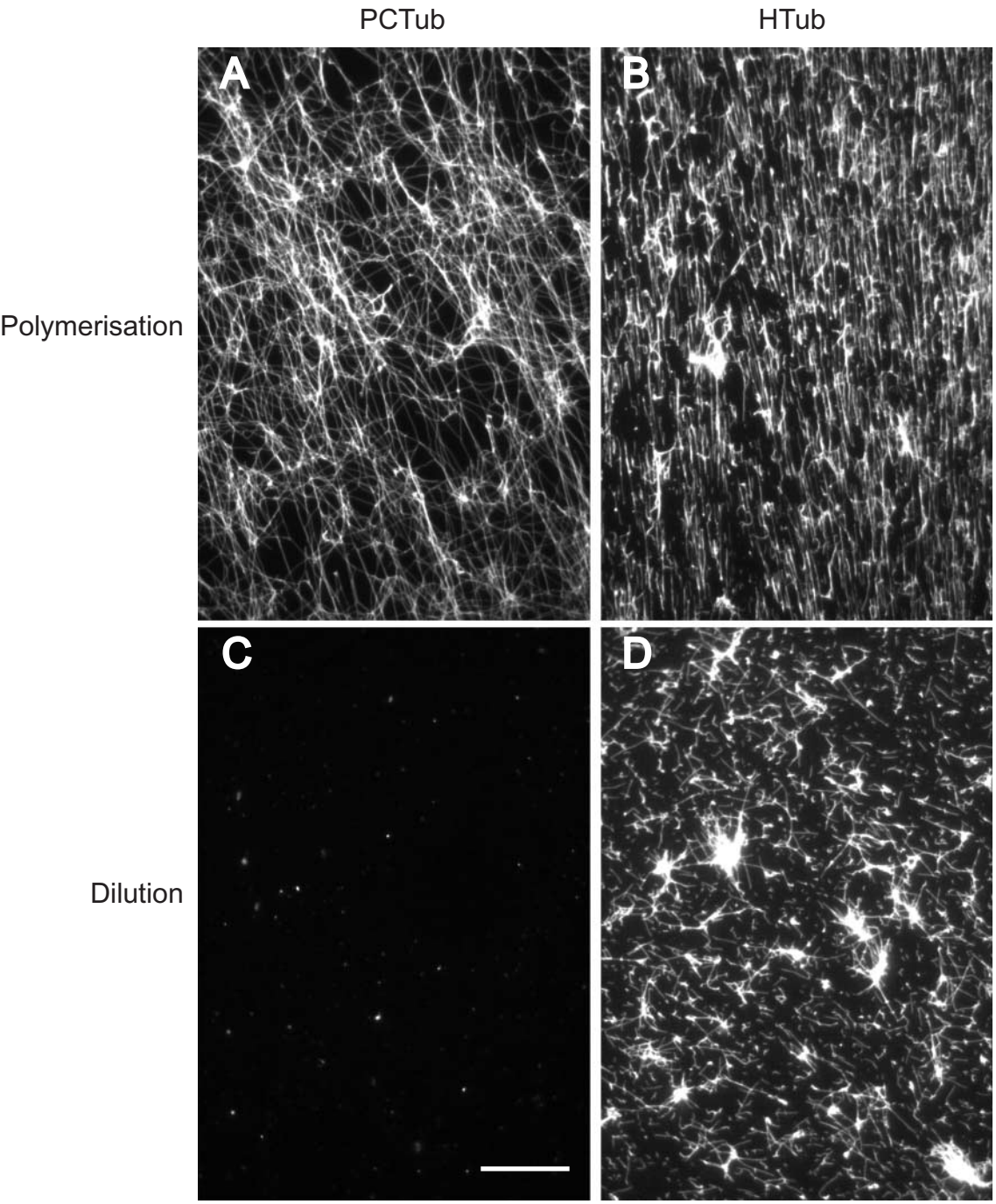


Fig. 4, Fourest-Lieuvin

Fig 5 purification of tubulin

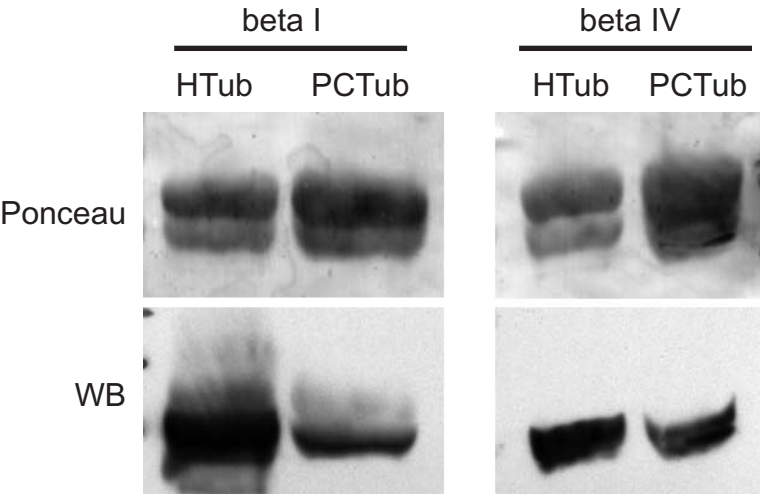


Fig. 5, Fourest-Lieuvin