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Title:

Semi-retentive cytoskeletal fractionation (SERCYF): a novel method for the biochemical analysis of the organization of microtubule and actin cytoskeleton networks

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Abstract

A variety of biochemical fractionation methods are available for the quantification of cytoskeletal components. However, each method is designed to target only one cytoskeletal network, either the microtubule (MT) or actin cytoskeleton, and non-targeted cytoskeletal networks are ignored. Considering the importance of MT–actin crosstalk, the organization of both the targeted and non-targeted cytoskeletal networks must be retained intact during fractionation for the accurate analysis of cytoskeletal organization. In this study, we reveal that existing fractionation methods, represented by the MT-sedimentation-method for MTs and the Triton X-100 solubility assay-method for actin cytoskeletons, disrupt the organizations of the non-targeted cytoskeletons. We demonstrate a novel fractionation method for the accurate analysis of the cytoskeletal organizations using a taxol-containing PEM-based permeabilization buffer, which we name “semi-retentive cytoskeletal fractionation (SERCYF)-method”. The organizations of both MTs and actin cytoskeletons were retained intact even after permeabilization with this buffer. By using the SERCYF-method, we analyzed the effects of nocodazole on the cytoskeletal organizations biochemically and showed promotion of the actin cytoskeletal organization by MT depolymerization.

Keywords.

cytoskeleton, microtubule, actin, nonmuscle myosin II, fractionation

Abbreviations:

MT, microtubule; NMHC, nonmuscle myosin heavy chain; FA, focal adhesion; MTS, MT sedimentation; TSA, Triton X-100 solubility assay; PEM, PIPES-EGTA-magnesium; PEMT, PEM-Triton X-100; PEMTT, PEMT-taxol; SERCYF, semi-retentive cytoskeletal fractionation

1. Introduction

Microtubules (MTs) and actin cytoskeletons are abundant subcellular components that regulate cell shape and motility [1,2]. Their organizations change spatiotemporally through dynamic assembly/disassembly events in response to various signaling cues. The dynamic behaviors of the MTs and actin cytoskeletons are mediated by their binding proteins, post-translational modifications, in addition to their polymerization/depolymerization. Moreover, MTs and actin cytoskeletons crosstalk to regulate their dynamics and/or stability with each other [3], which is important for cell motility. MT–actin crosstalk is mediated by many factors, including linker proteins and small GTPases. The loss of MT–actin crosstalk impairs cell motility.

We can analyze the cytoskeletal organizations biochemically by estimating the percentage of cytoskeletal components that are incorporated into the assembled cytoskeletons. This relies on several fractionation methods that prepare cytoskeletal (insoluble) and cytoplasmic (soluble, free) fractions of MTs [4–6] and actin cytoskeletons [7–10]. In these methods, cells are permeabilized with a surfactant-containing buffer, and then subcellular components are separated into cytoskeletal and cytoplasmic fractions through a method-specific procedure. In most cases, the fractionated proteins are quantified by immunoblot analysis.

To perform an accurate analysis of MT and actin cytoskeleton networks, the integrity of both cytoskeletons must be maintained throughout the experiment because they affect each other. However, the targeted cytoskeleton of the existing methods is either the MT or actin cytoskeleton. In other words, the organization of the non-targeted cytoskeletons is ignored, thus, they may exhibit abnormal organization. Irregularities in the non-targeted cytoskeletons may affect the organization of the targeted cytoskeletons due to the existence of MT–actin crosstalk, leading to data misinterpretation.

In this study, we aimed to perform an accurate analysis of the organization of MT and actin cytoskeleton networks. We report the establishment of a novel fractionation method for the accurate biochemical analysis of MTs and actin cytoskeletons simultaneously, which is not possible using

existing methods.

2. Materials and methods

2.1. Cell culture and drug treatment

MRC-5 TG1 SV1 cells (SV40-transformants of MRC-5; human embryonic lung fibroblasts) (RIKEN Cell Bank, Tsukuba, Japan) were maintained in Minimum Essential Medium Eagle (MEM) alpha (GIBCO/Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (BioSource/Life Technologies), 50 U/mL penicillin and 50 µg/mL streptomycin (GIBCO). Cells were cultured at 37°C in humidified air containing 5% CO₂. Where indicated, nocodazole (Millipore Calbiochem, Billerica, MA, USA) was added to the culture medium at a final concentration of 10 µM 1 hour before analysis.

2.2. Immunofluorescence microscopy

A rapid fixation with methanol was adopted to avoid the MT depolymerization. Cells cultured on fibronectin- (10 µg/mL; Roche Diagnostics, Basel, Switzerland) coated coverslips (Matsunami, Kishiwada, Japan) were fixed and permeabilized with cold methanol for 5 min at -20°C. For the observation of pre-permeabilized cells, gentle permeabilization was performed prior to fixation. Buffer compositions and conditions are listed in Table 1. Fixed cells were incubated with blocking buffer (3% BSA in PBS) for 15 min, followed by incubation with antibodies in the blocking buffer. Each incubation step was performed for 1 hour at 25°C. The primary antibodies were diluted as follows: mouse anti-β-actin monoclonal antibody (1:5,000) (AC-15; SIGMA-Aldrich, St. Louis, MO, USA), rabbit anti-nonmuscle myosin heavy chain (NMHC)-IIA polyclonal antibody (1:5,000) [11], rabbit anti-NMHC-IIB polyclonal antibody (1:5,000) [12], and mouse anti-vinculin monoclonal antibody (1:5,000) (hVIN-1; SIGMA-Aldrich). The secondary antibodies (Jackson ImmunoResearch Laboratories; West Grove, PA, USA) were diluted as follows: Cy3-labeled anti-mouse IgG (1:500),

AMCA-labeled anti-rabbit IgG (1:200), and Alexa Fluor 488-labeled anti-rabbit IgG (1:200). Direct immunofluorescence of microtubules was performed using a FITC-labeled mouse anti- α -tubulin monoclonal antibody (1:1,500) (a kind gift from Dr. Keiju Kamijo, Tohoku Medical and Pharmaceutical University, Sendai, Japan). Immunofluorescence images were obtained as previously described [8]. Image processing was performed using ImageJ software.

2.3. Fractionation by MTS-method

The MT sedimentation (MTS)-method was performed as described by Nagae *et al.* [4] (see also Table 1). Cells were washed with pre-warmed (37°C) PBS and gently permeabilized with pre-warmed MTS-buffer, followed by incubation at 37°C for 15 min with gentle shaking every 5 min. The permeabilized cells were scraped with a cell scraper and collected into a plastic tube, followed by centrifugation ($110,000 \times g$, 1 hour, 37°C). The resulting supernatant was transferred into a new plastic tube and mixed with 2 $\times$ SDS sample buffer [100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.2% bromophenolblue] supplemented with β -mercaptoethanol (β -ME) at a final concentration of 5% (hereafter, the 2 $\times$ SDS sample buffer always contained β -ME) (free fraction). The resulting pellet was resuspended with SDS lysis buffer [10 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1% SDS] and mixed with 2 $\times$ SDS sample buffer (cytoskeletal fraction). The samples were further denatured by boiling for 3 min.

2.4. Fractionation by TSA-method

The Triton X-100 solubility assay (TSA)-method was performed as previously described [8] (see also Table 1). Cells were washed with pre-warmed PBS and gently permeabilized with TSA-buffer, followed by incubation at 25°C for 3 min with gentle shaking every 1 min. The permeabilized cell extract was collected into a plastic tube, followed by centrifugation ($22,000 \times g$, 3 min, 4°C). The resulting supernatant was transferred into a new plastic tube and mixed with 2 $\times$ SDS sample buffer. The permeabilized cells remaining in the well were lysed with 2 $\times$ SDS sample buffer and collected into

a plastic tube. The samples were further denatured by boiling for 3 min.

2.5. Fractionation by SERCYF-method

Cells were cultured on separate plates when two or more samples were prepared, to avoid excessive permeabilization or the peel-off of cells from wells during the handling of other samples. Cells were washed with pre-warmed PIPES-EGTA-magnesium (PEM)-buffer [100 mM PIPES-NaOH (pH 6.8), 1 mM EGTA, 2 mM MgCl₂] and gently permeabilized with pre-warmed PEMTT-buffer [0.2% Triton X-100, 100 nM taxol in PEM], followed by incubation at 37°C for 1 min without shaking. The permeabilized cell extract was collected into a plastic tube. Pre-warmed PEM-buffer was gently added to the residual cells, and the resulting rinsed buffer was immediately collected into the same tube. This fraction was mixed with 2×SDS sample buffer. After rinsing, the residual cells were lysed with 2×SDS sample buffer and collected into a plastic tube. The samples were further denatured by boiling for 3 min. During the development of semi-retentive cytoskeletal fractionation (SERCYF)-method, PEMT-buffer [0.2% Triton X-100 in PEM], instead of PEMTT-buffer, was used (see also the Results and Table 1 for detailed information).

2.6. Immunoblot analysis

SDS-PAGE was performed using standard techniques. Separated proteins were transferred to an Immobilon-P PVDF membrane (Millipore). The membrane was incubated with blocking buffer [5% skimmed milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.05% Tween-20)] for 30 min. The membrane was then incubated with the primary antibodies in blocking buffer overnight at 4°C, followed by the secondary antibodies for 1 hour at 25°C. The primary antibodies were diluted as follows: mouse anti- α -tubulin monoclonal antibody (1:2,000) (B-5-1-2; SIGMA-Aldrich), mouse anti-acetyl- α -tubulin monoclonal antibody (1:500) (6-11B-1; SIGMA-Aldrich), mouse anti- β -actin monoclonal antibody (1:2,000), mouse anti-vinculin monoclonal antibody (1:500), mouse anti-GAPDH monoclonal

antibody (1:30,000) (6C5; Millipore), rabbit anti-NMHC-IIA polyclonal antibody (1:60,000), and rabbit anti-NMHC-IIB polyclonal antibody (1:2,000). The secondary antibodies (Bio-Rad Laboratories, Hercules, CA, USA) were diluted as follows: HRP-labeled anti-mouse IgG (1:10,000) and HRP-labeled anti-rabbit IgG (1:10,000). The chemiluminescent signal was produced using the Immobilon Western Chemiluminescent HRP Substrate (Millipore) and detected using a LAS-3000 (Fujifilm, Tokyo, Japan). The percentages of cytoskeletal fractions were calculated by densitometry using ImageJ software.

3. Results

3.1. Cell permeabilization with MTS- or TSA-buffer induces cytoskeletal disruption

First, we examined whether the MT and actin cytoskeleton networks are retained intact after permeabilization with existing fractionation buffers. Although several investigators have performed cytoskeletal fractionations, there is no established protocol using the common buffer composition and procedure. Here, we tested whether the MTS- [4] and TSA- [8] methods, the representatives of the existing methods which are designed for MTs and actin cytoskeletons, respectively, can retain both cytoskeletons (Table 1). SV1 cells were used in this study because they exhibit prominent MTs and stress fibers (contractile structures mainly consisting of actin filaments and nonmuscle myosin II filaments [13]) (Fig. 1A). Cells were permeabilized with MTS- or TSA-buffer prior to fixation and immunostaining, according to method-specific conditions. After treatment with MTS-buffer, MTs were visible, however, their morphology was abnormal; they were bundled, a characteristic of hyperstable MTs (Fig. 1A). Furthermore, actin cytoskeletons were also abnormal, with thick and winding bundles. This defect could be related to the removal of NMHC-IIA, a major isoform of NMHC-II in SV1 cells [11], from the actin cytoskeleton. Consistent with these results, immunoblot analysis showed that subpopulations of α -tubulin and β -actin, but not NMHC-IIA, were present in the cytoskeletal fraction (Fig. 1B-C). Acetylated α -tubulin, a modified form that accumulates in stable

MTs [14], was mainly detected in the cytoskeletal fraction. In the case of the TSA-method, immunofluorescence microscopy showed that, while the distribution of actin cytoskeletons was retained intact, most MTs were depolymerized during permeabilization (Fig. 1A). Immunoblot analysis showed similar results; while subpopulations of β -actin and NMHC-IIA were present in the cytoskeletal fraction, almost all α -tubulin was present in the cytoplasmic fraction (Fig. 1B-C). In addition, acetylated α -tubulin was detected in cytoskeletal and cytoplasmic fractions, indicating that a subset of MTs was improperly fractionated into the cytoplasmic fraction. Altogether, these results demonstrate that the MTS- and TSA-methods do not reflect the precise cytoskeletal organization in cells because of their partial disruption during permeabilization.

3.2. PEM-based permeabilization buffer has a potential for non-disruptive cytoskeletal fractionation

To overcome the disadvantages of the existing fractionation methods, we investigated an appropriate buffer for non-disruptive cytoskeletal fractionation. PEM-buffer, which is commercially available as a “general tubulin buffer”, is often used to analyze MTs. Furthermore, Gundersen and his colleagues used PEM-Triton X-100 (PEMT)-buffer for cell permeabilization and subsequent MT observation [15]. Although they made no mention of the integrity of the actin cytoskeletons after the PEMT-buffer treatment, the overall cell shape appeared to be unaffected, implying that the cells maintained actin cytoskeletons properly. To test this possibility, we performed immunofluorescence microscopy after permeabilization with PEMT-buffer (Fig. 2A). After a 60 sec permeabilization, we found that the organizations of the actin cytoskeletons as well as MTs were retained intact, indicating that PEMT-buffer has a potential for the non-disruptive fractionation of the both cytoskeletons. However, we also found that, while the overall organization of the actin cytoskeletons was not affected by a 180 sec permeabilization, the MT density decreased with permeabilization time, indicating that MTs were gradually depolymerized. For biochemical analysis, we fractionated cells into cytoskeletal and cytoplasmic fractions as illustrated in Fig. 2B. We analyzed the permeabilization-time

dependencies of the fractionated proteins and found that MTs were depolymerized during permeabilization; the percentage of cytoskeletal α -tubulin was constant (~50%) from 30 to 90 sec and significantly decreased (~40%) at 120 sec after permeabilization (Fig. 2C, E), which was comparable to the results of immunofluorescence microscopy.

3.3. Semi-retentive cytoskeletal fractionation is achieved by addition of low-dose taxol to PEMT-buffer

To block MT depolymerization during PEMT-buffer treatment, we added a low dose (100 nM) of taxol to the PEMT-buffer (termed PEMTT-buffer). Immunofluorescence microscopy revealed that permeabilization with PEMTT-buffer did not induce MT depolymerization, even after a 180 sec treatment (Fig. 2A). Notably, bundled MTs were not observed after the PEMTT-buffer treatment, confirming that this concentration of taxol did not induce MT hyperstabilization. Immunoblot analysis revealed that the percentage of cytoskeletal α -tubulin was constant (~70%) from 30 to 120 sec after permeabilization (Fig. 2D-E), supporting the idea that neither MT depolymerization nor hyperstabilization was induced by the addition of low-dose taxol. Importantly, permeabilization with PEMTT-buffer did not affect the distribution of actin cytoskeletons (Fig. 2A, D-E). Altogether, these results show that the fractionation procedure using PEMTT-buffer enabled the immunofluorescence and biochemical analysis of intact MT and actin cytoskeleton networks. We then defined “60 sec” as the ideal permeabilization time for the new method because the time-dependent percentage changes of α -tubulin, β -actin, and NMHC-IIA were small around 60 sec. Furthermore, acetylated α -tubulin was reasonably detected only in the cytoskeletal fraction (Fig. 2F). Thus, we named this improved fractionation method “semi-retentive cytoskeletal fractionation (SERCYF)-method”, by which we can perform the simultaneous and accurate analysis of the cytoskeletal organization.

We then tested whether the SERCYF-method could be used to study other cell types and/or other types of actin cytoskeletons. For this purpose, we used U-2 OS human osteosarcoma cells because these cells exhibit an extensive lamellipodial actin network during migration. After permeabilization

with PEMTT-buffer for 60 sec, we observed intact MTs and stress fibers by immunofluorescence microscopy (Supplementary Fig. S1). We also observed lamellipodia, indicating that the SERCYF-method did not disrupt the organization of lamellipodia as well as stress fibers. These results strongly support the validity of the SERCYF-method for the analysis of various types of cells and cytoskeletons.

3.4. Biochemical analysis of MT–actin crosstalk by SERCYF-method

MTs and actin cytoskeletons affect each other's organization via crosstalk mediators [3]. For example, GEF-H1, a MT-associated guanine-nucleotide exchange factor for RhoA, is involved in MT–actin crosstalk [16]. Specifically, MT depolymerization induces the release of GEF-H1 from MTs into the cytoplasm, resulting in the reorganization of the actin cytoskeletons through RhoA activation [17,18]. To determine whether this crosstalk can be studied by the SERCYF-method, we assessed the effects of nocodazole, a MT-depolymerizing agent, on the cytoskeletons. Before biochemical analysis, we performed immunofluorescence microscopy of SV1 cells, with or without PEMTT-buffer permeabilization prior to fixation (Fig. 3A). In addition to α -tubulin, β -actin, and NMHC-IIA, we also examined NMHC-IIB (another isoform of NMHC-II) and vinculin, a component of focal adhesions (FAs). Without permeabilization, nocodazole-treated cells lost almost all MTs and exhibited denser stress fibers at the cell center compared with control cells. Vinculin was partially localized to FAs, but mostly dispersed into the cytoplasm of control and nocodazole-treated cells. With permeabilization, control cells exhibited a normal distribution of MTs and actin cytoskeletons. Notably, vinculin was localized only to FAs, indicating that cytoplasmic vinculin was removed during permeabilization. Nocodazole-treated cells exhibited only a few MTs, dense stress fibers, and many FAs. The excessive development of FAs is a characteristic of MT depolymerized cells [19]. These results indicated the existence of MT–actin crosstalk, which is probably mediated through the GEF-H1/RhoA pathway, in SV1 cells. Moreover, permeabilization with PEMTT-buffer prior to fixation is an effective procedure for the removal of cytoplasmic components, without disruption of cytoskeletal organizations.

Finally, we investigated the effects of nocodazole on the cytoskeletons biochemically (Fig. 3B-C). Consistent with the immunofluorescence results, the percentage of cytoskeletal α -tubulin decreased to nearly background level, whereas that of β -actin significantly increased in nocodazole-treated cells. Interestingly, the effects of MT depolymerization on each NMHC-II isoform were different; the percentage of cytoskeletal NMHC-IIA was not affected, whereas that of NMHC-IIB increased in nocodazole-treated cells. These results imply that nonmuscle myosin II-mediated MT-actin crosstalk exists, and that each isoform (IIA and IIB) contributes differently to this crosstalk. Vinculin was mainly detected in the cytoplasmic fraction of both control and nocodazole-treated cells without an apparent difference between groups, indicating that the SERCYF-method is not suitable for the biochemical analysis of FA components. Altogether, these results confirm the usefulness of the SERCYF-method for the simultaneous quantification of MT, actin filament, and nonmuscle myosin II.

4. Discussion

The purpose of this study was to establish a fractionation method for the accurate quantification of the organization of MT and actin cytoskeleton networks. We demonstrated that PEMTT-buffer, but not MTS- or TSA-buffers, was suitable for the non-disruptive cytoskeletal fractionation, which led us to ask why MTS- and TSA-buffers failed to retain cytoskeletal organizations.

The major difference between MTS- and PEMTT-buffers is the presence of glycerol (Table 1). MTS-buffer contains a high concentration of glycerol that prevents MT depolymerization during fractionation. Although glycerol stabilizes MTs by decreasing the critical concentration of tubulin for polymerization *in vitro* [20], which resulted in abnormal bundling of MTs after the MTS-buffer treatment, indicating that it produces an artificial effect on MTs (Fig. 1A). Glycerol may also excessively stabilize actin filaments in a similar way, resulting in the formation of thick and winding actin bundles. In contrast, myosin II was likely destabilized by glycerol; almost all NMHC-IIA molecules were extracted from the cells (Fig. 1B-C). Although the reason is unclear, the interaction

between actin and myosin II might be reduced by glycerol. Instead of glycerol, we added a low dose of taxol to the PEMT-buffer to prevent MT depolymerization during permeabilization. This resulted in the inhibition of MT depolymerization without MT hyperstabilization or any effect on the actin cytoskeletons (Fig. 2). Notably, the percentage of cytoskeletal α -tubulin quantified by the SERCYF-method was significantly higher than that quantified by the MTS-method (~70% vs 40%). The results obtained using the SERCYF-method appear to better reflect the situation in cells, judging from the normal cytoskeletal organization after the PEMTT-buffer (but not MTS-buffer) treatment by immunofluorescence microscopy.

There are some significant differences between TSA- and PEMTT-buffers, namely, the PIPES concentration, salt concentration, and temperature during permeabilization (Table 1). Olmsted and Borisy reported that the buffer containing a high concentration of PIPES (maximal at 100 mM) and a physiological concentration of salt (150 mM) is best for the purified tubulin polymerization *in vitro* [21]. TSA-buffer contains PIPES at a standard concentration (20 mM); however, its ability to maintain MT organization is likely insufficient (Fig. 1). Although PEM-buffer does not contain additional salts, such as NaCl, 100 mM PIPES-NaOH (pH 6.8) already provides a considerable sodium ion concentration. Thus, PEM-buffer might meet the requirements of both PIPES and salt concentrations. The addition of a specific amount of salt to the PEM-based permeabilization buffer might further improve the method. Furthermore, it is critical to maintain the temperature at 37°C during fractionation because MTs undergo depolymerization at lower temperatures. TSA-buffer treatment at 25°C would also cause MT depolymerization.

Although previous studies used PEMT-buffer for the cytoskeletal fractionation [6,10], our procedure differs from theirs. Nejedla *et al.* applied PEMT-buffer to the cells collected by scraping and centrifugation [6]. Because our goal was to establish a fractionation method that does not affect cytoskeletal organization, we avoided any step that could affect cell shape (scraping and centrifugation). Dahan *et al.* used PEMT-buffer for the fractionation of NMHC-IIA in a procedure

similar to our method, except that they incubated cells with PEMT-buffer on ice for 5 min [10]. They probably used a low temperature to prevent protein denaturation; however, we maintained the temperature at 37°C for two reasons. Firstly, as mentioned above, MTs are depolymerized at lower temperatures. Secondly, low temperatures are likely to decrease the permeabilizing activity of surfactants. Indeed, large molecules, such as NMHC-IIA, were difficult to extract from cells at low temperatures (Supplementary Fig. S2). Thus, the reduced surfactant activity requires a longer time for sufficient permeabilization. We therefore adopted a warm temperature (37°C) and a short time (1 min) for permeabilization.

Based on these arguments for the buffer compositions and experimental procedures, we established the SERCYF-method as illustrated in Figure 2B. By using this method, we achieved simultaneous fractionation of intact MTs and actin cytoskeletons. The usefulness of this method was evaluated by analyzing the effects of MT depolymerization on the actin cytoskeletons (Fig. 3). This method was successful in analyzing the crosstalk between MTs and actin cytoskeletons and revealed that the percentages of cytoskeletal β -actin and NMHC-IIB increase after MT depolymerization. However, the percentage of cytoskeletal vinculin was unaffected, which is in conflict with the increase in the number of FAs in nocodazole-treated cells observed by immunofluorescence microscopy. The rinsing step during the fractionation might cause this discrepancy; the binding of vinculin to FAs after permeabilization was probably too weak to resist rinsing with PEM-buffer. Despite this disadvantage, permeabilization with PEMTT-buffer prior to fixation is an effective procedure for the immunofluorescence observation of FAs because they became more visible due to the removal of cytoplasmic components.

In this study, we established the SERCYF-method as a cytoskeletal fractionation procedure in which biochemical analysis can be performed without significant disruption of MTs or actin cytoskeletons. Thus, using this novel method, it is possible to analyze a specific cytoskeleton for study without having to change the buffer composition or experimental procedure. Because of this high

versatility, our method will accelerate the studies of MTs, actin cytoskeletons, and also their crosstalk.

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Conflict of interests

The authors declare no competing financial interests.

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Figure Legends

Fig. 1. Permeabilization with MTS- or TSA-buffer induces cytoskeletal disruption.

(A) Immunostaining of α -tubulin, β -actin, and NMHC-IIA in non-pre-permeabilized and pre-permeabilized cells under the indicated conditions. Scale bar, 20 μ m. (B) Immunoblots of fractionated proteins prepared by the MTS- or TSA-method. “Fr.” indicates free, cytoplasmic fractions, “Sk.” indicates cytoskeletal fractions. GAPDH was used as an indicator of cytoplasmic fractions. (C) The percentages of cytoskeletal fractions, calculated from (B). Data represent mean $\pm$ SEM from three independent experiments.

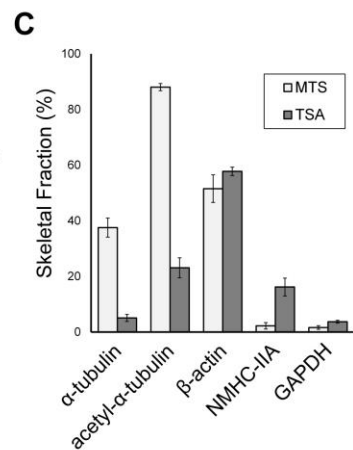
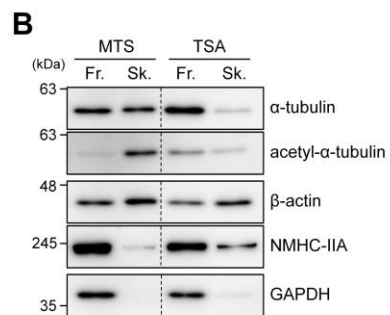
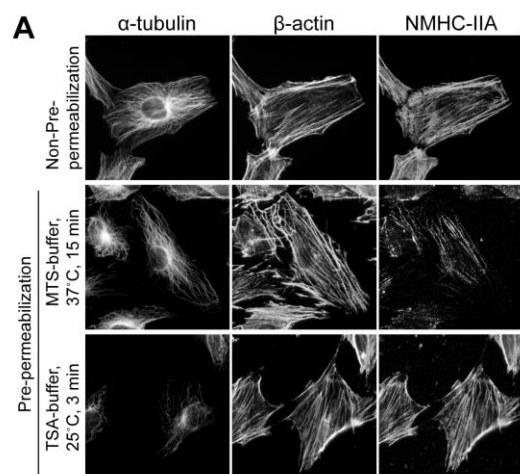
Fig. 2. Development of a fractionation method with PEM-based permeabilization buffer.

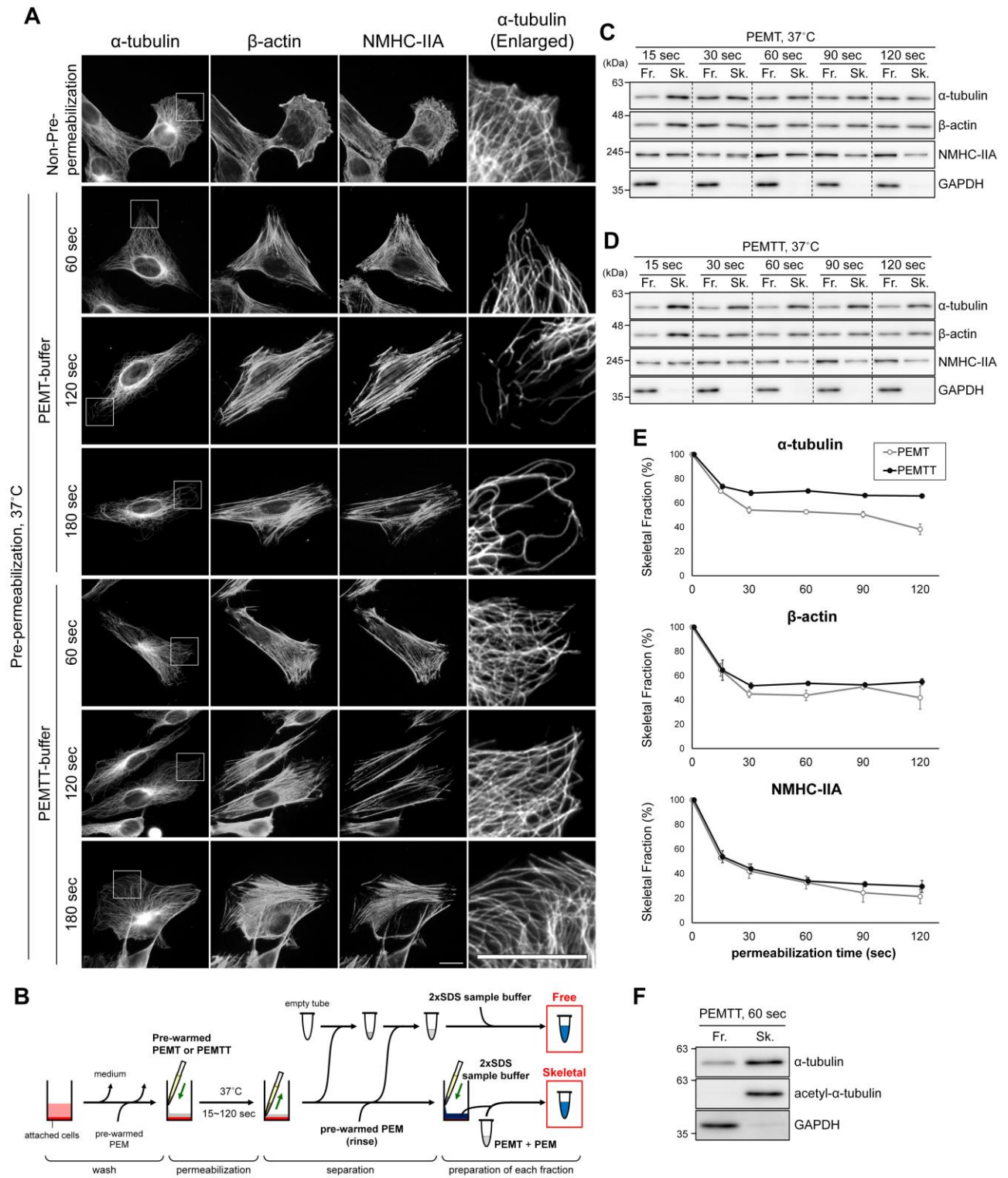
(A) Immunostaining of α -tubulin, β -actin, and NMHC-IIA in non-pre-permeabilized and pre-permeabilized cells under the indicated conditions. Areas indicated by squares in each α -tubulin image

were enlarged. Scale bar, 20 μm . (B) A schematic illustration of the cytoskeletal fractionation with PEMT- or PEMTT-buffer. (C, D) Immunoblots of fractionated proteins prepared with PEMT- (C) or PEMTT-buffer (D) from cells permeabilized at the indicated time points at 37°C. (E) The percentages of cytoskeletal fractions, calculated from (C; gray lines) and (D; black lines). Data represent mean $\pm$ SEM from three independent experiments. The values at 0 sec (without permeabilization) were regarded as 100%. (F) Immunoblots of fractionated proteins prepared by the SERCYF-method (PEMTT-buffer, 60 sec permeabilization at 37°C).

Fig. 3. Analysis of the effects of MT depolymerization on the actin cytoskeletons by the SERCYF-method.

(A) Immunostaining of α -tubulin, β -actin, NMHC-IIA, NMHC-IIB, and vinculin in non-pre-permeabilized and pre-permeabilized cells with PEMTT-buffer for 60 sec at 37°C. Before fixation or pre-permeabilization, cells were treated with DMSO or 10 μM nocodazole for 1 hour. Scale bar, 20 μm . (B) Immunoblots of fractionated proteins prepared by the SERCYF-method. The cells were treated in the same way as in (A). (C) The percentages of cytoskeletal fractions, calculated from (B). Data represent mean $\pm$ SEM from five independent experiments. *, $p < 0.05$, ***, $p < 0.001$, ns, non-significant, calculated by unpaired Student's *t*-test.





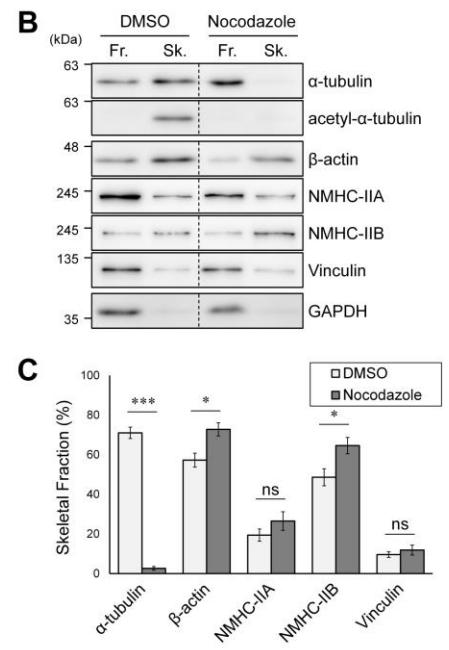
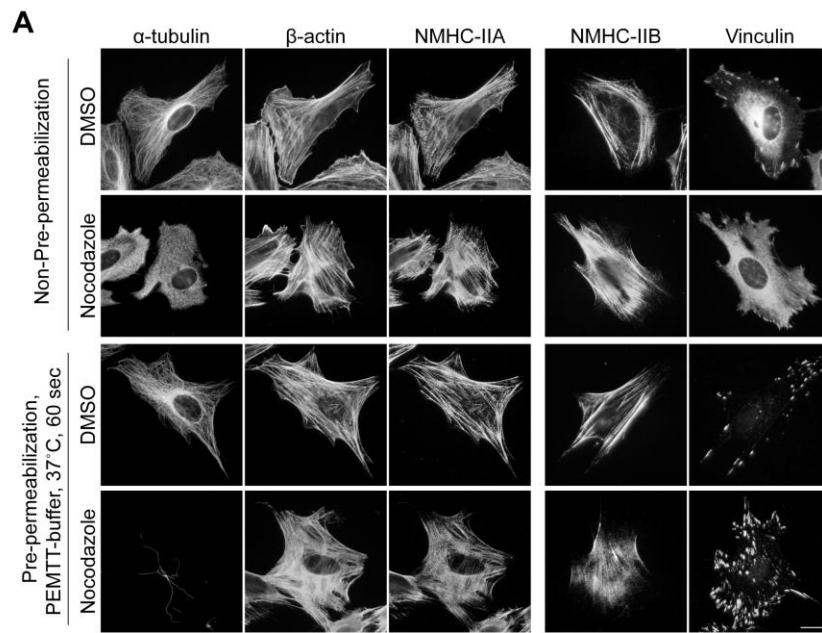


Table 1. Comparison of the fractionation methods.

Method	Buffer composition	Permeabilization condition	Separation step	Fractionation efficiency (assessed in this study)			Reference
				MTs	Actin	NMHC-II	
Microtubule sedimentation (MTS)	100 mM PIPES-NaOH (pH 6.8) 2 mM EGTA 5 mM MgCl ₂ 2 M Glycerol 2.5 mM GTP 0.1% NP-40 1× Protease inhibitor cocktail	37°C 15 min	Centrifugation (111,000 × g, 1 h)	△	△	×	4
Triton X-100 solubility assay (TSA)	20 mM PIPES-NaOH (pH 6.8) 150 mM KCl 10 mM Imidazole 1 mM EGTA 1 mM MgCl ₂ 1 mM DTT 0.05% Triton X-100 1× Phosphatase inhibitor 1× Protease inhibitor cocktail	25°C 3 min	Collection of cell extract as the cytoplasmic fraction	×	○	○	8
PIPES-EGTA-magnesium-Triton (PEMT)	100 mM PIPES-NaOH (pH 6.8) 1 mM EGTA 2 mM MgCl ₂ 0.2% Triton X-100 1× Protease inhibitor cocktail	37°C 15–120 sec (see text)	Collection of cell extract as the cytoplasmic fraction	△	○	○	15
Semi-retentive cytoskeletal fractionation (SERCYF)	100 mM PIPES-NaOH (pH 6.8) 1 mM EGTA 2 mM MgCl ₂ 100 nM Taxol 0.2% Triton X-100 1× Protease inhibitor cocktail	37°C 1 min (see text)	Collection of cell extract as the cytoplasmic fraction	○	○	○	-