Chapter 2

General experimental methods

2.1 Introduction

The majority of the assays in this thesis involved \textit{in vitro} reconstitutions with dynamic microtubules and pre-polymerized actin filaments. These were typically immobilized on a glass surface and imaged by total internal reflection fluorescence (TIRF) microscopy. This chapter outlines the general methods applicable to all the \textit{in vitro} assays described in this thesis.

2.2 Flow cell preparation and surface functionalization

To minimize non-specific binding of fluorescent proteins to the glass, hence to avoid too much background signal, we made use of thoroughly cleaned glass slides and coverslips, which were then functionalized through a variety of techniques.

2.2.1 Glass cleaning

Glass coverslips were cleaned in \textit{base piranha} solution, a safer alternative to \textit{acid piranha} solution that only becomes reactive when heated at or above 60°C. Storing the cleaned coverslips in a 0.1 M KOH aqueous solution activated hydroxyl (OH\textsuperscript{−}) groups on the
glass surface [436], giving the surface a net negative charge which was needed for later functionalization steps.

Base piranha was prepared under the fume hood by mixing Milli-Q water, 30% H$_2$O$_2$, and 30% NH$_4$OH at a 5:1:1 volume ratio in a glass beaker sufficiently large to fit a Teflon® rack holding the coverslips. The mixture was then heated to 75°C on a hot plate with continuous stirring using a magnetic bar. Meanwhile, the coverslips were rinsed twice, 5 min each, with Milli-Q water (Merck Millipore, Billerica, MA, USA) in a bath sonicator. Once the base piranha solution reached a temperature of 75°C, the Teflon® rack holding the coverslips was inserted into the beaker. After a 15 min incubation at 75°C, the coverslips were removed and rinsed in Milli-Q water in a bath sonicator for 5 minutes. The coverslips were finally stored at room temperature (RT) in a 0.1 M KOH solution until use, for up to one month.

Glass slides were cleaned in a slightly less rigorous fashion, by sequential bath-sonication steps (20 min each) in 0.1% (v/v) aqueous solution of Hellmanex™, 100% acetone, and 70% ethanol, with two 5 min rinses in Milli-Q water in between each step. Thereafter, the Teflon® rack holding the coverslips was transferred to a beaker containing a 0.1 M KOH aqueous solution for storage, and the slides were used for up to one month.

2.2.2 Flow cell preparation

Flow cells with ~10 μl channels were assembled with strips of Parafilm® sandwiched between a cleaned glass slide and coverslip (Fig. 2.1).

![Figure 2.1: Two types of flow cells.](image)

**Figure 2.1: Two types of flow cells.** Schematic of the two types of flow cells used. The flow cell in (b) can be used to monitor the effects of solution exchange on an inverted microscope stage.

First, a cleaned glass slide and coverslip were retrieved from the 0.1 M KOH storage solution and rinsed in a bath sonicator for 5 min in Milli-Q water. Meanwhile, using
a glass slide as a ruler, three ∼2 mm-wide and ∼2.5 cm-long channels were cut out from a piece of Parafilm®. The channels were spaced far enough so that they fit comfortably within the coverslip. Thereafter, the glass was retrieved and blow-dried with pressurized N₂ gas, and the Parafilm® placed between coverslip and glass slide. To melt the Parafilm®, the assembled flow cell was placed for ∼10 s on a 120°C hot-plate, while applying even pressure by placing a glass slide and a small metal weight on top. After this step, the surface functionalization was started right away.

For assays in which solutions had to be exchanged during the experiment, glass slides perforated with ∼1 mm-diameter holes (spaced 20 mm apart to fit within a 2" coverslip, Fig. 2.1 b) were used. The Parafilm® channels and coverslip were mounted on these glass slides such that the holes became the entry and exit points of the channel. In this way, all solutions could be flowed in and out from the side of the glass slide, while the flow cell was already mounted on the microscope stage (Fig. 2.2).

**Figure 2.2: Monitoring solution exchange on the microscope stage.** Schematic of solution exchange on an inverted microscope stage using the flow cell type described in Fig. 2.1 b.

### 2.2.3 Biotinylated and centrosome-coated surfaces

Biotin and streptavidin linkages were the main way by which we immobilized individual proteins, microtubules or actin filaments onto the coverslip surface (Fig. 2.3 a). First, the assembled flow cell channels were coated with the block co-polymer poly(L-lysine)-graft-poly(ethylene glycol) functionalized with a biotin tag (PLL-PEG-Biotin, SuSoS AG, Dübendorf, Switzerland), at a concentration of 0.2 mg/ml in MRB80 (80 mM PIPES pH 6.8 with KOH, 1 mM EGTA and 4 mM MgCl₂). After a 30 - 60 min incubation, the channel was rinsed with 50 - 100 μl of MRB80. Thereafter, a 50 - 100 μg/ml solution of streptavidin (or NeutrAvidin™) in MRB80 was added. After a 10 min incubation, the channel was rinsed as above. Finally, any hydrophobic patches left on the glass surface
were blocked by adding a 0.5 mg/ml solution of $\kappa$-casein in MRB80, followed by a 1% (w/v) solution of Pluronic® F-127 also in MRB80. The flow cell was incubated with each solution for 10 min, with 50 - 100 µl MRB80 rinses in between. After this step, the glass surface was ready to bind biotinylated microtubule seeds or actin filaments (Fig. 2.3 a).

![Figure 2.3: Different surfaces for microtubule nucleation.](image)

For assays with centrosomes, since they can adhere non-specifically to the glass surface, the flow cell surface preparation was different (Fig. 2.3 b). First, the centrosome solution, normally stored at $-80^\circ$C, was warmed to $37^\circ$C for 10 min and gently diluted in MRB80 at RT to the desired concentration. Thereafter, centrosomes were added to the flow cell, and incubated for 5 min to allow them to adhere to the surface. After a 50-100 µl rinse in MRB80, a 0.2 mg/ml PLL-PEG (SuSoS AG, Switzerland) solution in MRB80 was added, and incubated for 10 min. Finally, hydrophobic patches were blocked by flowing in 50-100 µl of a solution containing 0.1 mg/ml $\kappa$-casein and 1% (w/v) Pluronic® F-127 in MRB80. After a 15 minute incubation, the flow cell channel was rinsed one last time and used straight away.
2.3 Buffer conditions to work with actin filaments and dynamic microtubules

Traditionally, *in vitro* assays with F-actin and microtubules have been performed in radically different buffers [11, 437]. We found that MRB80 (80 mM PIPES pH 6.8 with KOH, 1 mM EGTA and 4 mM MgCl$_2$), a variant of BRB80 (otherwise known as Brinkley Reassembly Buffer) that is generally used for microtubules [8], had sufficiently high ionic strength to trigger F-actin polymerization. Conversely, we found that microtubule polymerization did not proceed in the canonical F-buffer (20 mM HEPES pH 7.4 with KOH, 1 mM EGTA, 2 mM MgCl$_2$ and 50 mM KCl), which is likely due its lower ionic strength and higher pH [11]. We thus used MRB80 as the buffer for all the assays described here. For excellent guides on buffers suitable for *in vitro* biological research, we refer the reader to the following references: [438, 439].

2.3.1 Phalloidin-stabilized actin filaments

In all the *in vitro* assays presented here, we made use of pre-polymerized F-actin containing a small fraction of labeled G-actin. For the experiments with single actin filaments (Chapters 3, 5 and 6) or actin bundles (Chapters 4 and 5), phalloidin-stabilized F-actin was polymerized at a 1.0 – 1.5 μM final G-actin concentration. For the experiments with centrosomes and single filaments (Chapter 5), G-actin was polymerized at 30 μM G-actin concentration.

To produce fluorescently labeled actin filaments, two separate solutions were prepared: First, a G-actin mix containing $\sim 12 - 15\%$ G-actin labeled with Alexa Fluor® 488, 594 or 647 (Molecular Probes, Life Technologies, Carlsbad, CA, USA), which was kept on ice for 5 min. Second, an F-actin polymerization mix containing 5 mM DTT, phalloidin at an equimolar ratio to the final G-actin concentration, 50 mM KCl and 0.2 mM ATP in MRB80. To trigger F-actin polymerization, the F-actin polymerization mix was added to the G-actin mix, vigorously pipetted, and incubated for $\sim 1$ hr at RT in the dark, to avoid photo-bleaching. The resulting solution of actin filaments was used up to five days after polymerization.
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2.3.2 GMPCPP-stabilized microtubule seeds and centrosomes

With the exception of the experiments with centrosomes (Chapter 5), in all assays presented here we nucleated microtubules from surface-immobilized microtubule seeds. These microtubule seeds contained 12% of fluorescently-labeled and biotinylated tubulin subunits, and were stabilized against depolymerization with the slowly-hydrolyzable GTP analog guanylyl-(α,β)-methylene-diphosphonate (GMPCPP [50]).

To obtain seeds of approximately 5 μm in length, the following steps were followed: a 20 μM tubulin mix was prepared in MRB80 which contained 70 molar percent of unlabeled tubulin (Cytoskeleton, Denver, CO, USA), 18% biotinylated tubulin (Cytoskeleton, USA) and 12% tubulin labeled with either TRITC Rhodamine, HiLyte® 488 or, HiLyte® 635 (Cytoskeleton, USA). To remove aggregates, the tubulin mix was centrifuged at 149,000 g for 5 min at RT with the use of an Airfuge® ultracentrifuge (Beckman Coulter, Brea, CA, USA). To trigger microtubule polymerization, GMPCPP was added to the supernatant to a final concentration of 1 mM, and the tubulin mix was incubated at 37°C for 30 – 40 min. After this incubation step, the microtubule seeds were pelleted as above and resuspended in MRB80 to a final 20 μM tubulin concentration, assuming that 80% of the tubulin was recovered.

More stable seeds that can be frozen and stored at −80°C were produced by adding a cycle of depolymerization (≈ 20 min on ice) after the second centrifugation step, followed by re-polymerization at 37°C with fresh 1 mM GMPCPP added, in order to replace more of the GDP-tubulin with GMPCPP-tubulin [436]. After the second polymerization step, these ’doubley-cycled’ seeds were pelleted as before, and resuspended in MRB80 containing 15% (v/v) glycerol, to a final concentration of 5 μM tubulin (assuming 80% recovery after each centrifugation step). Thereafter, the seeds were aliquoted in ~ 5 μl volumes, flash-frozen in liquid N2 and stored at −80°C until use.

For daily use, the GMPCPP-stabilized microtubule seeds were kept in the dark at RT, and were used within two days of preparation (or thawing), since with time they tended to anneal and get longer. In a typical assay, GMPCPP-stabilized microtubule seeds were added to the flow cell at the equivalent concentration of 50 – 60 nanomolar tubulin concentration, in a solution containing 0.1% (v/v) methyl-cellulose in MRB80, to help bring them close to the glass surface.

Centrosomes were purified by Florian Huber and Sophie Roth, with the generous help of Claude Celati (Institut Curie, Section Recherche, UMR144-CNRS, 75005 Paris, France),
from human lymphoblastic KE37 cell lines. They were stored in a 30% (w/v) sucrose solution in MRB80, in a liquid N\textsubscript{2} tank.

2.4 Microtubule polymerization and tip tracking assays

A minimal mixture for microtubule polymerization should contain tubulin, GTP, a surface blocking agent, an oxygen scavenging system to prevent photo-damage, and a reducing agent. When working with EB3 and other +TIPs, we used KCl to tune the ratio of their microtubule tip-to-lattice binding [82, 98]. Finally, methyl-cellulose was always included to confine out-of-plane microtubule and F-actin bundle fluctuations, as needed for TIRF microscopy. Thus, the core microtubule polymerization reaction (always made in MRB80) consisted of:

- 0.2-0.5 mg/ml \(\kappa\)-casein
- 0.1% (v/v) methyl-cellulose
- 1 mM GTP
- Oxygen scavenging and reducing system: 4 mM DTT, 0.2 mg/ml catalase, 0.4 mg/ml glucose oxidase and 25-50 mM D-glucose
- 50-75 mM KCl
- Tubulin (with 6 % of fluorescently-labeled subunits)

The tubulin concentration was varied depending on the experiment (Table 2.1). Typically, high tubulin concentrations were used when we needed to have long microtubules [12], such as in the actin-microtubule co-alignment experiments studied in Chapter 4.

In addition to the core microtubule polymerization reaction, additional proteins such as EBs, +TIPs, actin-binding proteins, and F-actin were typically included in the microtubule polymerization mix:

- **EB3 and TipAct**: in most experiments the concentration of EB3 (unlabeled, or GFP- and mCherry-labeled) was kept in the nanomolar range, in order to enhance the ratio of microtubule tip-to-lattice binding [104]. For most experiments the
EB3 concentration was kept at 100 nM, and the GFP-TipAct concentration at 25-50 nM. The only exceptions being in the actin-microtubule co-alignment assays in Chapter 4 in which the TipAct concentration was varied between 6.5 – 50 nM, and in the actin-transport assays of Chapter 6, in which the EB3 and TipAct concentrations were varied between 40 – 200 nM and 10 – 50 nM, respectively.

- **GFP-Tip**: For the actin-microtubule co-alignment experiments in Chapter 4, where we worked with the actin-binding deficient GFP-Tip, its concentration was kept at 50 nM.

- **Fascin**: For the experiments in Chapter 4 where we worked with fascin-bundled F-actin, the microtubule polymerization reaction was supplemented with 200 – 500 nM fascin.

- **F-actin**: For experiments with actin filaments weakly bound to the coverslip (Chapters 3 and 5), phalloidin-stabilized actin filaments were added to the flow cell along with the microtubule seeds, at the equivalent of 100 – 200 nM G-actin concentration. Even without biotinylation, we found that the actin filaments could weakly and non-specifically bind the glass surface when streptavidin was present. For the experiments with centrosomes, phalloidin-stabilized F-actin was incorporated directly into the microtubule polymerization mix at the equivalent of 1 μM G-actin concentration. Finally, for the experiments of actin-filament transport in Chapter 6, phalloidin-stabilized actin-filaments were first sheared by vigorous pipetting, and also incorporated directly to the microtubule polymerization mix at the equivalent of 30 nM G-actin concentration.

After mixing (but before the addition of actin filaments), the microtubule polymerization reaction mix was always clarified at 149,000 g for 5 min in an Airfuge® ultracentrifuge (Beckman Coulter, USA) and immediately added to the flow cell channel. Finally, the channels were sealed either with wax or vacuum grease to avoid solvent evaporation while imaging.

Table 2.1 summarizes the key components of the microtubule polymerization reactions, as well as the imaging temperatures, for most of the assays described in this thesis.

### 2.5 Proteins used in this thesis

Lyophilized porcine brain tubulins (unlabeled, or labeled with TRITC Rhodamine, HiLyte®488, HiLyte®635 and biotin) were obtained from Cytoskeleton (Denver, CO,
General experimental methods

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<td>10-50</td>
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Table 2.1: Main experimental conditions for all the in vitro assays described in this thesis.

USA), resuspended at 50-100 μM in MRB80, snap-frozen and stored at −80°C until use.

G-Actin was purified from rabbit skeletal muscle acetone powder [437, 440] and kept at −80°C for long-term storage [440]. For daily use, G-actin was stored at 4°C in G-buffer (2 mM Tris-HCl pH 7.8, 0.2 mM Na₂ATP, 0.2 mM CaCl₂, 5 mM dithiothreitol (DTT)). After four weeks, the protein was clarified by centrifugation at 149,000 g for 10 min and dialysis overnight against fresh G-buffer. Alexa Fluor® 647 and 594 succinimidyl ester dyes (Molecular Probes, Life Technologies, Carlsbad, CA, USA) were used to produce labeled G-actins [440].

GST-tagged recombinant human fascin 1 (the plasmid was a generous gift from Dyche Mullins, University of California, San Francisco, U.S.A) was expressed and purified via affinity chromatography on Glutathione Sepharose 4 Fast Flow matrix (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Cleavage of the GST tag was performed with the PreScission protease system (GE Healthcare), followed by a gel-filtration step through a Superdex-200 column (GE Healthcare), [440].

6×His-tagged recombinant human EB3, GFP-EB3, mCherry-EB3 [86, 120], and GFP-Tip (elsewhere called GFP-MACF43, [107]) were kind gifts from Michel Steinmetz (Laboratory of Molecular Research, Paul Scherrer Institut, Switzerland) and Anna Akhmanova (Department of Biology, Utrecht University, the Netherlands).
The homogeneity of all recombinant proteins used in this study was confirmed by SDS-PAGE (Fig. 2.4).

![SDS-PAGE analysis of the recombinant proteins used in this study.](image-url)

**Figure 2.4:** SDS-PAGE analysis of the recombinant proteins used in this study. The corresponding molecular weights are: fascin 55 kDa, GFP+TIP 30 kDa, mCherry-EB3 65 kDa, EB3 35 kDa, 6His-GFP-TipAct 76 kDa, and GFP-TipAct 74 kDa.

### 2.6 Buffers and stocks

We provide a list with all the key reagents and stock solutions used in this thesis in alphabetical order. Unless stated otherwise, chemicals were obtained from SIGMA Aldrich (Sigma-Aldrich Chemie B.V., the Netherlands). All stock solutions were prepared in MRB80 unless otherwise noted. For stock solutions, information is provided as follows: name (catalog number, company), concentration, storage temperature and additional details:

- **ATP** (disodium salt hydrate, A2383), 50mM, $-80^\circ$C
- **D-Glucose** (G7528), 1M, filter, $-80^\circ$C
- **G-actin** (labeled and unlabeled), for long term storage keep concentration $\geq$ 48 μM in G-buffer, $-80^\circ$C
- **Alexa Fluor® 488, Alexa Fluor® 594, and Alexa Fluor® 647 succinimidyl ester dyes** (A-20000, A-20004, A-20006, Molecular Probes, Life Technologies, Carlsbad, CA, USA), $-20^\circ$C
- **GMPCPP** (10mM solution, NU-405, Jena Biosciences, Jena, Germany), $-80^\circ$C
• GTP (G8877), 50mM, −80°C
• H$_2$O$_2$ (30% in water, non-stabilized, 95313), 4°C
• κ-casein (C0406), 5 mg/ml, filter, −80°C
• Methyl-cellulose (M0512), 1% (w/v), −80°C
• NeutrAvidin™ (31055, Thermo Scientific, Pierce Protein Biology Products, Rockford, IL, US), 5 mg/ml, −80°C
• NH$_4$OH (30% in water, 320145), RT
• Oxygen scavenging and reducing mix (50X): 200 mM DTT, 10 mg/ml catalase and 20 mg/ml glucose oxidase in MRB80, filter, −80°C
• DTT (D0632), 1M, −80°C
• Catalase (powder, C9322), −20°C
• Glucose oxidase (powder, G7016), −20°C
• Phalloidin (P2141), 1mM in methanol, −20°C
• PLL-PEG (PLL(20)-g[3.5]-PEG(2), Susos AG, Dübendorf, Switzerland), 2mg/ml, −80°C
• PLL-PEG-Biotin (PLL(20)-g[3.5]-PEG(2)/PEG(3.4)-Biotin (50%), Susos AG), 2 mg/ml, −80°C
• Pluronic® F-127 (P2443), 10% (w/v) in DMSO, RT
• Streptavidin (85878), 5 mg/ml, −80°C
• Tubulin (T240, Cytoskeleton Inc., Denver, CO, USA), 50 μM, −80°C
• Tubulin HiLyte 488-labeled, HiLyte 647-labeled, TRITC Rhodamine-labeled, and Biotin-labeled (TL488M, TL670M, TL590M, T333P, Cytoskeleton Inc), 50 μM, −80°C

2.7 Total internal reflection fluorescence (TIRF) microscopy

The best signal-to-noise ratio for in vitro surface assays with fluorescently-labeled proteins in solution is achieved with TIRF microscopy [441]. Since we often had to visualize two or even three proteins within one experiment, we used up to three different wavelengths: 488 nm, 561 nm and 635 nm. We found the optimal fluorophore/laser
combinations using TRITC Rhodamine-labeled tubulin, Alexa 647-labeled actin, and GFP-labeled EBs (or +TIPs). If unlabeled EBs were used, Alexa 488-labeled G-actin worked equally well.

TIRF microscopy and fluorescence recovery after photo-bleaching (FRAP) experiments were performed on an Nikon Eclipse Ti-E inverted microscope (Nikon Corporation, Tokyo, Japan) equipped with an Apo TIRF 100x1.49 N.A. oil objective, a motorized stage, Perfect Focus System (PFS), a motorized TIRF illuminator (Roper Scientific, Tucson, AZ, USA) and a QuantEM:512SC EMCCD camera (Photometrics, Roper Scientific). For excitation, we used a 561 nm (50 mW) Jive (Cobolt, Solna, Sweden) and a 488 nm (40mW) Calypso (Cobolt) diode-pumped solid state laser, and a 635 nm 28 mW Melles Griot laser (CVI Laser Optics & Melles Griot, Didam, Netherlands). For FRAP experiments the microscope was equipped with a MAG Biosystems FRAP-3D system (Photometrics, Roper Scientific) that could either be used to do FRAP-on-the-fly (point FRAP) or region of interest (ROI) FRAP experiments with diffraction-limited spots.

Most of the imaging of dynamic microtubules was performed at two seconds per frame with 100 – 200 ms exposure time at 10-15% laser power (unless otherwise noted). FRAP experiments on mCherry-EB3 and GFP-TipAct at growing microtubule ends and actin bundles (Chapter 4) were performed at video rate (33 ms exposure time per frame), at 10-15% laser power for imaging, and 100% laser power for photo-bleaching.

To ensure consistent microtubule dynamics and control their growth speeds and average lengths [12], the sample temperature was regulated with the use of a home-built objective heater/cooler with a range of 15 – 40 ± 1°C. The temperature was set depending on the desired range of microtubule lengths. For the experiments without actin, or with single actin filaments weakly bound to the coverslip the temperature was kept at 25 ± 1°C, for the assays with linear arrays of actin bundles it was kept between 32 – 34 ± 1°C, for the experiments with centrosomes it was kept at 30 ± 1°C, and for the assays of actin-filament transport between 28 – 30 ± 1°C (Table 2.1).

### 2.8 Data analysis

All image processing and data analysis were performed using plugins for FIJI [442] or ImageJ [443] and custom-written programs in MATLAB (MathWorks Inc., Natick, MA,
USA). Each chapter contains a detailed explanation of the image analysis techniques developed for each specific experiment.

We thank Anna Akhmanova (Utrecht University, The Netherlands) and Michel O. Steinmetz (Paul Scherrer Institute, Switzerland) for providing us with the EB3 and GFP-Tip proteins, Dyche Mullins (UCSF, San Francisco, USA) for providing us with the fascin plasmid, and Marjolein Vinkenoog (FOM Institute AMOLF, The Netherlands) for purifying and labeling actin. We furthermore thank Henk-Jan Boluijt and the mechanical workshop at the FOM Institute AMOLF for the design and production of the glass slides we used to work directly on the microscope stage. Finally, we thank Marko Kamp (FOM Institute AMOLF, The Netherlands) and Roland Dries (TU Delft, The Netherlands) for maintaining the TIRF microscope.