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MECHANISM OF MICROTUBULE ROD FORMATION IN 13-LINED GROUND SQUIRREL (*ICTIDOMYS TRIDECEMLINEATUS*) PLATELETS

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

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MECHANISM OF MICROTUBULE ROD FORMATION IN 13-LINED GROUND SQUIRREL (*SPERMOPHILUS TRIDECEMLINEATUS*) PLATELETS

By Xingxing Lin

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ABSTRACT

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Blood platelets play a critical role in blood clotting, or hemostasis. Platelets from human, baboon, and mouse are sensitive to cold, resulting in rapid removal from blood circulation by macrophages in the liver. Short shelf life at room temperature has led to a worldwide shortage of platelets. An understanding of how to prolong platelet shelf life is a high priority of platelet research. 13-lined ground squirrel (*Ictidomys tridecemlineatus*) platelets undergo significant morphological changes, forming rod shapes, when exposed to cold temperature. However, to date, little research has been done to uncover the correlation between the morphological changes and ground squirrel platelets' survival during hibernation. In this study, we confirmed that microtubules are responsible for ground squirrel platelet shape changes when exposed to different temperatures. Microtubules from ground squirrel platelets are maintained in equilibrium between rod and ring conformations. Microtubule polymerization leads platelets to form rods. Microtubule depolymerization leads platelets to form rings. Low temperature acts as an inhibitor of microtubule depolymerization shifting platelet equilibrium to rod shapes.

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Platelet Size

Mammalian platelets are the smallest blood cells. Platelets are about 20% the diameter of red blood cells. Most papers and books describe platelets with a diameter 1-5 μm with considerable variation; 2-3μm (Tinmouth, 2007; Campbell, 2008; Lochowicz & Curtis, 2011), 2-5μm (Michelson, 2013), 1-4μm (Semple, 2013), and 1-2 μm (Thon & Italiano, 2010; Chiang & Postlethwaite, 2008).

Platelet Structure

Although platelets are anucleate, they possess typical characteristics of cells including peripheral, membranous, organellar, and cytoskeletal structures (Zucker-Franklin, 1996; Schwertz et al, 2006; Qian et al, 2008; Shashkin et al, 2008; Fritsma, 2015; Figure 1). These structures contribute a functional role in hemostasis (Spencer & Becker, 1997). Resting platelets are flat and discoid in shape, which is maintained by marginal bands of microtubules (White & Rao, 1998). When activated, platelets will change into a more spherical shape with emission of multiple pseudopodia (Maurer-Spurej et al, 2001).

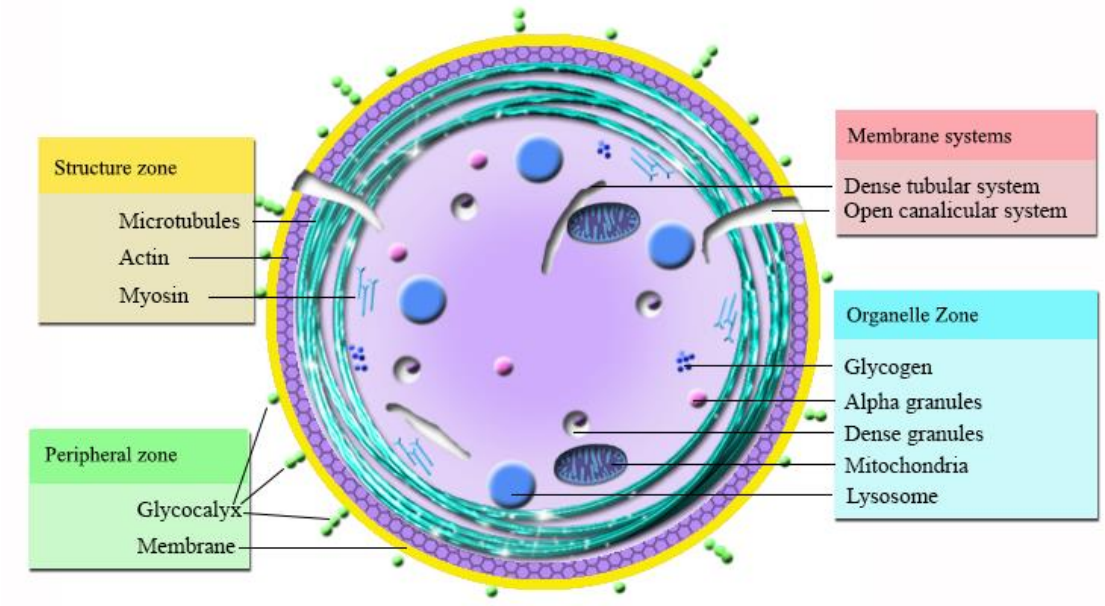


Figure 1. Diagram of the Ultrastructural Structure of Thin Sections of Resting Human Platelets.

Platelet Production

Platelets are produced by bone marrow megakaryocytes (Pease, 1956; Wright, 1910; Schulze & Shivdasani, 2005; Junt et al, 2007; Machlus & Italiano, 2013; Figure 2). Megakaryocytes (50-100μm) are the largest but rarest nucleated cells in bone marrow accounting for 0.01 % of nucleated bone marrow cells (Nakeff & Maat, 1974). After maturation and development, megakaryocytes will extend 10-20 proplatelet protrusions of cytoplasm packed with organelles into a blood vessel (Richardson et al, 2005; Thon et al, 2010; Machlus & Italiano, 2013). Proplatelets thicken at their tips and release anucleate discoid particles called preplatelets with a diameter of 2-10μm. Preplatelets will continue to convert into smaller size proplatelets and finally, will release highly specialized and undividable platelets with neither a nucleus nor microtubule organizing center. One megakaryocyte will eventually produce 100-1000 individual platelets (Thon & Italiano, 2010).

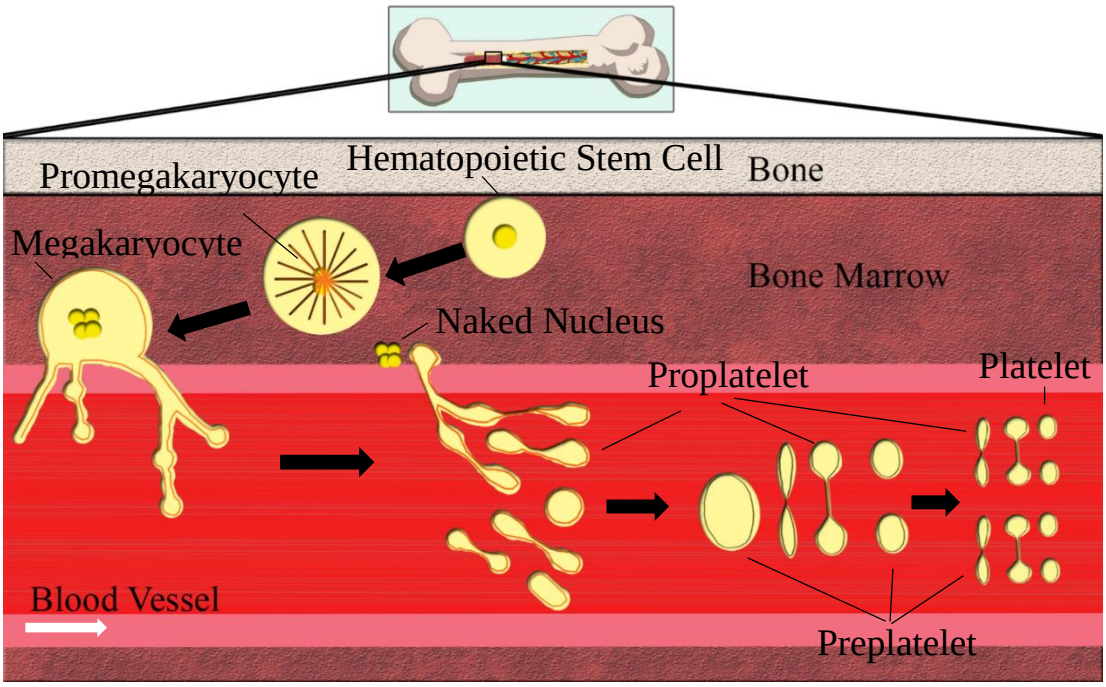


Figure 2. Simulation of Platelet Formation. Hematopoietic stem cells develop into promegakaryocytes which later develop into megakaryocytes in bone marrow. Megakaryocytes extrude into blood vessel to form a proplatelet. Proplatelets split into preplatelets until reaching the size of platelets. Black solid arrows represent the direction of development. The white solid arrow represents blood circulation.

Platelet Life Span

In order to keep blood from clotting, platelets should remain at a level of 150,000 to 450,000 platelets/μl. Human platelets circulate in the bloodstream for roughly 7-10 days (Harker & Finch, 1969; Kaushansky, 1995), compared with 3 to 5 days in mice (Liu et al, 2014), and 5 to 6 days in rats (Odell, Tausche, & Furth, 1954). In contrast, 13-lined ground squirrel (*Ictidomys tridecemlineatus*), hereafter referred to as ground squirrels, platelets can be stored up to 6 months during hibernation, and circulate for 5-6 days in non-hibernating animals (Cooper et al, 2012).

Platelet Storage Problems

Platelet Function

Many functions of platelets have been discovered, such as immune defense, inflammation, wound healing, and particularly hemostasis (Weiss, 1975a; Weiss, 1975b; Jurk & Kehrel, 2005). These functions are mediated by preformed cell surface and soluble mediators contained in platelet granules (Weyrich & Zimmerman, 2004; Semple, Italiano, & Freedman, 2011; Jenne, Urrutia, & Kubes, 2013). Platelets contain three types of granules: α-granules, dense bodies, and lysosomes (Nierodzik, Klepfish, & Karpatkin, 1995; Rendu & Brohard-Bohn, 2001; Denis et al, 2005; Italiano et al, 2008; Whiteheart, 2011; Kraemer et al, 2011). These granules are reservoirs of mediators including adhesion molecules, coagulation and angiogenic factors, cytokines, chemokines, mitogenic proteins and growth factors (Whiteheart, 2011).

Platelets in hemostasis. The primary contribution of platelets is to hemostasis (Jiravanichpaisal, Lee, & Söderhäll, 2006; Jenne, Urrutia, & Kubes, 2013). Under normal conditions, circulating platelets flow in the blood vessels to monitor the integrity of the vasculature (Ho-Tin-Noé, Demers, & Wagner, 2011). Collagen is an abundant thrombogenic protein located in the subendothelial matrix (Lodish et al, 2000). Platelets cannot bind to collagen beneath endothelial cells until the endothelium is exposed by a cut or trauma (Ruggeri & Mendolicchio, 2007). Platelets will bind directly to collagen by glycoprotein (GP) IIb/IIIa and GP VI receptors located in the platelet plasma membrane (Brass, 2003; Offermanns, 2006; Davi & Patrono, 2007; Varga-Szabo, Pleines, & Nieswandt, 2008). The initial tethering and binding of the platelet onto exposed collagen at injured blood vessels is called platelet adhesion (Rumbaut & Thiagarajan, 2010). Von

Willebrand factor (vWF) is a large multimeric glycoprotein in blood plasma that binds to collagen through the platelet GP Ibα receptor (Savage, Saldivar, & Ruggeri, 1996; Lopez & Dong, 1997; Du, 2007; Thijs et al, 2010). Platelet adhesion to collagen also activates platelets (Rumbaut & Thiagarajan, 2010). Activated platelets secrete dense granule contents (calcium, ADP, ATP, GTP, thromboxane and serotonin) and alpha granule contents (fibrinogen, vWF, thrombospondin and fibronectin) (Blair & Flaumenhaft, 2009; Italiano & Battinelli, 2009; Whiteheart, 2011). Some of these adhesive proteins, such as vWF and collagen, or soluble platelet agonists, such as ADP, thrombin, and thromboxane A_2, promote further platelet activation (Du, 2007; Ren & Whiteheart, 2008; Li et al, 2000). The activated platelets then facilitate platelet aggregation and activate the coagulation cascade (Brass, 2003).

Platelet Transfusions Are Critical in Clinical Settings

High demand for platelets. Platelet transfusions are used in clinical practice to prevent and treat hemorrhage in diagnosed thrombocytopenic patients or patients with severe platelet function defects (British committee for standards in haematology, 2003; Miller et al, 2007). Low platelet count and platelet dysfunction can result in severe, life-threatening bleeding problems (Sharma, McDonald, & Banji, 1982). More than two million platelet units are transfused annually in the United States for hematological disorders, cancer treatment, transplants, surgery, and intensive care unit (ICU) patients (Whitaker, 2013). The demand for platelet components has seen a considerable increase of 32% between 2004 and 2011 in the United States (United States Department of Health and Human Services, 2011). In the United Kingdom, the platelet clinical demand increased by 24% between 2002 and 2012 (Taylor et al, 2010; Bolton-Maggs et al, 2013). Increasing

platelet demand has also been reported in Europe and Australia (van der Poel, Janssen, & Behr-Gross, 2011a; van der Poel, Janssen, & Behr-Gross, 2011b; van der Poel, Janssen, & Behr-Gross, 2011c; van der Poel, Janssen, & Behr-Gross, 2011d; Commonwealth of Australia, 2013).

Thrombocytopenia patients have lower than normal platelet counts (150,000 to 400,000 platelets per microliter), and often suffer excessive bleeding (Drachman, 2004). When the platelet count is less than 50,000 platelets/μL, mild bleeding will sometimes occur (George et al, 1996). Patients with a severely low platelet count, less than 10,000 or 20,000 platelets/μL, are at risk for serious bleeding (George et al, 1996). In the United States, 1-5% of newborns are born with thrombocytopenia (Burrows & Kelton, 1988; Hohlfeld et al, 2000), and 0.1-0.5% of these have severe thrombocytopenia (Dreyfus et al, 1997, Sainio et al, 2000). 8% of preterm and 6% of all neonates admitted to a neonatal intensive care unit have severe thrombocytopenia (Murray et al, 2002). When they are close to delivery, 5% of healthy pregnant women develop mild thrombocytopenia (Burrows & Kelton, 1988; Burrows & Kelton, 1990; McCrae, Samuels, & Schreiber, 1996). Many other factors could induce thrombocytopenia, such as toxic chemicals, alcohol, viruses and/or bacterial infection, transplant, and surgery (Gauer & Braun, 2012). The treatments for thrombocytopenia can last from days to years due to different causes (NIH, 2014). Severe thrombocytopenia patients who are having major bleeding may require 1.5-2 units of platelets every 4-8 hours (Slichter, 2007). Most platelet transfusions are given to the patients to prevent bleeding, including 67% of patients with hematological disorders, 7-10% of patients after cardiac surgery and 5-9% of patients in an intensive care unit (ICU) (Cameron et al, 2007; Greeno, McCullough, & Weisdorf, 2007; Pendry & Davies, 2011;

Estcourt et al, 2012; Jones et al, 2013; Charlton et al, 2014). More units of platelets are required annually to meet the growing demand (Estcourt, 2014).

Short shelf-life problem. A five-day platelet shelf-life makes it challenging to meet the increasing demand for platelets (Crowe et al, 1999). In 2011, a total 2.738 million units of platelets were collected for transfusions in the United States (Whitaker, 2013). About 21% of the platelet products expired before use due to a short shelf life (Whitaker, 2013).

Storing platelets at 22℃ (room temperature) leads to a 56-58% reduction of platelet aging compared with storage at 37℃ (Holme & Heaton, 1995). Platelet preservation at temperatures above 24℃is detrimental, due to accumulation of metabolic by-products and the risk of platelet contamination (Murphy & Gardner, 1969). Currently, clinics are uniformly using 20-24℃ for their platelet storage temperature with only 5 days of shelf life (Council of Europe, 2011).

Theoretically, lower storage temperature can reduce the bacterial contamination rate to prolong platelet shelf life. However, platelets undergo morphological, biochemical and functional changes which would lead to a rapid clearance during transfusion after chilling to 4℃, even if re-warmed to 37℃ (Becker et al, 1973; Melaragno et al, 1981; Berger, Hartwell, & Wagner, 1998; Valeri et al, 2004). At 4℃ human and mouse platelets lose their disc shape and extend out multiple pseudopods (Loftus, Choate, & Albrecht, 1984; White & Krivit, 1967; White & Rao, 1998). Low temperature promotes microtubule disassembly loss of the circumferential ring (White & Krivit, 1967; White, 1968; White, 1982). Under low temperature, actin filaments detach from the platelet membrane. At the same time, cooling increases the concentration of cytosolic calcium, leading to actin filament assembly (Nishio et al, 1992; Authi et al, 1993; Winokur & Hartwig, 1995; Oliver

et al, 1999; Hoffmeister et al, 2003). The increasing actin filaments assembly leads to cytoskeleton reorganization and a change in shape of chilled platelets (Pribluda & Rotman, 1982; Winokur & Hartwig, 1995; White & Rao. 1998; Oliver et al, 1999; Hoffmeister et al, 2001). Cold also induces dephosphorylation of Lyn, phosphorylation of both PLCγ2 and Src (Gousset et al, 2004) and rearrangement of vWF receptors (GPIbα) (Hoffmeister, Felbinger et al, 2003).

After rewarming from 4℃ to 37℃, human platelets form a spherical shape rather than returning to a discoid shape (Winokur & Hartwig, 1995). Microtubule rings reassemble, but with a diameter twice the size of resting platelets (White, Krumwiede, & Sauk, 1985; Figure 4). Newly assembled actin filaments in the chilled platelets disassemble after rewarming to 37℃ (White & Rao, 1998; Behnke, 1970a). Chilled and rewarmed platelets show similar adhesion rates to sinusoidal regions with high macrophage density in liver lobules (Bioulac-Sage et al, 1996; Hoffmeister et al, 2003). The cluster of GP Ibα receptors on both chilled and rewarmed platelets can be recognized by either hepatic macrophage complement 3 type receptor (CR3) on Kupffer cells (hepatic macrophages) or hepatic lectin receptors on hepatocytes and result in a clearance of chilled and rewarmed platelets out of blood circulation (Hoffmeister et al, 2003; Rumjantseva & Hoffmeister, 2009a; Rumjantseva et al, 2009b). Therefore, studying a mammal whose platelets can be chilled to 4℃ without clearance would be useful toward building an understanding of how to store human platelets in cold. Hibernating mammals like ground squirrels may provide such a model system.

13-lined Ground Squirrel as a Model Organism to Study Platelets

Torpor

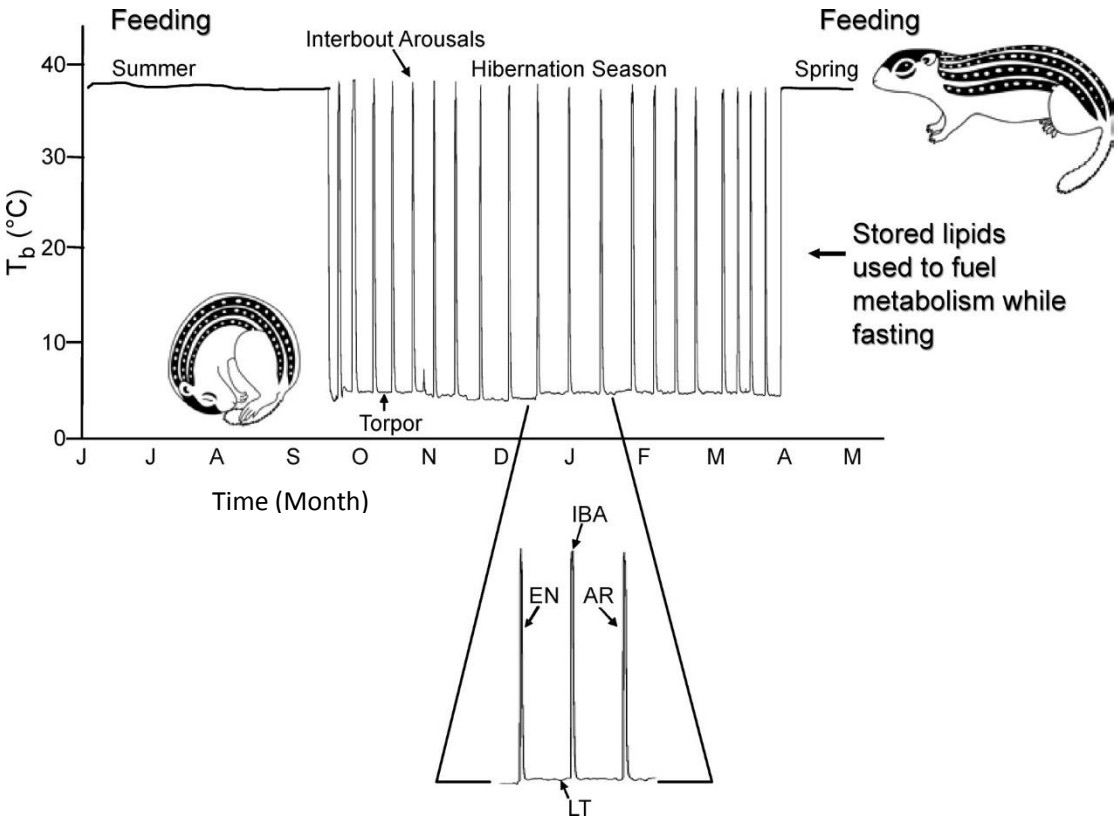


Figure 3. 13-lined Ground Squirrel Hibernation Cycle. IBA, interbout arousal. EN, entering torpor. AR, arousing from torpor. LT, late torpor. (Nelson, Otis, & Carey, 2009)

With five to seven months of low body temperature during hibernation, 13-lined ground squirrels are true hibernators and are an interesting model to explore the mechanism of long-term platelet preservation (McGee-Lawrence et al, 2011). To survive the extreme condition of torpor, 13-lined ground squirrels have developed several physiological adaptations. Hibernation consists of 5-7 months alternating between 10-20 periods of low temperature, or torpor, lasting 7 to 10 days, interspersed by 12-24 hour interbout arousals (IBA) (Carey, Andrews, & Martin, 2003; Lechler & Penick, 1963; Reddick, Poole, & Penick, 1973; Zatzman, 1984). Before entering torpor, ground squirrels dramatically increase food consumption and accumulate white adipose (Schwartz, Hampton, &

Andrews, 2013). During torpor, ground squirrel body temperature is maintained at around 4-8℃ versus 37℃ in active animals (Carey, Andrews, & Martin, 2003). Hibernating ground squirrels' heart rates drop from 300-400 beat per minute (bpm) when active to 3-5 bpm in torpor (Lyman et al, 1982; Zatzman, 1984). In torpor, ground squirrel cerebral blood flow (CBF) is ～10% compared to normal CBF (Frerichs et al, 1994). Ground squirrels' respiration reduces from 100–200 breaths per minute in the active state to 4–6 breaths per minute in torpor (McArthur & Milsom, 1991; Carey, Andrews, & Martin, 2003). During torpor, basal metabolic rate (BMR) decreases to 2–4% of normothermic rates (Popovic, 1964; Hampton, Nelson, & Andrews, 2010). Ground squirrel renal function is greatly reduced or ceased altogether in torpor (Zatzman, 1984).

It takes an average of 2.8 hours for an animal to warm from torpor to IBA (Hampton, Nelson, & Andrews, 2010). During the IBA, ground squirrels' average body temperature maintains at 35.0℃ (Wade, 1930; Hampton, Nelson, & Andrews, 2010). The heart rate ranges from 200-450 bpm during IBA versus 300-400 bpm in euthermia (Hampton, Nelson, & Andrews, 2010). During IBA, ground squirrels' CBF returns to normal (Frerichs et al, 1994; Carey, Andrews, & Martin, 2003). Ground squirrel respiration rate increases rapidly to over 60 breaths per minute during the early IBA (Wu & Storey, 2012; Brooks, Myburgh, & Storey, 2015). A short burst of metabolism occurs during the middle of the arousal which is five times the active state BMR in the summer time and six times the BMR of a normothermic animal at rest at an ambient temperature of 5℃ (Popovic, 1964; Hampton, Nelson, & Andrews, 2010). Renal function returns to an active state during IBA (Lesser et al, 1970). The mechanisms of regulating spontaneous arousals are still not clear (Harris & Milsom, 2000; Drew et al, 2007). The popular assumption of the reason for spontaneous

arousals is a need for gluconeogenesis or removal of metabolic wastes (Galster & Morrison, 1975; Carey, Andrews, & Martin, 2003; Drew et al, 2007).

In a nonhibernating mammal, extended periods of such low body temperature and low circulation would cause blood clotting in vessels and subsequent organ injury (Watts et al, 1998; DeLoughery, 2004), however, ground squirrels have evolved mechanisms to avoid the damage from low body temperature (Lechler & Penick, 1963; Reddick, Poole, & Penick, 1973; Zatzman, 1984). Platelet and leukocyte levels drop 90% during torpor (Lechler & Penick, 1963; Pivorum & Sinnamon, 1981). After ground squirrels wake up from hibernation, their platelet levels return to normal within 2 hours without new platelet formation (Pivorun & Sinnamon, 1981; Reddick, Poole, & Penick, 1973). Newly generated platelets could not be found until 2-3 days after arousal from hibernation (Cooper et al, 2012). Therefore, platelets must be sequestered out of the blood during torpor. The released platelets post-arousal are functional and do not undergo rapid clearance from the circulation (Cooper et al, 2012). The mechanism of long lasting cold storage of ground squirrel functional platelets may help with preserving human platelets.

Ground Squirrel Platelets Changing Shapes during Temperature Changes

Ground squirrel platelets form into elongated rods when chilled to 4 ℃ (Figure 4). Cold induced rod shaped ground squirrel platelets return to normal disc shapes after rewarming to 37 ℃ (Cooper et al, 2012). The circumferential microtubules from human platelets disassemble when to exposed to cold, and reassemble into a larger ring after rewarming to 37 ℃ (White, Krumwiede, & Sauk, 1985). The differences between ground squirrel and human platelets indicate that the mechanism of ground squirrel platelet rod formation might be the key to avoiding cold induced problems for human platelets.

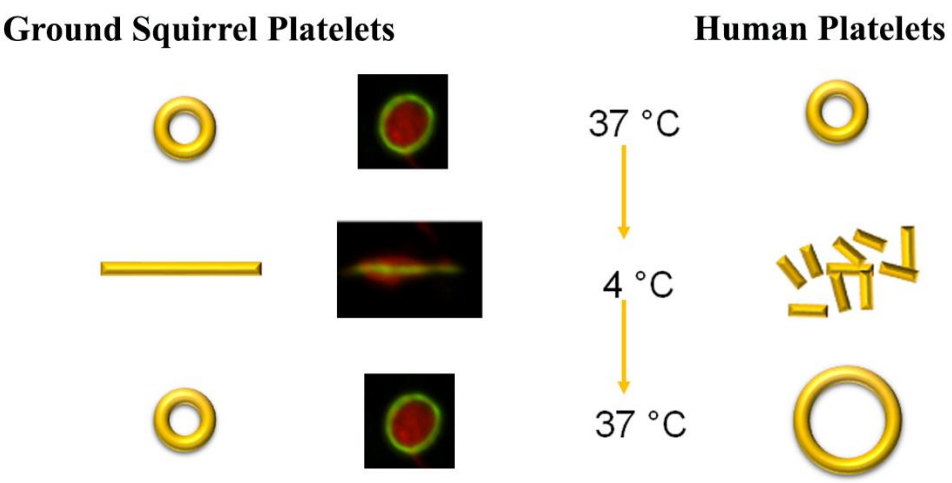


Figure 4. Simulation of the Differences between Ground Squirrel Platelets and Human Platelets. Platelet microtubules maintain ring shapes in all species at 37℃. Ground squirrel platelet microtubules form rod shapes after exposure to cold. Return to a similar sized ring after rewarming to body temperature. Microtubules from human platelets disassemble after exposure to low temperature, and form larger rings after rewarming to 37℃.

Cytoskeleton Plays a Role in Platelet Shape Changes

Mammalian cells maintain their shape with a cytoskeleton composed of microtubules, actin filaments, and intermediate filaments (Bernard, Gunning, & Kavallaris, 2009; Hoffmeister et al, 2001; Pertuy et al, 2014; Diagouraga et al, 2014)). Microtubules in human platelets disassemble when platelets are exposed to cold (White & Rao, 1998). Instead of disassembling, microtubule sfrom ground squirrel platelets form rods in response to low temperature (Fox, 1993; Winokur & Hartwig, 1995; Cooper et al, 2012; Cerecedo et al, 2013). The actual cause of ground squirrel platelet shape changes could be the reorganization of microtubules.

Microtubules

Microtubules are ubiquitous filamentous component of the cytoskeleton contributing to motility in all eukaryotic cells (Cooper, 2000). In resting platelets, microtubules form a marginal band to support a discoid shape (Behnke, 1965). A microtubule is a polymer

consisting of a hollow 25nm diameter cylinder of 10-15 protofilaments (usually 13 in mammalian cells) composed of α-tubulin and β-tubulin heterodimers associated with quantitatively minor non-tubulin components, or microtubule-associated proteins (Behnke, 1970b; Feit, Slusarek, & Shelanski, 1971; Bryan & Wilson, 1971; Ludueńa, Shooter, & Wilson, 1977; Desai & Mitchison; 1998). Microtubules grow faster on one end (the plus end) and grow slower on the other end (the minus end) (Behnke, 1965; Mitchison, 1993; Nogales et al, 1999). The polarity of a microtubule defines the direction of movement of cargo-carrying motor proteins along the microtubule (Howard & Hyman, 2003; Behnke, 1965).

In most cell types, microtubules initiate from centrosomes at a microtubule organizing center through gamma-tubulin (a member of tubulin superfamily) mediated nucleation on the minus end to overcome the kinetic barrier inside the cells (Pickett-heaps, 1969). Without centrosomes, gamma tubulin ring complex (γ-TuRC) acts alone as a nucleator of microtubules in platelets (Patel-Hett et al, 2008; Patel et al, 2005). Plus-end tracking proteins, such as end binding protein-1 (EB1), are one group of microtubule-associated proteins that are conserved in all eukaryotes and selectively accumulate at the growing microtubule plus ends (Schuyler & Pellman, 2001). EB1 regulates microtubule elongation by causing growth on the plus end of the microtubule (Galigart, 2010; Komarova et al, 2009; Vitre et al, 2008). In mitosis, microtubules have to disassemble first and reassemble into a mitotic spindle at metaphase (Kitanishi-Yumura & Fukui, 1987; Kline-Smith & Walczak, 2004). In human and mouse platelets, microtubule polymerization and depolymerization contribute to temperature-induced platelet shape changes (White & Rao, 1998). We suspected that microtubule polymerization and depolymerization could also be

responsible for temperature-induced ground squirrel platelet shape changes.

In addition to polymerization and depolymerization, microtubule dynamics are also driven by motor proteins, dynein and kinesin families (Cooper, 2000). During human platelet formation, dynein slides microtubules apart to form extrusions of megakaryocytes and to elongate preplatelets as a result of forming proplatelets (Patel et al, 2005; Diagouraga et al, 2014). Kinesin also plays a role, carrying organelles walking over microtubule into the bubble ends of proplatelets, during human platelet formation. Additionally, both dynein and kinesin are detected in human platelet lysates. (Rothwell & Calvert, 1997; Miki, Okada, & Hirokawa, 2005; Patel et al, 2005; Diagouraga et al, 2014). In resting human platelets, kinesin balances the opposite direction force from dynein to maintain microtubule ring shape by anchoring microtubules to the actin cortex instead of sliding paralleled microtubule apart. (Patel et al, 2005; Bartoli, Bishop, & Saunders, 2011; Flaumenhaft, 2011; Diagouraga et al, 2014; Sadoul, 2015; Figure 5). Therefore, dynein, which is involved in microtubules sliding apart, is possibly responsible for ground squirrel platelet shape changes during temperature changes rather than kinesin.

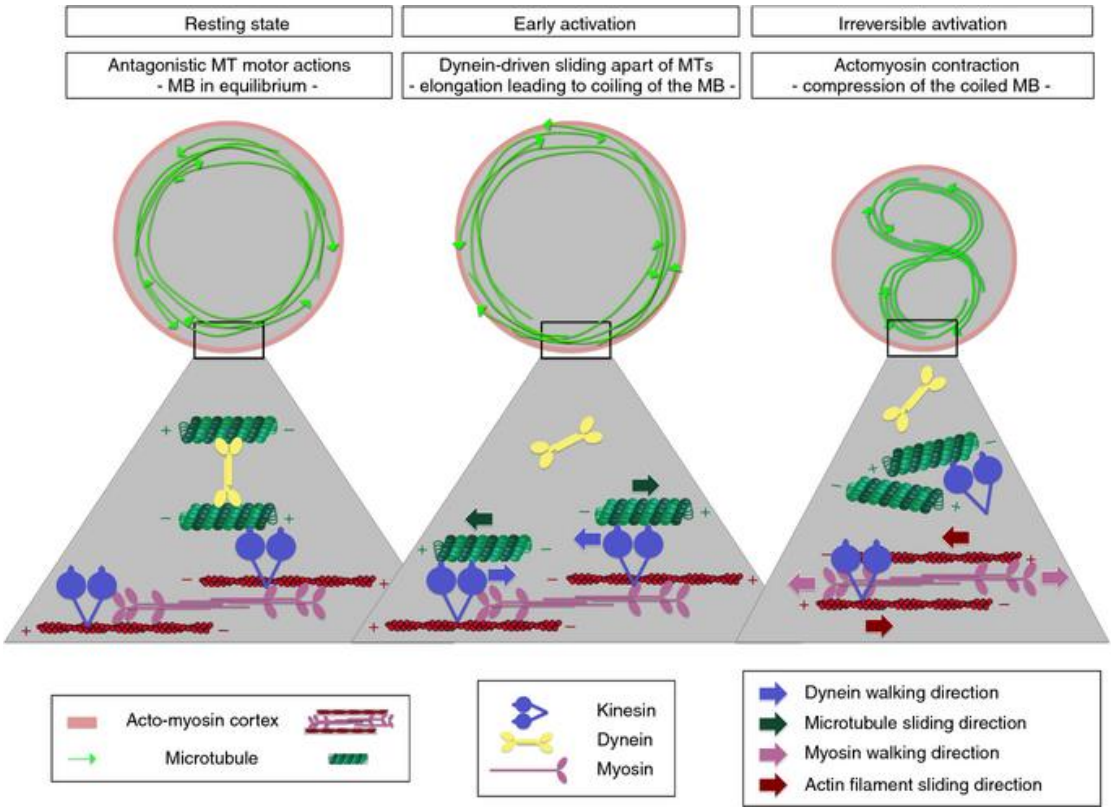


Figure 5. Motor Proteins Network in Platelet Activation (Sadoul, 2015)

Hypothesis

Ground squirrel platelets change into rod shapes when exposed to cold, and back to rings when rewarmed. Platelet shape changes under different temperatures could be the reason for platelets to survive cold storage during hibernation (Cooper et al, 2012). Three models have been proposed for how microtubule function might contribute to platelet shape changes: 1) microtubule polymerization (with or without depolymerization first), 2) cytoplasmic motor protein (dynein and/or kinesin) based sliding of overlapping, antiparallel microtubules past each other, which would extend protrusions on platelet ends, 3) a combination of both mechanisms.

SPECIFIC AIMS

Taxol and nocodazole have been employed to determine the mechanism of ground squirrel platelet shape changes under different temperatures, specifically, the potential role of microtubule polymerization/ depolymerization driven motility. And also anti-EB1, and anti-gamma tubulin were applied to explain the role of motor protein induced microtubule sliding in ground squirrel platelet shape changes. The specific aims of the study are to:

1. Determine if microtubule polymerization/depolymerization is required for ground squirrel platelet shape changes induced by temperature changes.
2. Determine the role of motor protein in ground squirrel platelet shape changes.

EXPERIMENT DESIGN

In order to determine if microtubule depolymerization was necessary for ground squirrel platelet shape changes under different temperatures, the microtubule-stabilizing agent taxol was employed to suppress microtubule depolymerization.

In order to determine if microtubule polymerization was required for ground squirrel platelet shape changes when exposed to different temperatures, nocodazole was applied to suppress microtubule polymerization.

In order to preliminarily determine the role of microtubule motor proteins during ground squirrel platelet changes, microtubule plus end and minus end labeling was performed. Anti-EB1 antibody was used to label the plus end, anti-gamma tubulin antibody was used to label the minus end. Anti-dynein antibody was used to label motor protein dynein.

Immunofluorescence and differential interference contrast (DIC)/phase contrast microscopy were used to collect images of the platelets in each experiment.

MATERIALS AND METHODS

Platelet Preparation

Ground squirrel blood samples were collected by arterial tail puncture with a 27-gauge needle from anesthetized healthy 13-lined ground squirrels. All animals in this study were born in captivity and housed in the University of Wisconsin-La Crosse Laboratory Animal Facility following protocols submitted to and approved by the institutional IACUC. Blood samples were collected in acid-citrate-dextrose (ACD), at 15% of the total volume. Whole blood was processed by low-speed centrifugation (200 x g for 8 minutes) to collect the supernatant and buffy coat. Platelet-rich plasma (PRP) was obtained by subsequent low-speed centrifugation of the supernatant and the buffy coat (100 x g for 6 minutes). Prostaglandin E1 (PGE1) (6 μM) was added to prevent platelet activation. Platelet counts were performed using a Hemocytometer on samples diluted 1:10 in Phosphate-buffered saline (PBS).

Taxol Treatment

Prior to treatment, all platelets were incubated at 37℃for one hour. Platelet samples received taxol (Life technologies, Carlsbad, CA), dissolved in dimethyl sulfoxide (DMSO, stock concentration 10mM)，to a final concentration of 1μM were incubated at 37℃ for 20 minutes. Control platelet stored in PBS solution were also incubated at 37℃ for an

extra 20 minutes after the pre-incubation. Both taxol treated and control samples were incubated in the following temperatures: 37℃ for 0 hour, 37℃ for 2 hours, 37℃ for 4 hours, 4℃ for 2 hours, and 4℃ for 2 hours and followed by 37℃ for 2 hours. 2×10^6 platelets of each treatment were fixed onto polylysine-coated glass slides with 4% paraformaldehyde and 0.5% glutaraldehyde by centrifugation at 2,100 x *g* for 4 min in a Cytofuge2 (StatSpin, Norwood, MA). Platelets were permeablized with 0.5% Triton X-100 for 2 minutes. Bovine serum albumin (1% BSA in 0.01M PBS) was used to block non-specific binding sites and reduce background fluorescence for 10 minutes. Actin filaments were labeled with Texas red phalloidin (Invitrogen, Carlsbad, CA) (1:40 dilution). Beta tubulins were stained with mouse anti-beta tubulin primary antibody (Life technologies, Eugene, OR) (1:1000 dilution) bound with Alexa Fluor 488 goat anti-mouse IgG (Life technologies) (1:200 dilution). Fluorescence and phase contrast images were obtained on Nikon Eclipse 80i fluorescence microscope with QImaging ExiAqua camera and NIS Elements D software and processed using ImageJ software (NIH, http://imagej.nih.gov/ij) to create merged picture overlays.

Nocodazole Treatment

All platelets were incubated at 37℃ for one hour prior to treatment. Nocodazole (Thermo Fisher. Scientific®) stock solution was prepared in DMSO (stock concentration 10mg/ml). Nocodazole treatment was organized into four groups at 37℃ for 1 hour: 0nM (control group), 250nM, 500nM, and 1000nM nocodazole treatment. All four groups of nocodazole-treated platelets were incubated as following temperatures treatments: 37℃ for 2hours, 37℃ for 4 hours, 4℃ for 2 hours, and 4℃ for 4hours, and 4℃ for 2hours followed by 37 ℃ for 2hours. Platelets were centrifuged onto polylysine-coated

microscope slides at 2,100 x *g* for 4 min in a Cytofuge2 after nocodazole treatment and different temperature incubation followed same procedure of taxol treatments.

Gamma Tubulin, EB1, and Dynein Labeling

All platelets were incubated at 37℃ for two hours before treatment. Platelets were fixed at three time points: 37℃ for 0 hour, 4℃for 2 hours, and 37℃ for 2 hours after chilling at 4℃ for 2 hours. 2×10^6 platelets were centrifuged on polylysine-coated microscope slides at 2,100 x *g* for 4 min in a Cytofuge2 for gamma tubulin, EB1, and dynein labeling. Endogenous Biotin-Blocking kit (Invitrogen, Carlsbad, CA) and 1% BSA were used for blocking. A 1:100 dilution of mouse anti-gamma tubulin (Life technologies), and 1:200 dilution of rabbit anti-beta tubulin primary antibody (Life technologies, Eugene, OR) were used as primary antibodies in gamma tubulin labeling experiment. 1: 100 rabbit anti- EB1 (Life technologies) and 1:1000 dilution of mouse anti-beta tubulin primary antibody (Life technologies, Eugene, OR) were used as primary antibodies in EB1 labeling experiment. 1:100 mouse anti-dynein (Life technologies) and 1:200 dilution of rabbit anti-beta tubulin primary antibody (Life technologies, Eugene, OR) were used as primary antibodies in dynein labeling experiment. A 1:200 dilution of Streptavidin-Alexa 488 goat anti-mouse IgG (Life technologies) was used as secondary antibody for gamma tubulin and dynein. 1:1000 dilution of goat anti-rabbit IgG Alexa 594 (Life technologies) was used as secondary antibody for beta-tubulin in gamma tubulin and dynein experiment. 1:1000 dilution of Streptavidin-Alexa 594 goat anti-rabbit IgG (Life technologies) was used as secondary antibody for EB1, and 1:200 dilution of goat anti-mouse IgG Alexa 488 (Life technologies) was used as secondary antibody for beta-tubulin in EB1 labeling experiment.

Statistical Analysis

In order to assess the different shapes of microtubules in platelets, they were categorized into three sets: ring, transition between ring and rod, and rod. Results were also analyzed a second time based on classifying the images into two sets: rod-like and ring-like. In order to assess the statistical significance of the difference, Student's t-test was applied to the number of platelets within each group at different temperatures and treatments.

RESULTS

Categorizing Microtubule Shapes

Platelets with multiple shapes of microtubules in addition to straight rods or rings have been observed (Cooper et al, 2012). In order to study the mechanism of platelets shape changes objectively, ground squirrel platelets were categorized in two different ways according to the various shapes of their microtubules, named SET 1 and SET 2. In SET 1, ground squirrel platelets were separated into three groups: rod (Figure 6A and B), transition (Figure 6C-L), or ring (Figure 6M and N). In SET 2, ground squirrel platelets were separated into two groups: rod-like (Figure 6A-H) or ring-like (Figure 5I-N).

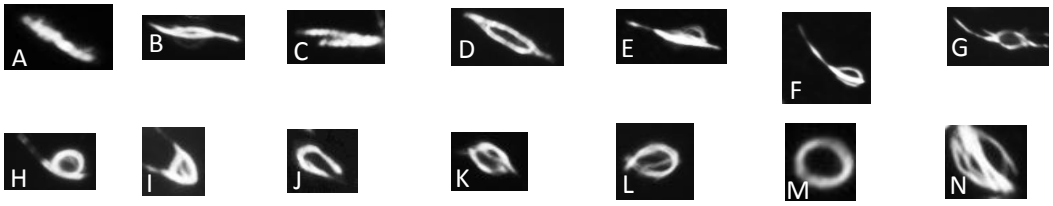


Figure 6. Categorization of Microtubule Shapes in Cold Induced Ground Squirrel Platelets. SET 1: (A) and (B) represent rod shape microtubules; (C) to (L) represent microtubules in transition state; (M) and (N) represent ring shape microtubules. SET 2: (A)-(H) represent rod-like microtubules; (I)-(N) represent ring-like microtubules.

Ground Squirrel Platelet Microtubule Shape Changes Associated with Platelet Shape Changes

Due to the observation and analysis from previous research, a hypothesis that microtubule shape changes correlate with ground squirrel platelet shape changes was proposed. The hypothesis was tested by analyzing the shapes of fluorescently labeled

microtubules under different temperature and chemical treatments. The study of control platelets under different temperatures first confirmed that the temperature sensitivity of the platelet shape changes can be relected by microtubule conformation (Cooper et al, 2012).

When ground squirrel platelets were stored at 37℃, ring shapes predominated (Figure 7). When grouped by SET1, the ratio of ring to rod shaped microtubules in platelets incubated at 37℃ changed from 3:1 (t-test, $p=6.5\times10^{-6}$) at 0 hour to 4:1 (t-test, $p=1.4\times10^{-6}$) after two hours and 9:1 (t-test, $p=1.1\times10^{-7}$) after four hours (Figure 6A). When grouped by SET 2, the ratio of ring to rod shaped microtubules changed from 2:1 (t-test, $p=3.7\times10^{-5}$) to 4:1 (t-test, $p=4.2\times10^{-8}$) to 6:1 (t-test, $p=6.0\times10^{-8}$) at the same time points (Figure 6B).

In contrast to 37℃, when ground squirrel platelets were stored at 4℃, rod shapes predominated (Figure 7). When grouped by SET 1, the ratio of ring to rod shaped microtubules was 1:2 (t-test, $p=3.1\times10^{-7}$) and 1:2.5 (t-test, $p=10\times10^{-8}$) at 4℃ for 2 or 4 hours, respectively (Figure 7A). When grouped by SET 2, the ratio of ring-like to rod-like shape microtubules was 9:10 (t-test, p=0.001) and 8:10 (t-test, $p=3.6\times10^{-8}$) at 4℃ for 2 hours and for 4 hours, respectively (Figure 7B). When chilled at 4℃ for 2 hours and rewarmed to 37℃ for 2 hours, the platelets transition from a rod to ring shape (Figure 7). When grouped by SET 1, the ratio of ring to rod shaped microtubules is 3:1 (t-test, $p=3.3\times10^{-6}$) when rewarmed to 37℃ for 2 hours after chilling at 4℃ for 2 hours (Figure 7A) while the ratio of ring-like vs. rod-like shapes microtubules is also 3:1 (t-test, $p=1.5\times10^{-6}$) when grouped by SET 2 (Figure 7B).

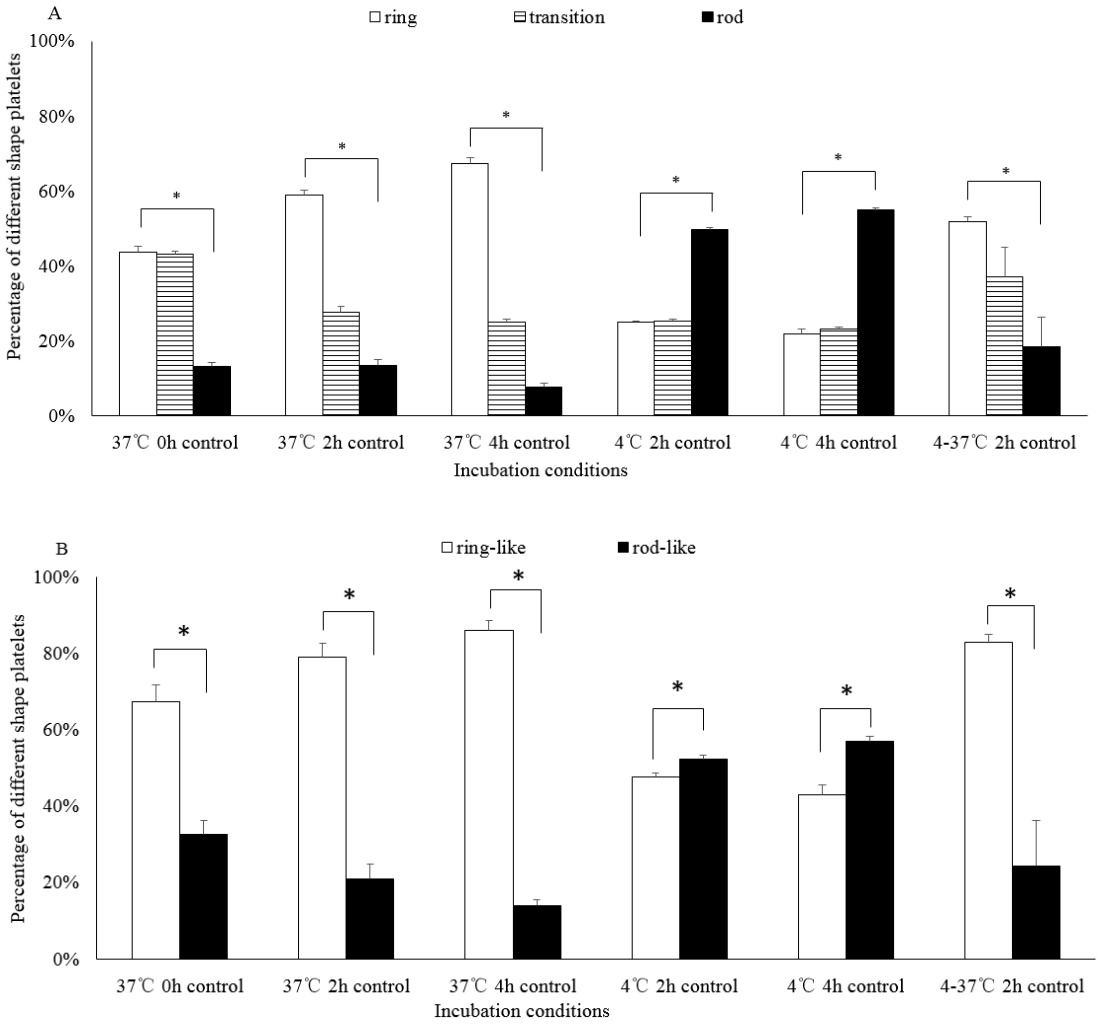


Figure 7. Ground Squirrel Control Platelets Shape Changes under Different Temperatures. (A) Trend of Set 1 control platelet shape changes with temperature. Control platelets have a tendency to form more ring shape platelets with longer time incubation time at 37℃. Control platelets formed more rod shapes after chilled to 4℃. Control platelets formed back to ring shapes after rewarm to 37℃. (B) Trend of Set 2 control platelet shape changes with temperature. Control platelet formed more ring-like shapes with longer incubation. Cold incubated platelets formed more rod-like platelets with longer incubation time. Rewarming cold control ground squirrel platelets, resulted in more ring-like platelets. Y-axis represents the percentage of platelets in each shape. X-axis represents incubation conditions. n=12 animals. n>1376 platelets have been counted for each treatment *Statistically significant different (t-test, $p<0.05$).

Taxol Treated Platelet Formed More Rod Shapes at both 37℃ and 4℃

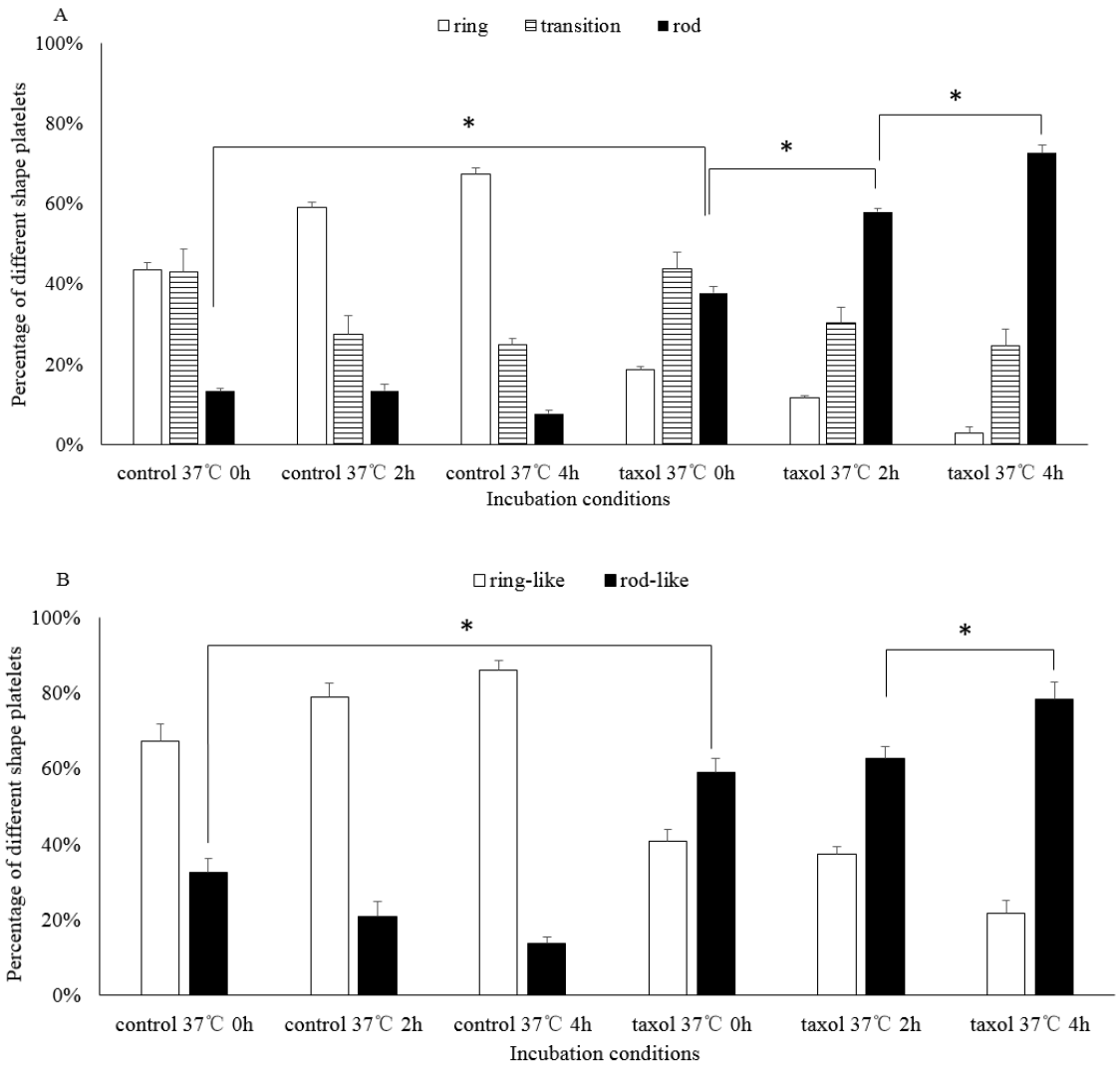
In order to test the role of microtubule depolymerization during cold induced ground squirrel platelet rod formation, platelets were pre-incubated at 37℃ for one hour, followed by treatment with taxol for 20 minutes to inhibit microtubule depolymerization.

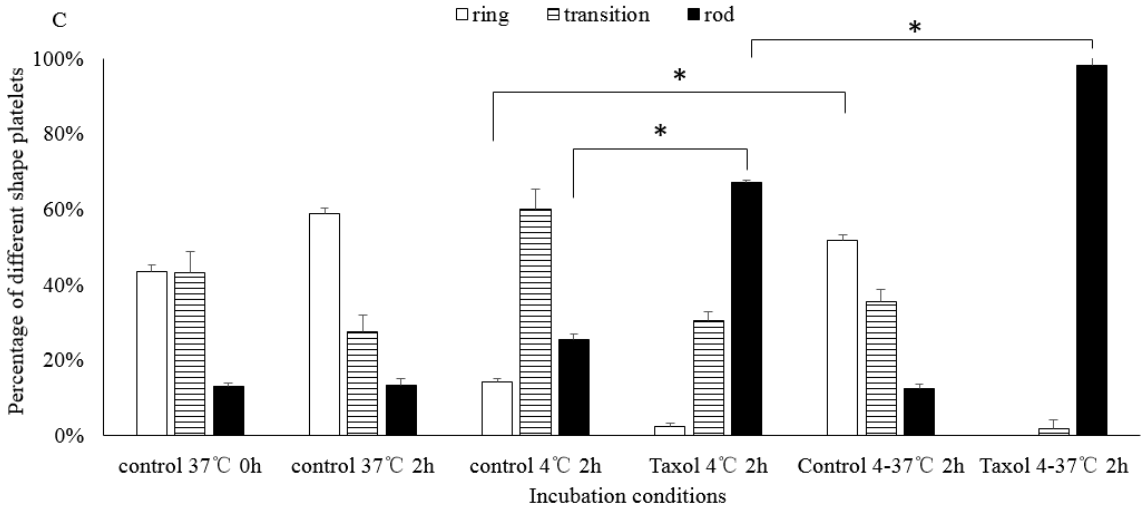
In taxol treated platelets, microtubules were able to form rods at 37℃, and the number of ring shaped microtubules decreased (t-test, $p<0.01$) (Figure 8A and B). The longer the exposure time to taxol, the more rod shaped platelets formed at 37℃(t-test, $p<0.01$) (Figure 8A and B). Platelets started to form more rod shapes right after 20 minutes exposure to taxol (at time 0), 24% more rod shape platelets (t-test, $p<0.01$) (Figure 8A) and 26% more rod-like shape platelets formed (Figure 8B) when compared with control platelets.

Taxol treated platelets formed more rod shapes at 4℃, and did not return to a ring shape. After taxol treatment, platelet microtubules formed 42% more rods (t-test, $p<0.02$)(Figure 8C) and 32% more rod-like structures (t-test, $p<0.01$) (Figure 8D) compared with control platelets at 4℃ incubation. Taxol treated platelets formed 31% more rods (Figure 8C) and 4% more rod-like structures (Figure 8D) when rewarmed to 37℃ for 2h after two hours exposed to cold than with 2h 4℃ incubation (t-test, $p<0.01$). Taxol treated platelets, formed more rod shapes when rewarmed to 37℃ after cold incubation. The trend, rewarmed platelets, under taxol treatment, that formed more rod shaps, was the opposite of control platelets which formed more ring shapes after rewarming to body temperature for 2h from 2h 4℃ incubation (t-test, $p<0.01$) (Figure 8C and D).

Platelets formed more rod shapes with longer exposure time to taxol (Figure 8E and F). At the same taxol exposure time, platelets exposed to low temperature formed more rod

shapes compared with platelets incubated at body temperature (t-test, $p<0.01$)(Figure 8E and F). In taxol treatment, two hours at 4℃ incubation let to formation of 9% more rod shape platelets (Figure 8E) and 33% more rod-like platelets (Figure 8F) compared with platelets incubated at 37℃ for 2h (t-test, $p<0.01$). Taxol treated platelets, formed 26% more rods (Figure 8E) and 22% more rod-like structures (Figure 8F) when rewarmed to 37℃ for 2h after a 2h 4℃ incubation compared with a 37℃ 4h incubation (t-test, $p<0.01$).

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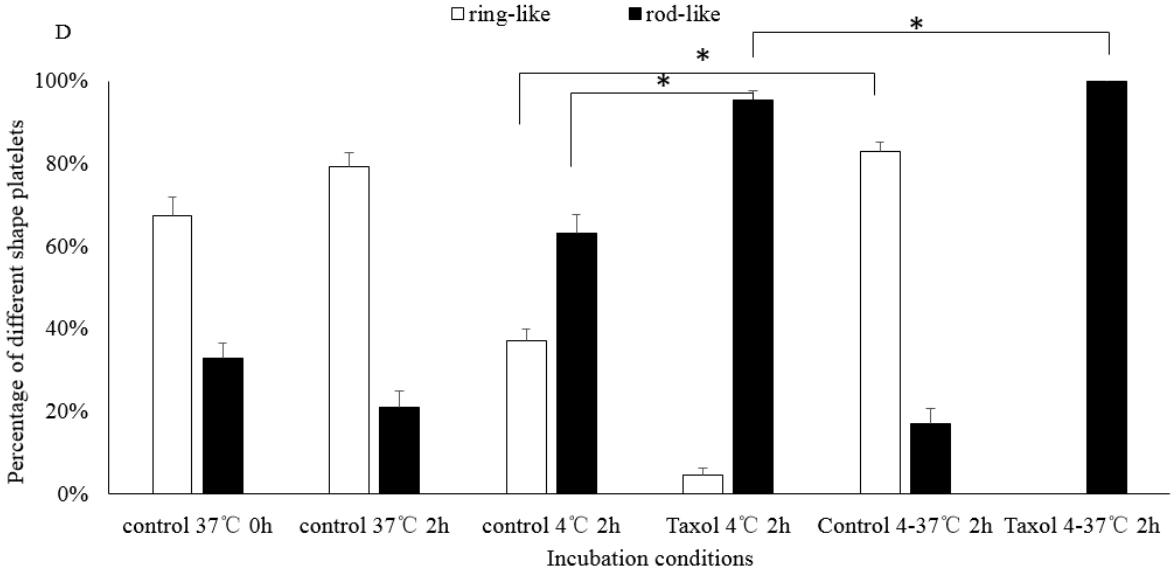
Control 4-37℃ 2h
Taxol 4-37℃ 2h
Incubation conditions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Taxol 4-37℃ 2h
Incubation conditions

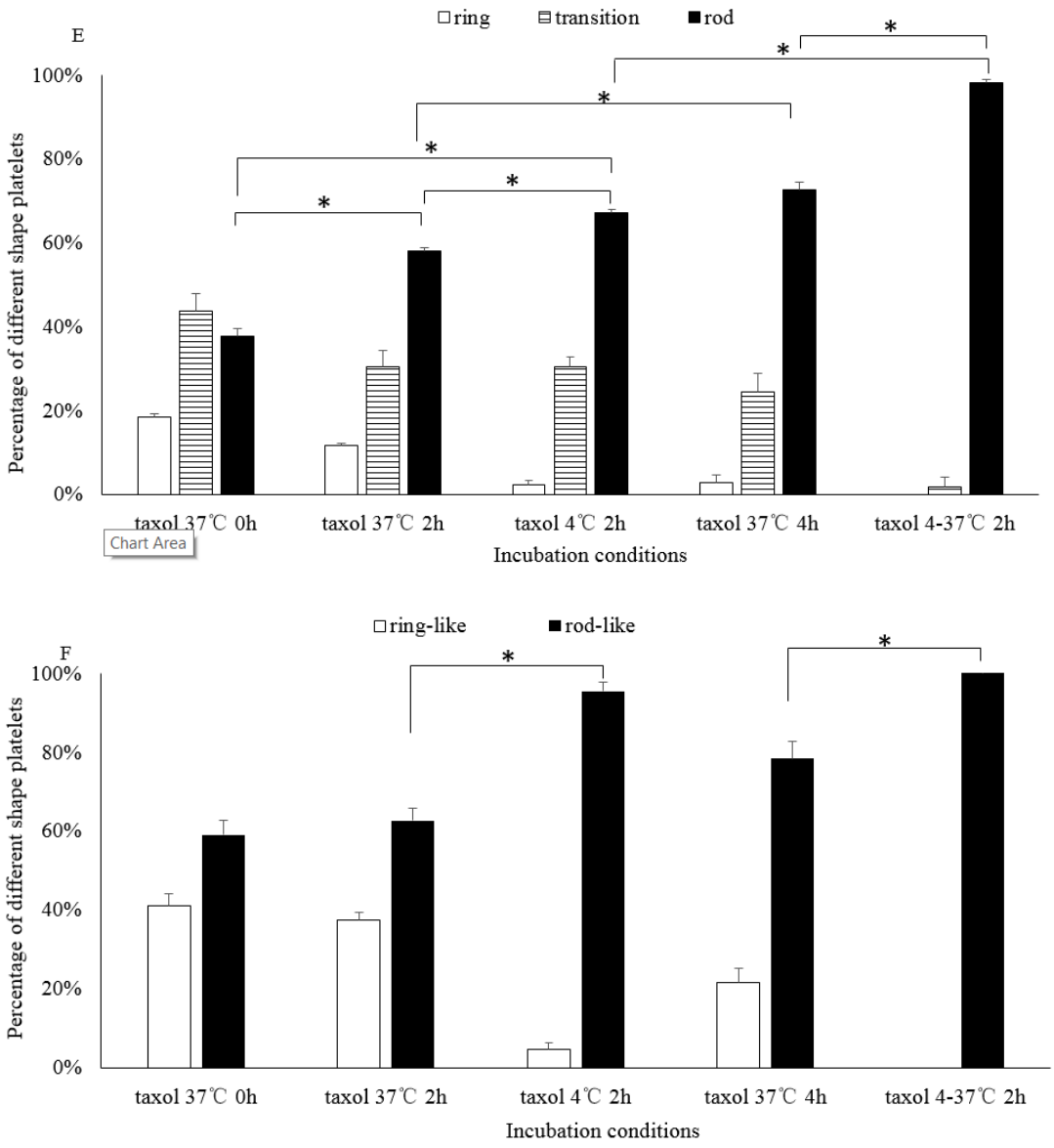


Figure 8. Taxol Treatment Is Enough to Form Rod Shape Platelets. (A) SET 1 Taxol Alone Is Enough to Form Rod Shape Platelets at 37 ℃. Taxol treated platelets have a tendency to form rod shape platelets under longer incubation time at 37℃. Platelets started forming more rod shapes after 20minutes exposure to taxol. (B) SET 2 Taxol Alone Is Enough to Form Rod Shape Platelets at 37 ℃. Platelets formed more rod-like under longer exposure time to taxol at 37℃. Platelets stated forming rod-like shapes right after 20minutes exposed to taxol. (C) SET 1 Disassembly Is Not Required to Form Rod Shape Platelets at 4 ℃. Taxol treated platelets formed more rod shapes at 4℃. Taxol treated platelets did not form ring shapes after rewarming to 37℃ from 4℃ incubation. (D) SET 2 Disassembly Is Not Required to Form Rod Shape Platelets at 4 ℃. Taxol treated platelets formed more rod-like shapes at 4℃. Taxol treated platelets cannot form ring-like shapes after rewarming from 4℃ to 37℃. (E) SET 1 Low Temperature Increase Taxol Treatment Forming Rod Shape Platelets. Longer exposure time to taxol caused platelets to form more rod shapes. After

the same exposure to taxol, platelets incubated in the cold formed more rod shapes. (F) SET 2 Low Temperature Increase Taxol Treatment Forming Rod Shape Platelets. The longer time of exposure to taxol, the more rod-like shaped platelets formed. With the same taxol exposure time, platelets incubated at low temperatures formed more rod-like shapes. Y-axis represents the percentage of platelets with different shapes. X-axis represents incubation conditions. n=6 animals. n> 220 platelets were counted for each treatment. *Statistically significant difference (t-test, $p<0.05$).

Nocodazole Treatment Delayed Rod Formation

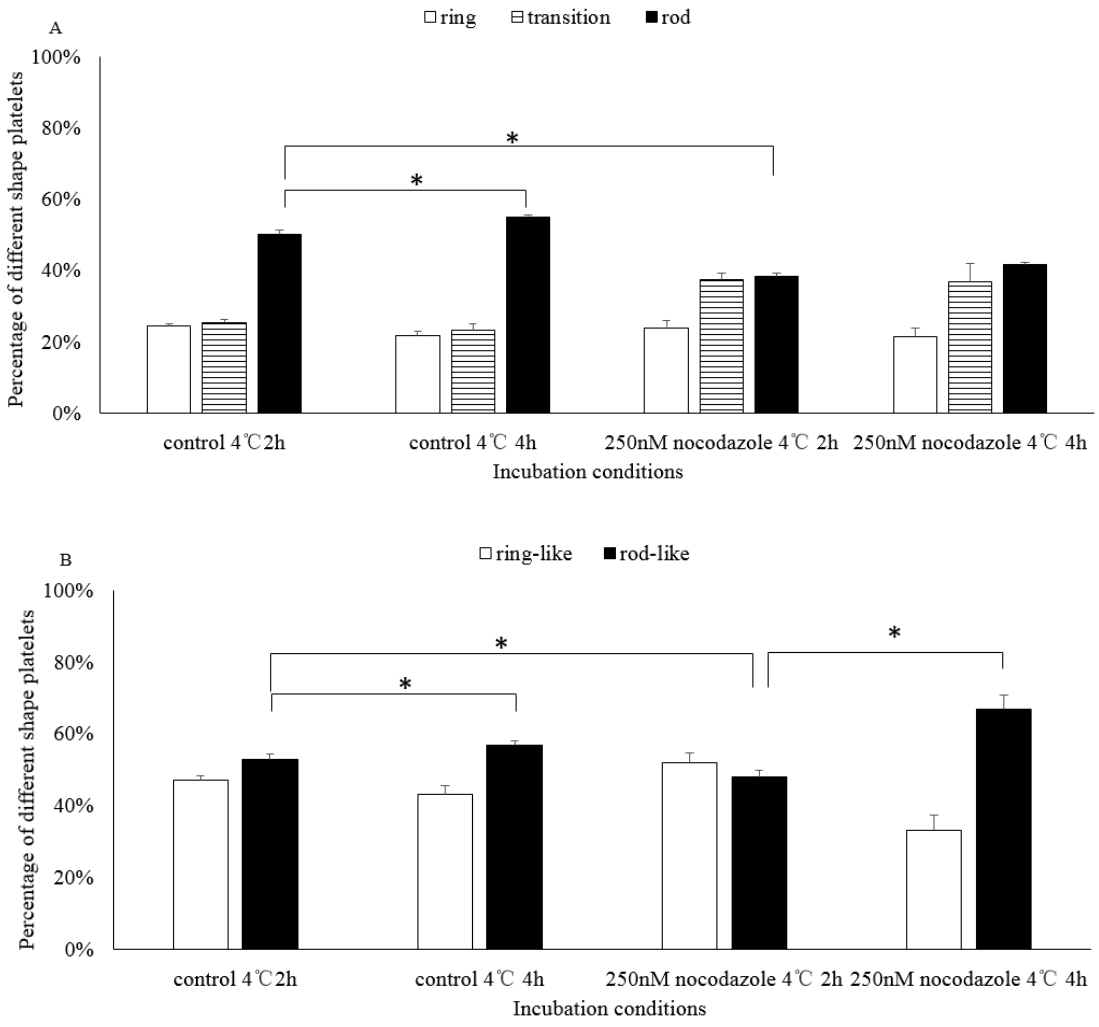
In order to determine whether microtubule polymerization is required for ground squirrel platelet shape changes, platelets were treated with 250nM, 500nM, 1000nM nocodazole to inhibit microtubule polymerization for 1h after 1h 37℃ pre-incubation. 250nM nocodazole treatment stabilized microtubules without moving towards microtubule depolymerization (Appendix V). A concentration of 250nM nocodazole was used for subsequent experiments.

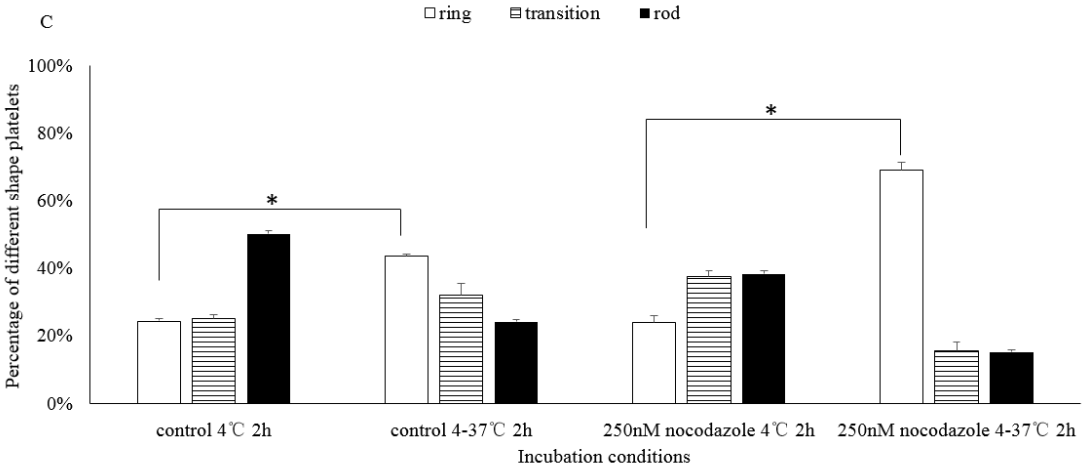
Low temperature delayed nocodazole treated platelets from forming rods with 12% fewer rod shaped microtubules (Figure 9C) and 5% fewer rod-like shaped microtubules forming (Figure 9D) than in control platelets incubated at 4℃ for 2h (t-test, $p<0.01$). Platelets treated with nocodazole had 3% more rod shaped microtubules (Figure 9A) and 19% more rod-like shaped microtubules (Figure 9B) after 4h 4℃ incubation compared with those with a 2h 4℃ incubation (t-test, $p<0.01$). Nocodazole treated platelets formed more rods than control samples but did not stop platelet rod formation after longer time 4℃ incubation (t-test, $p<0.01$) (Figure 9A and B).

Nocodazole treatment did not inhibit microtubules from forming ring shapes upon rewarming to 37℃ after a cold incubation (Figure 9C and D). Nocodazole treated platelets formed 15% more ring shaped microtubules (Figure 9C) and 12% more ring-like shaped microtubules (Figure 9D) when rewarmed to 37℃ for 2h after a 2h 4℃ incubation than

control platelets at the same temperature (t-test, $p<0.01$).

Low temperature increased nocodazole treated platelets rods formations (Figure 9 E and F). The ratio of rod-shaped to ring-shaped platelets at 37℃ over time was relatively stable. Nocodazole treatment did not stop microtubules from forming rods at 4℃. After incubation at 4℃ for 2h, nocodazole treated platelets formed 20% more rod shapes (Figure 9E) and 23% more rod-like shapes (Figure 9F) than nocodaozle treated platelet incubated at 37℃ for 4h (t-test, $p<0.01$).

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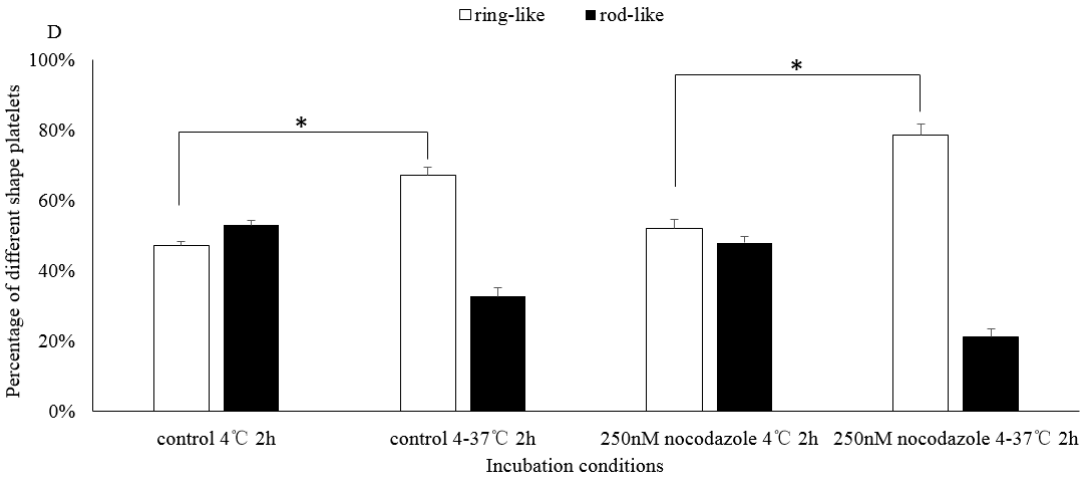
Incubation conditions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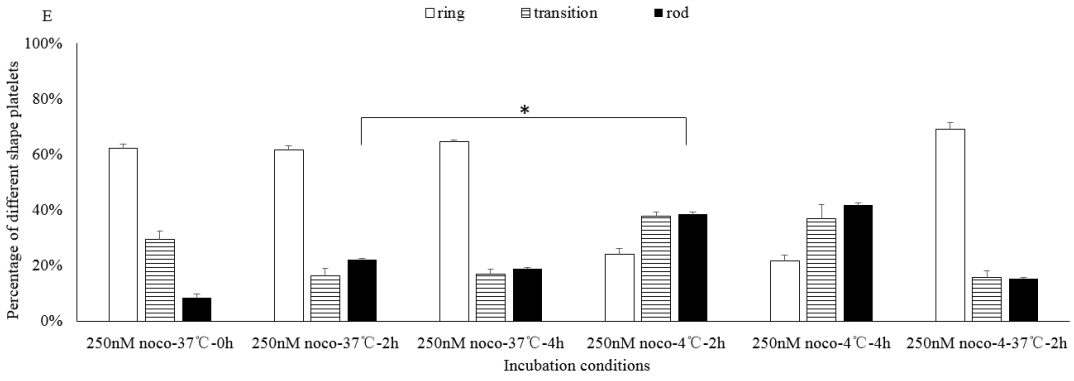
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Incubation conditions

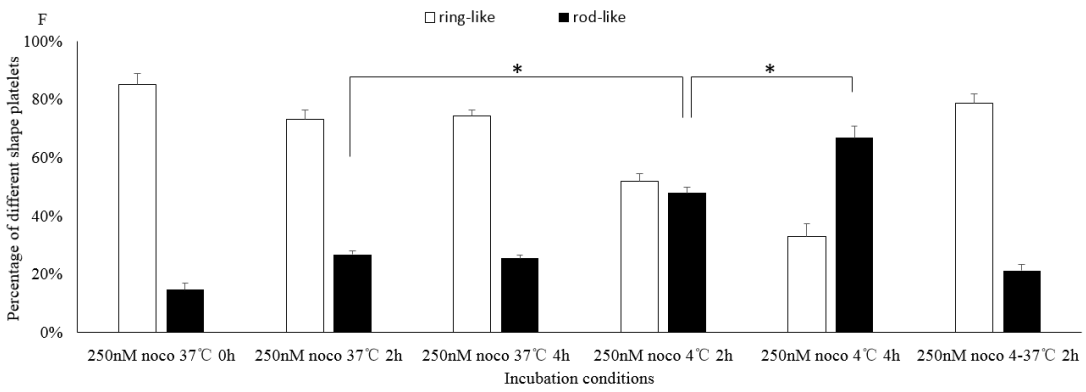


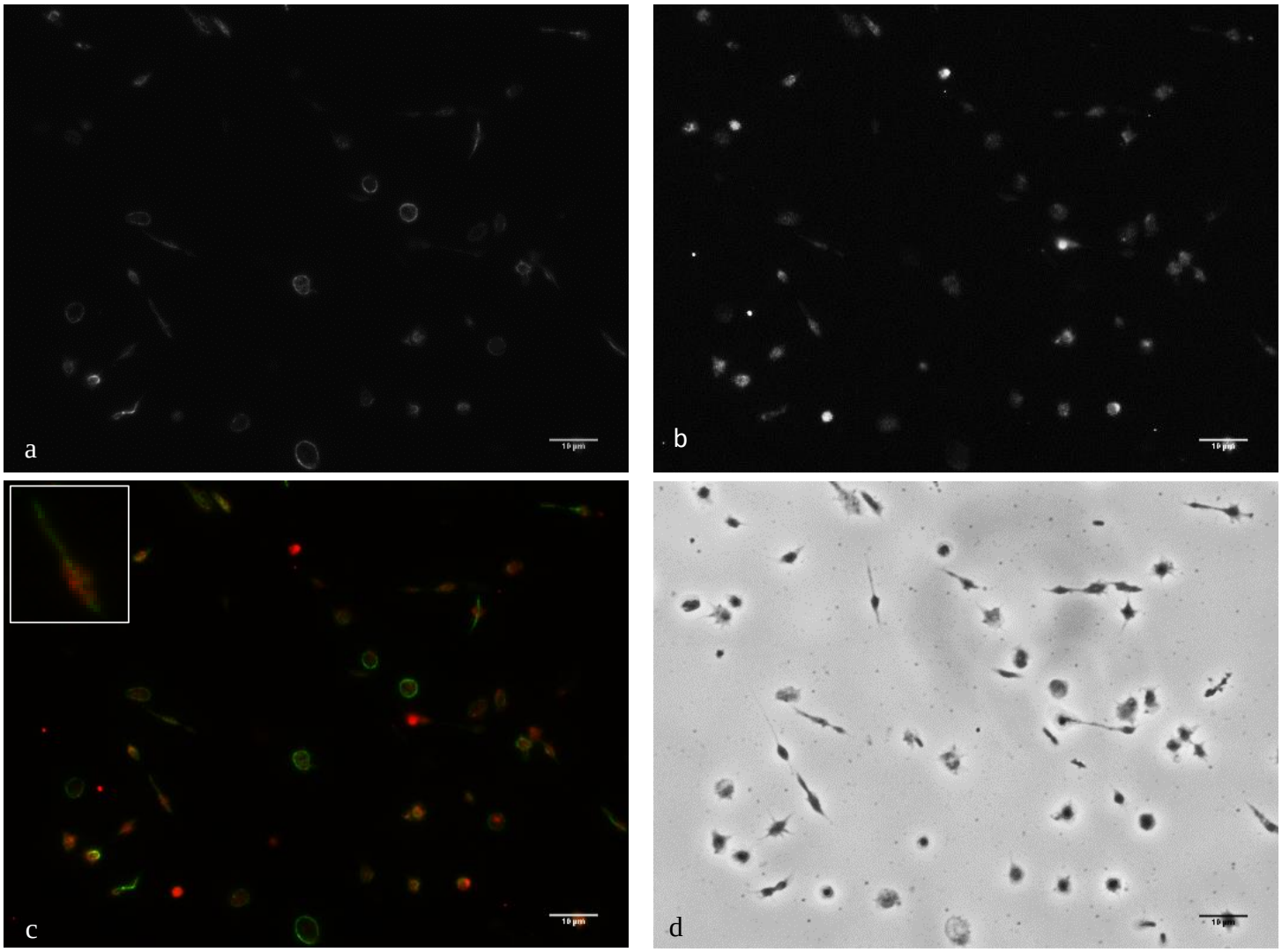
Figure 9. Nocodazole Treatment Delayed Microtubule Rod Formation. (A) SET1 4℃ Increased Nocodazole Treated Platelet Rod Formation. Fewer nocodazole treated platelets rod shapes than controls at 4℃. After longer incubation time at 4℃, nocodazole treated platelets formed more rod shapes. (B) SET2 Low Temperature Increased Nocodazole Treated Platelet Rod Formation. At 4℃, nocodazole treated platelets formed fewer rod-like shapes. Nocodazole treated platelets have a tendency to form more rod-like shapes with longer incubation at 4℃. (C) SET1 Nocodazole Treatment Did Not Inhibit Ring Shape Platelet Reformation. Nocodazole treated platelets formed back to ring shapes after rewarmed to body temperature. (D) SET2 Nocodazole Treatment Did Not Prevent Ring-like Shape Platelet Reformation. Nocodazole treated platelets formed back to ring-like shapes after rewarming to 37℃. (E) SET 1 Nocodazole Treated Platelets Formed Rod Shapes At 4℃. Nocodazole treated platelets formed more rod shapes when incubated to 4℃. (F) SET 2 Nocodazole Treated Platelets Formed Rod Shapes At 4℃. Nocodazole treatment did not stop platelets from forming rod-like shapes at 4℃. Y-axis represents the percentage of differently shaped platelets. X-axis represents incubation conditions. n=6 animals. n>388 platelets have been counted for each treatment. *Statistically significant difference (t-test, $p<0.05$).

Motor Proteins Involvement Cannot Be Determined

Microtubule motor protein sliding is another potential mechanism for ground squirrel platelet shape changes. Various shapes of microtubules were observed during ground squirrel platelet shape changes. The motor proteins dynein and/or kinesin could be responsible for the changes in shapes of microtubules during platelet chilling. Dynein slides microtubules apart to cause preplatelets to form proplatelets in human platelet formation. Dynein may be responsible of ground squirrel platelet shape changes. Anti-dynein immunofluorescence was employed to confirm the presence of dynein in platelets (Figure

10 i-l and Figure 10 I-L). Anti-EB1 (Figure 10a-d and A-D) and anti-gamma tubulin (Figure 10e-h and E-H) immunofluorescence were applied to examine the pattern of the distribution of microtubule plus ends and minus ends in rod/rod-like shaped platelets. Streptavidin and avidin-biotin were used to amplify the signal of EB1, gamma tubulin and dynein labeling which compensated for the limited amount of these three proteins.

EB1, gamma tubulin, and dynein were detected in ground squirrel platelets. The distribution of EB1, gamma tubulin and dynein showed that they all associated with microtubules in different shapes (rods, ring and transition) (Figure 10 A-L). Based on the compiled data, it is difficult to determine whether dynein or kinesin promoted microtubule rod formation.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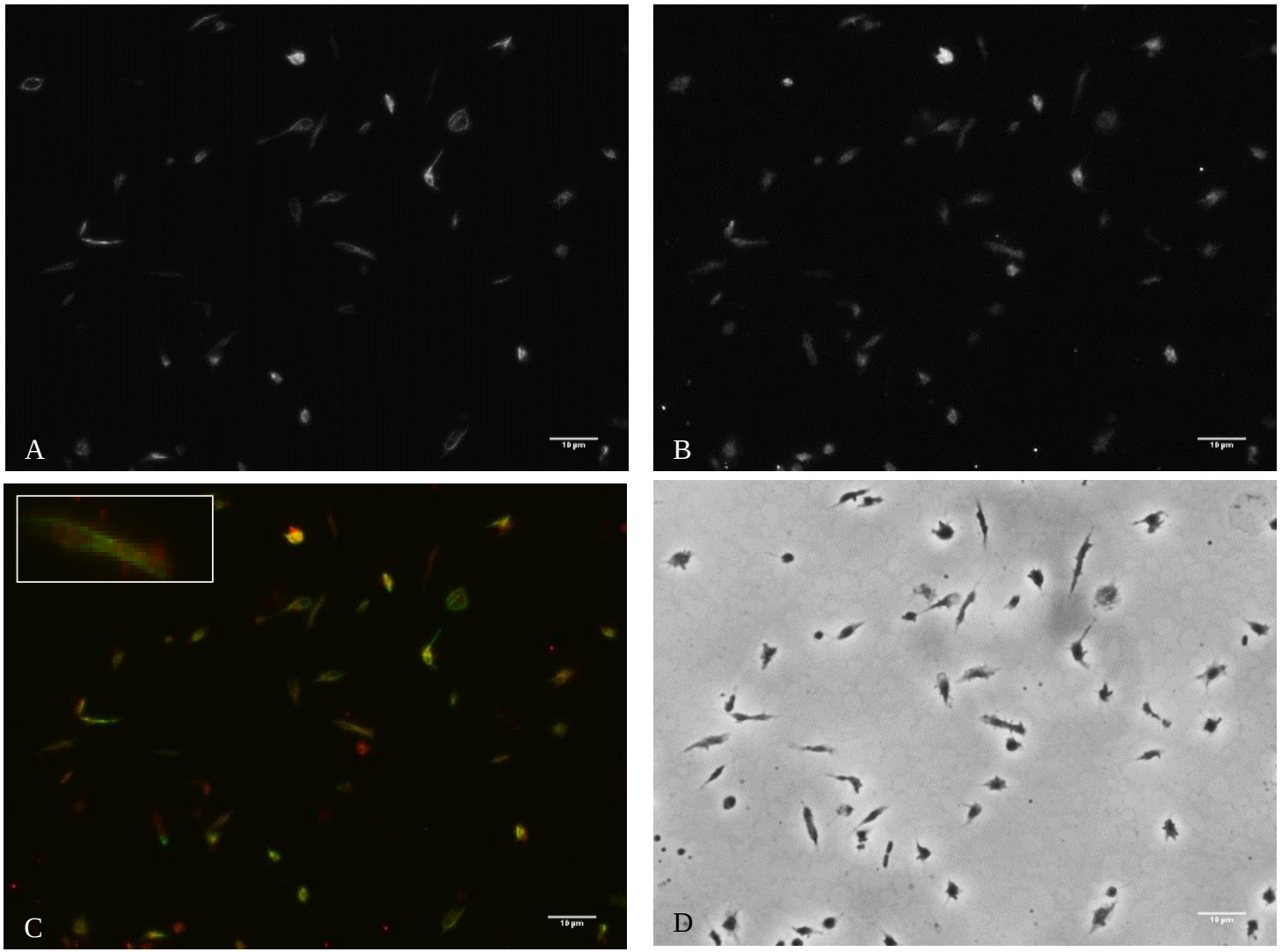
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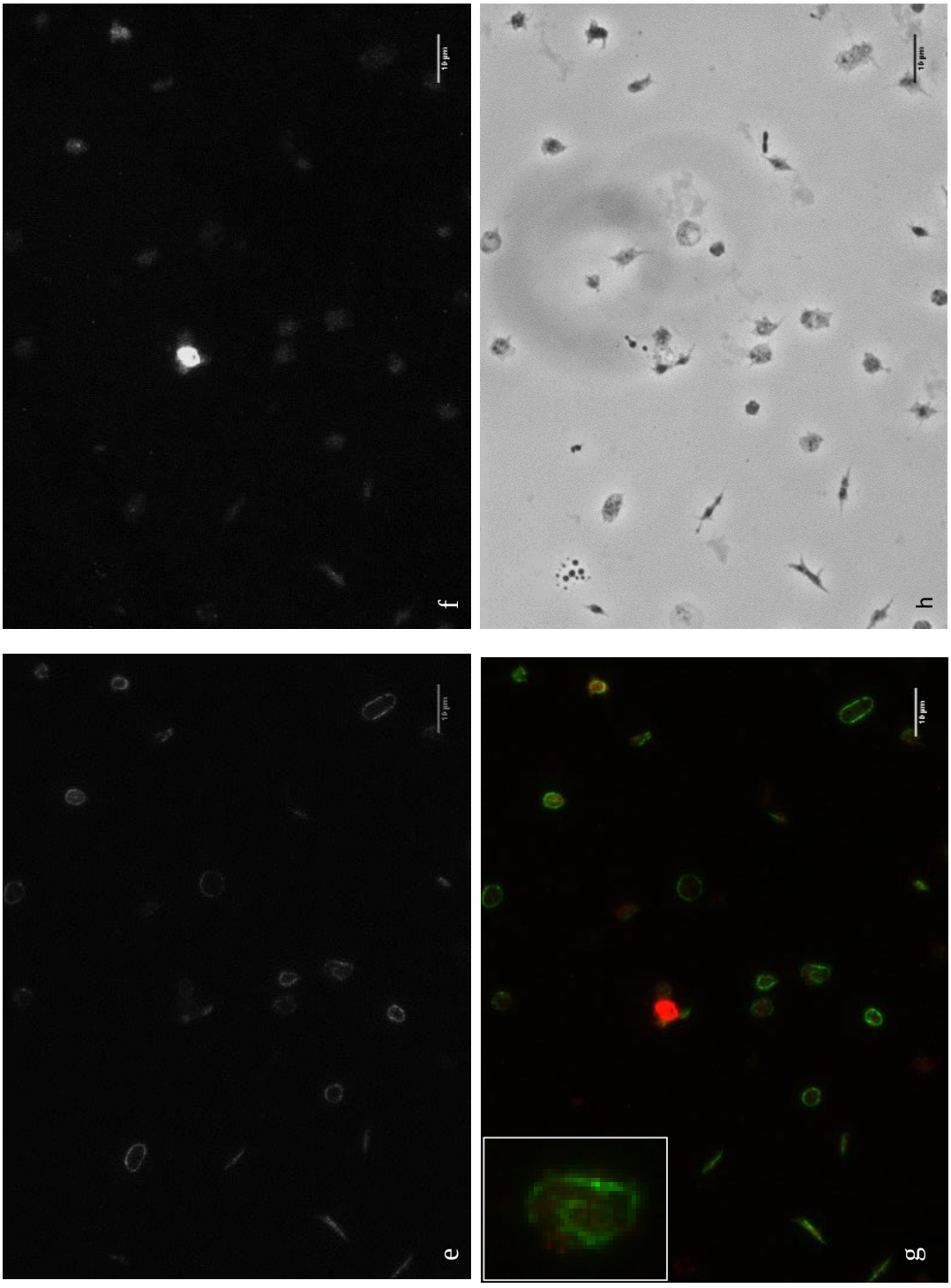
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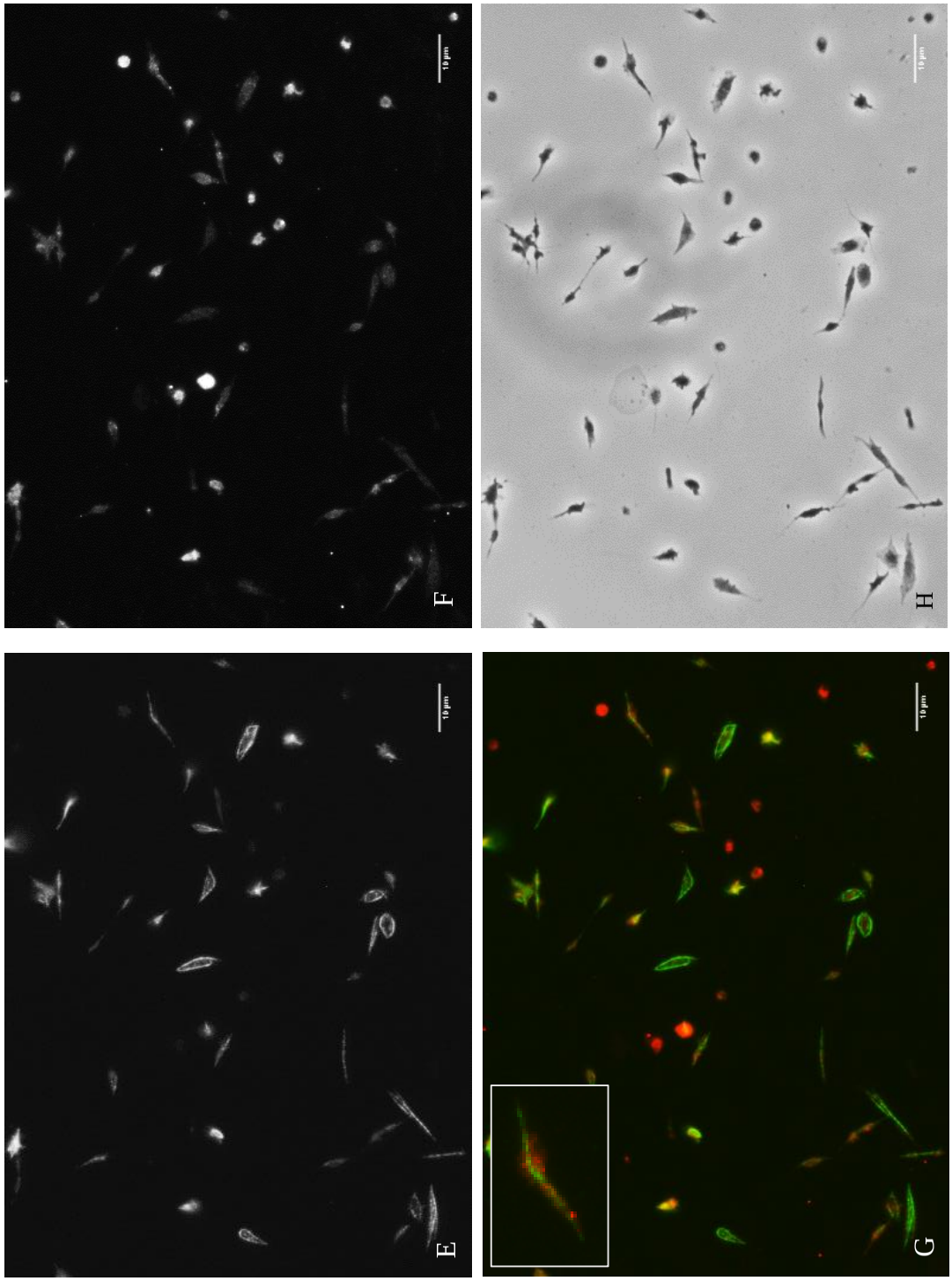
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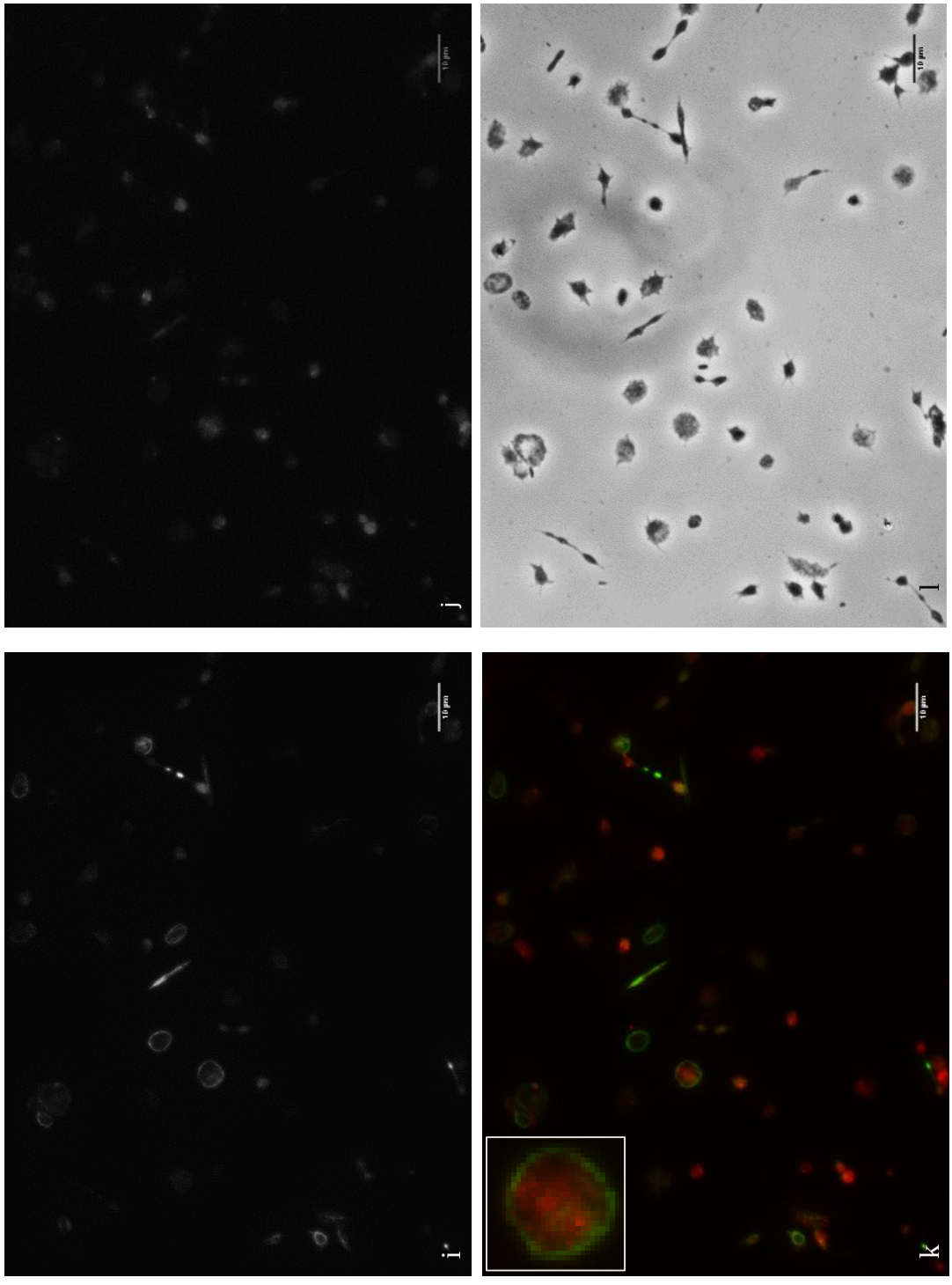
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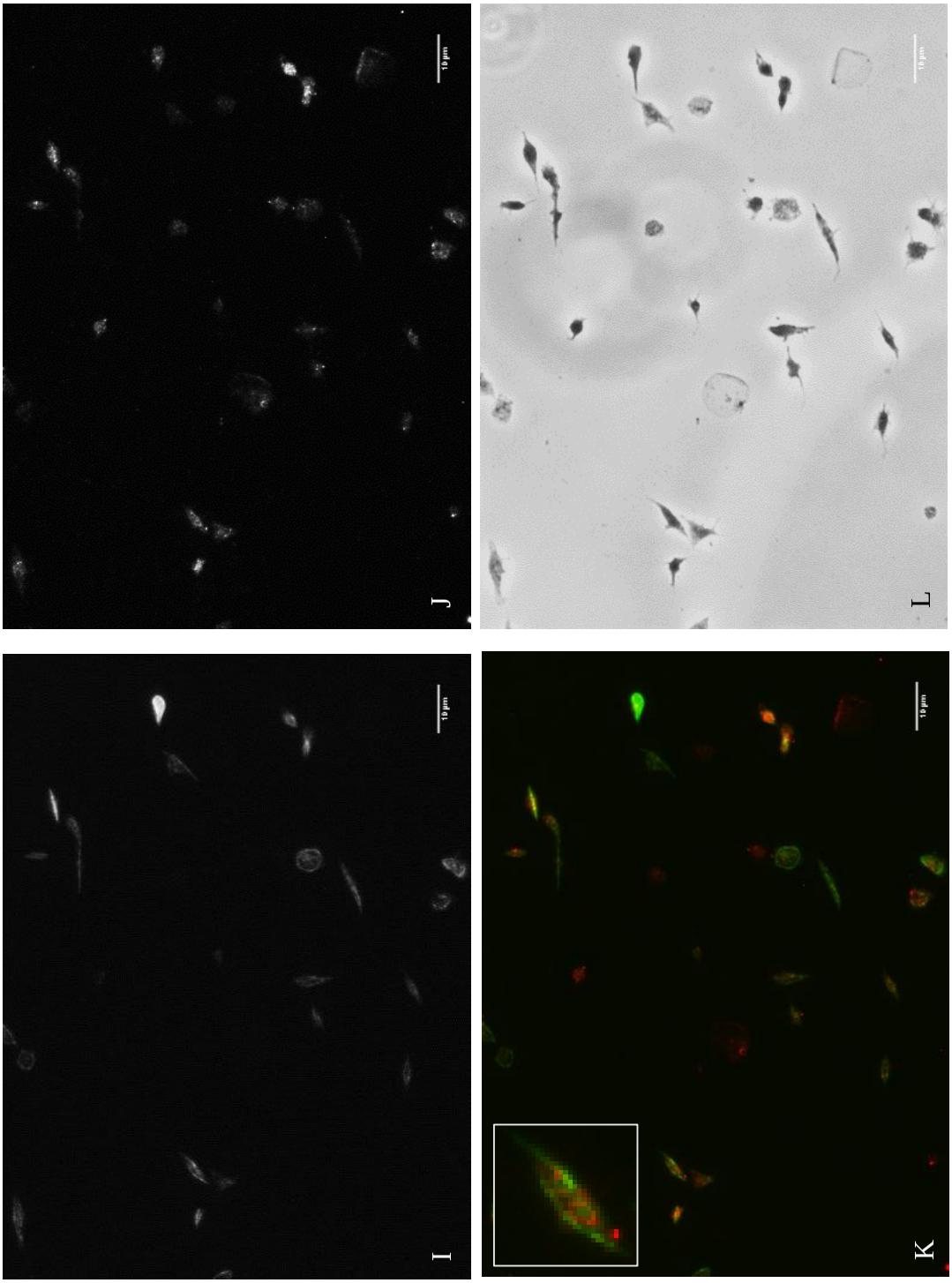
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Figure 10. Visualization of Platelets Detecting EB1 (a-d and A-D), Gamma Tubulin (e-h and E-H), and Dynein (i-l and I-L). Platelets were incubated at 37℃for 2h (a-d) to detect the presence of EB1 with anti-beta tubulin (a, and green in c) and anti-EB1(b, and red in c) labeling. Platelets were also incubated at 4℃ for 2h (A-D) with anti-beta tubulin (A, and green in C) and anti-EB1 (B, and red in C). Platelets were incubated at 37℃ for 2h (e-h) to detect the presence of gamma tubulin with anti-beta tubulin (e, and green in g) and anti-gamma tubulin (f, and red in g). Platelets were also incubated at 4℃ for 2h (E-H) with anti-beta tubulin (E, and green in G) and anti-gamma tubulin (F, and red in G). To detect the existence of dynein, platelets were incubated at 37℃ for 2h (i-l) with anti-beta tubulin (I, and green in k) and anti-dynein (j, and red in k). Platelets were also incubated at 4℃ for 2h (I-L) with anti-beta tubulin (I, and green in K) and anti-dynein (J, and red in K). In c, C, g, G, k and K, the white frames are enlarged platelet image. d, D, h, H, l and L are phase contrast image. n=6 animals for each treatment. Bar, 10μm.

DISCUSSION

Microtubule Polymerization alone Is Enough for Platelet Rods Formation

During mitosis, in order to form a mitotic spindle, microtubules first disassemble and then reassemble at metaphase (Kitanishi-Yumura & Fukui, 1987; Kline-Smith & Walczak, 2004). In ground squirrel platelets shape changes, microtubules were proposed to go through a similar process as in mitosis, to disassemble first and reassemble into different shapes. Therefore, taxol was employed to suppress microtubule depolymerization. Taxol treated platelets have a tendency to form more rod/rod-like shapes at 37℃ (Figure 8 A and B). Also platelets started to form rod/rod-like shapes right after exposure to taxol for 20minutes at 37℃. Taxol treated platelets formed more rod/rod-like shapes at 37℃ indicating that microtubule polymerization alone was enough to form rod/rod-like shape platelets at 37℃. In taxol treated ground squirrel platelets, suppressing microtubule depolymerization, led to formation of more rod/rod-like shapes after a two hours incubation at 4℃ than control platelets (Figure 8C and D). At 4℃, more rod/rod-like platelets formed after suppression of microtubule depolymerization by taxol. The result of rewamed platelets under taxol treatment showed that microtubule depolymerization was not required to form rod/rod-like shaped-platelets at 4℃. Therefore, microtubule polymerization alone can lead to platelet rod/rod-like shapes, and microtubule depolymerization is not required for platelet rod formation.

Microtubule Depolymerization Is Required for Ring Shape Platelet Formation

Control ground squirrel platelets were not only able to form rod/rod-like shapes in the cold but also able to return to ring/ring-like shapes after warming back to 37℃. Taxol treated ground squirrel platelets could not form back to ring/ring-like shapes when rewarmed to 37℃ for 2h after incubated at 4℃ for 2h (Figure 8C and D). Suppressing microtubule depolymerization by taxol resulted in prevention of reformation of ring/ring-like shape platelet which indicated that microtubules cannot form ring/ring-like shapes when microtubule depolymerization is suppressed. 250nM nocodazole was applied to restrict microtubule polymerization. Nocodazole treated platelets were able to form back from rods to ring/ring-like shapes when rewarmed to 37℃ for 2h after 2h incubation at 4℃ (Figure 9D and E). Suppressing microtubule polymerization by 250nM nocodazole led to ring/ring-like shape platelet reformation which confirmed that microtubule depolymerization was required for ground squirrel platelet ring/ring-like shape reformation.

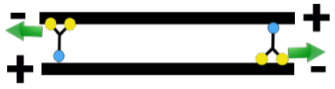
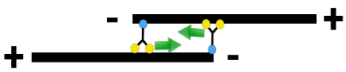
Low Temperature Increased Rod/Rod-like Platelets Formation

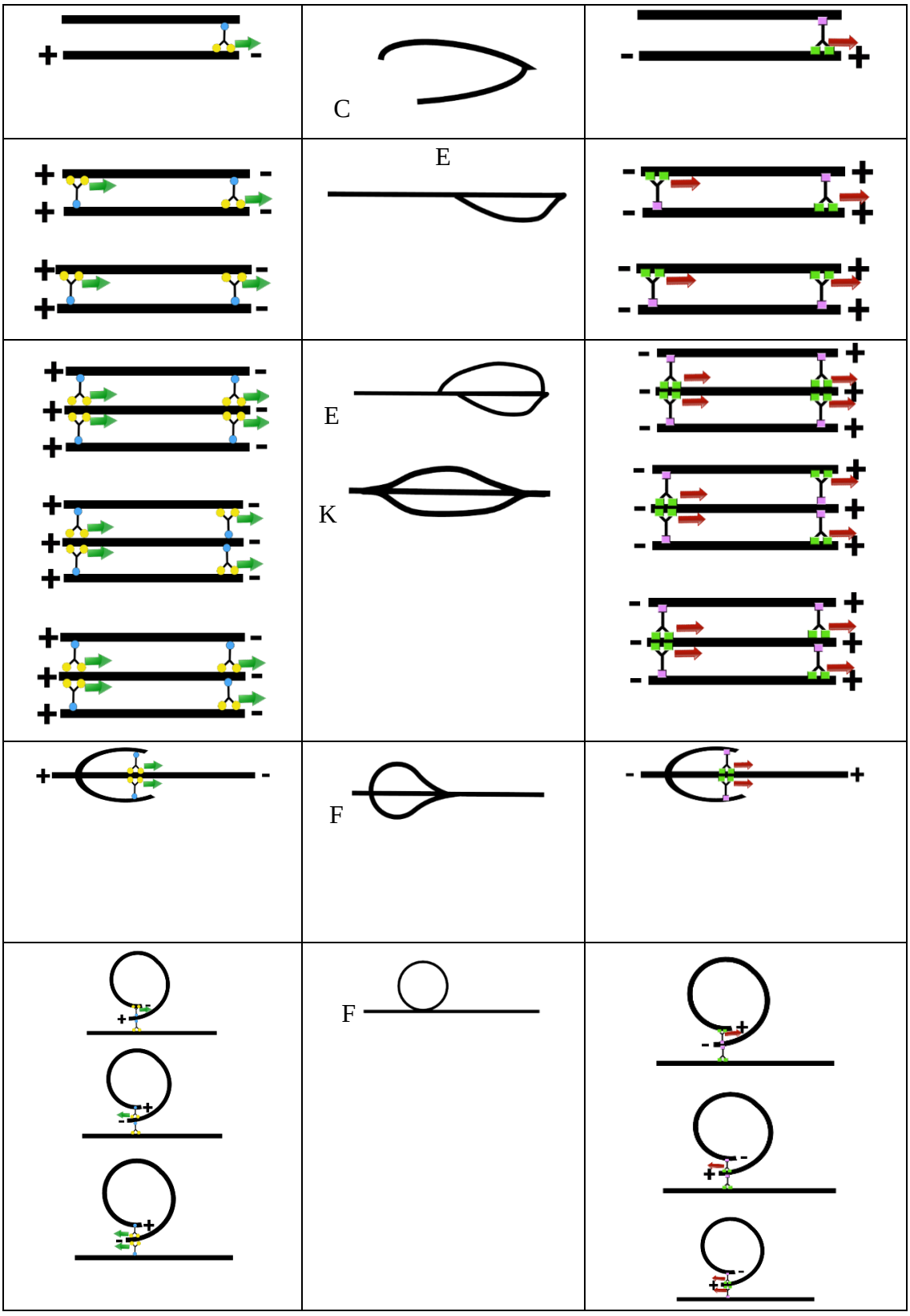
Low temperature increased both taxol and nocodazole treated platelet formation of rod/rod-like shapes (Figure 8C, D, Figure 9A and B). Taxol suppressed microtubule depolymerization, and platelets exposed to cold formed more rod/rod-like shapes than platelets only incubated at body temperature (Figure 8C and D). 250nM nocodazole treated platelets formed more rod/rod-like shapes (Figure 9A and B). Microtubules disassemble when human platelets are exposed to low temperature (White, Krumwiede, & Sauk, 1985). Therefore, in ground squirrel platelets, low temperature acted as microtubule depolymerization inhibitor to increase rod/rod-like shape formation.

Motor Protein Sliding Is another Potential Mechanism Regulating Platelet Shape Changes

During ground squirrel platelet shape changes, various shapes of microtubules have been observed (Figure 6). Microtubule motor proteins, particularly dynein, could be responsible for the multiple different shapes of microtubules during ground squirrel shape changes.

Dyneins are microtubule minus end-directed motor proteins. Most kinesins are microtubule plus end-directed motor proteins. Both dynein and kinesin are found in human platelets (Rothwell & Calvert, 1997; Miki, Okada, & Hirokawa, 2005; Patel, Richardson, Schulze, Kahle, Galjart, Drabek, Shivdasani, Hartwig, & Italiano, 2005; Diagouraga, Grichine, Fertin, Wang, Khochbin, & Sadoul, 2014). Theoretically, dynein and/or kinesin could regulate microtubule shape changes (Figure 11). Dynein sliding microtubules apart is responsible for preplatelets forming into proplatelelts during human platelet formation. Dynein, EB1 and gamma tubulin were detected in ground squirrel platelets (Figure 10). However, the responsible mechanism of dynein and/or kinesin cannot be determined by experiments.

Rod formation under dynein mechanism	Microtubule shape simulation	Rod formation under kinesin mechanism
		
		
		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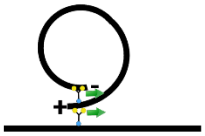
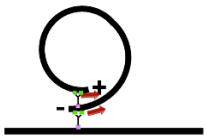
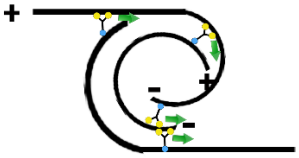
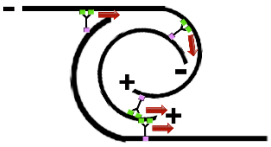
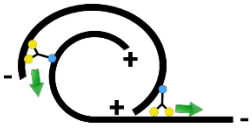
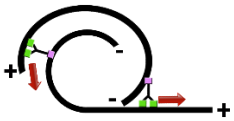
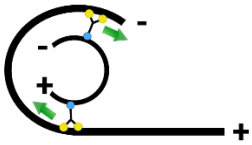
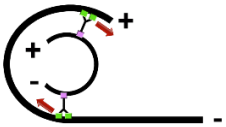
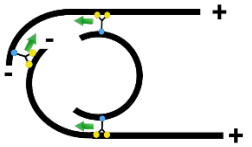
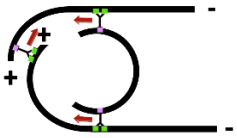
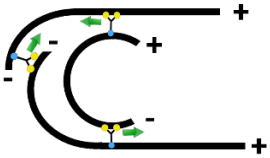
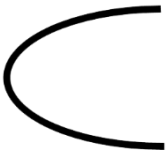
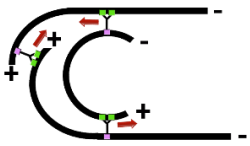
		
	G 	
	H   L	
	H 	
	I 	
	I  J 	

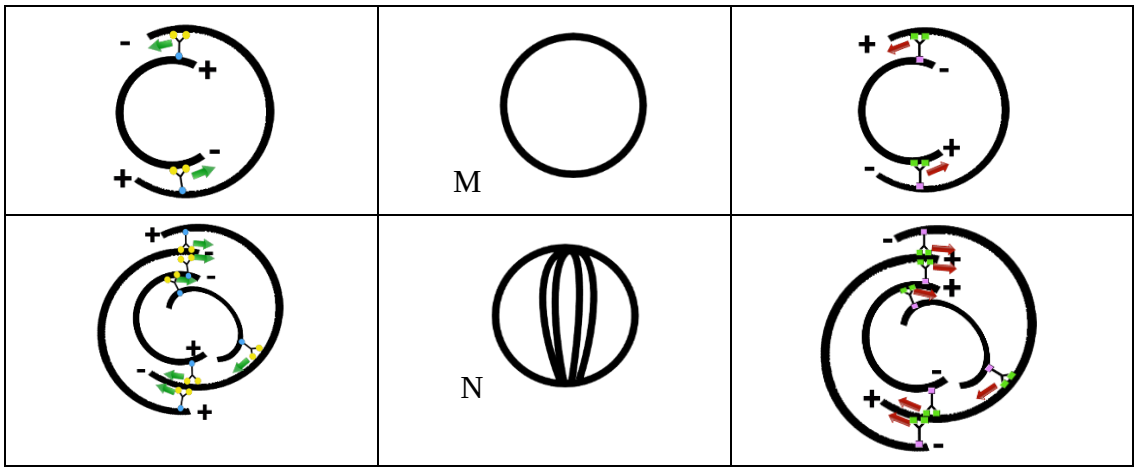


Figure 11. Possible Dynein and Kinesin Mechanisms Are Responsible for the Various Microtubule Shapes During Ground Squirrel Platelet Shape Changes. Under the dynein mechanism, the blue circle represents dynein base; yellow circles represent dynein head domains; green arrows represent the direction of dynein motion. Under the kinesin mechanism, the pink square represents the kinesin tail; green squares represent kinesin head domains; red arrows represent direction of kinesin motion. + represents microtubule plus end, – represents microtubule minus end. Microtubule shapes were simulated based on the microtubules in Figure 6. The letters correlate with the platelet shape categorization in Figure 6.

CONCLUSION

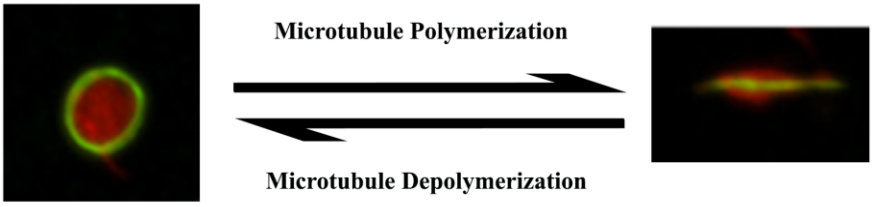


Figure 12. Mechanism of Ground Squirrel Shape Changes. Ground squirrel platelet microtubules are maintained an equilibrium between polymeriziation and depolymerization. Microtubule polymerization pushes platelets forming rod/rod-like shapes. Microtubule depolymerization leads to platelets form ring/ring-like shapes.

Taxol and nocodaole experiments lead to support a conclusion that ground squirrel platelets maintain an equilibrium between ring/ring-like shapes and rod/rod-like shapes (Figure 12). Microtubule polymerization leads to platelet rod/rod-like shape formation. Microtubule depolymerization leads to platelet ring/ring-like shape formation. Low temperature acted as an inhibitor of microtubule depolymerization, pushing the equilibrium to rod shaped platelets. Taxol, suppressed microtubule depolymerization, and also led to formation of more rod/rod-like platelets (Figure 8). At 37℃, nocodazole treated platelets maintained stable ratio between ring/ring-like and rod/rod-like, which confirmed that

250nM nocodazole is the right concentration to stabilize the kinetics of microtubule dynamics (Figure 9E and F). Low temperature inhibited microtubule depolymerization resulting in taxol and 250nM nocodazole treated platelets forming rod/rod-like shapes (Figure 8C, D, Figure 9A and B).

During ground squirrel platelet shape changes, various shapes of microtubules were observed. Motor proteins, especially dynein, were suspected to be responsible for the multiple microtubule shapes. Dynein, microtubule plus end marker EB1, and microtubule minus end marker gamma tubulin were detected in ground squirrel platelets distributed along with microtubules (Figure 10). The responsible mechanism between dynein and kinesin cannot determined.

In future studies, it will be interesting to verify dynein and kinesin involvement and to investigate how to use microtubule polymerization and depolymerization mechanism to regulate human platelet storage. Additionally, it will be worthwhile to study the mechanism of low temperature inhibiting microtubule depolymerization.

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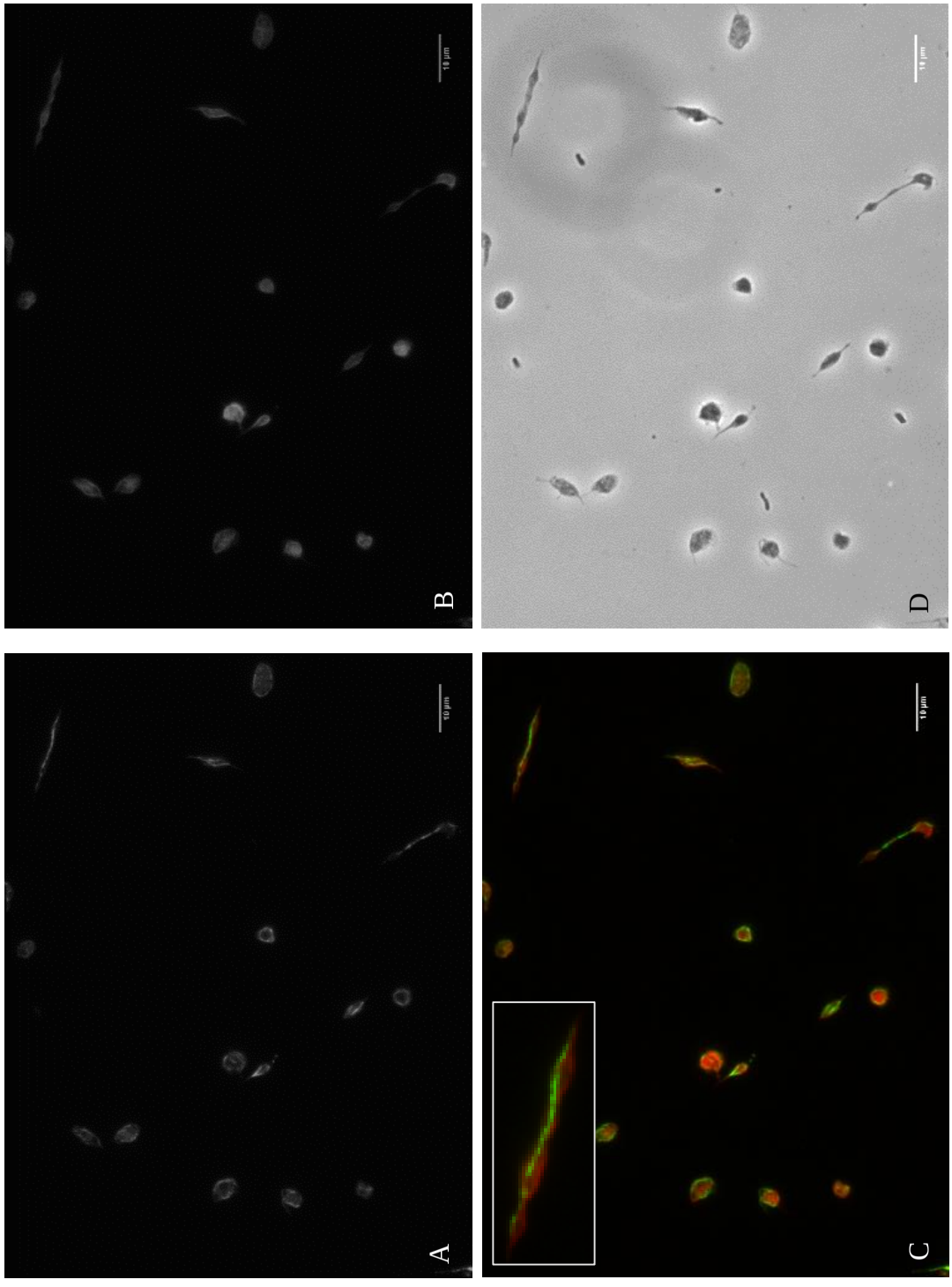
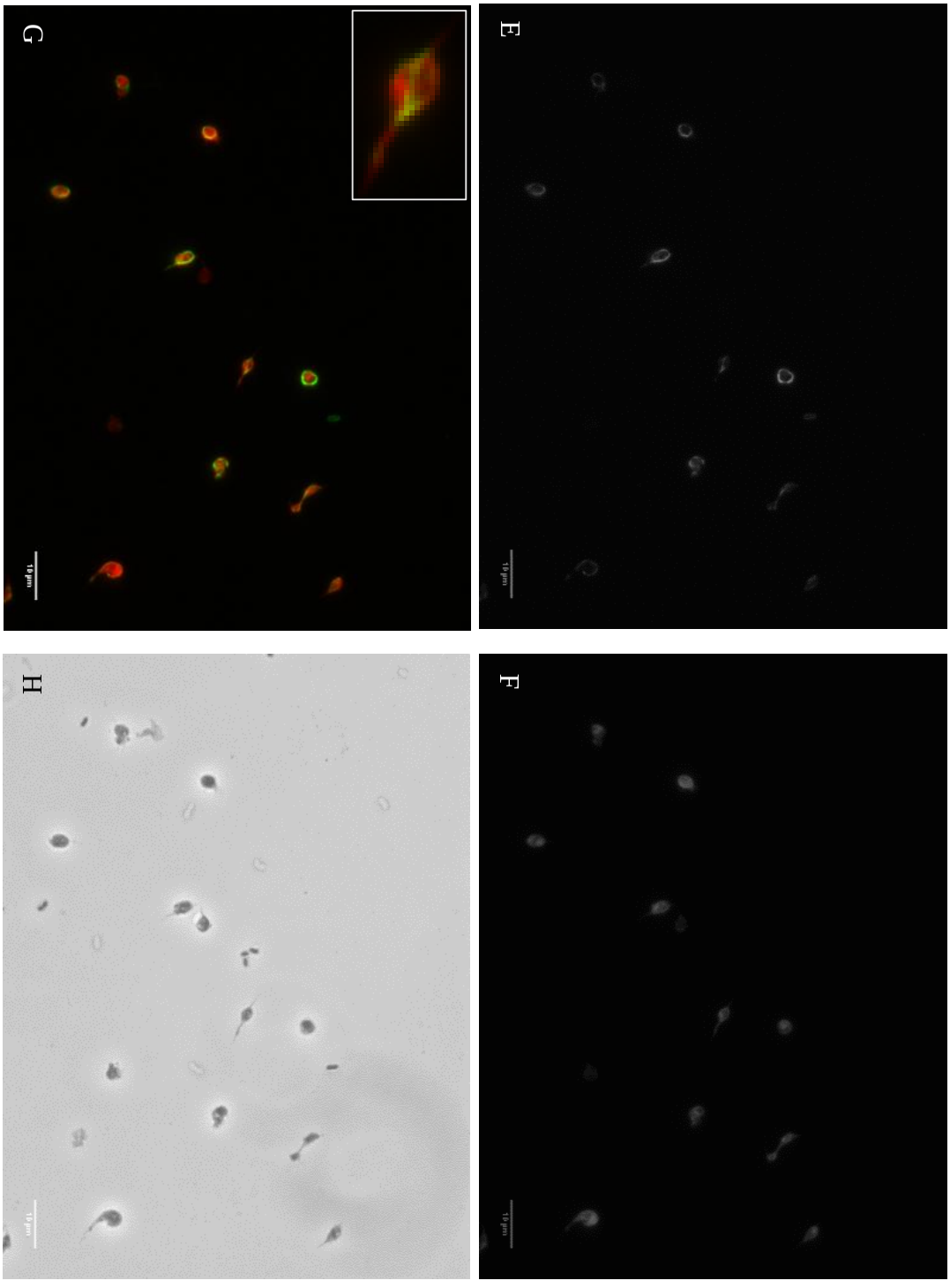
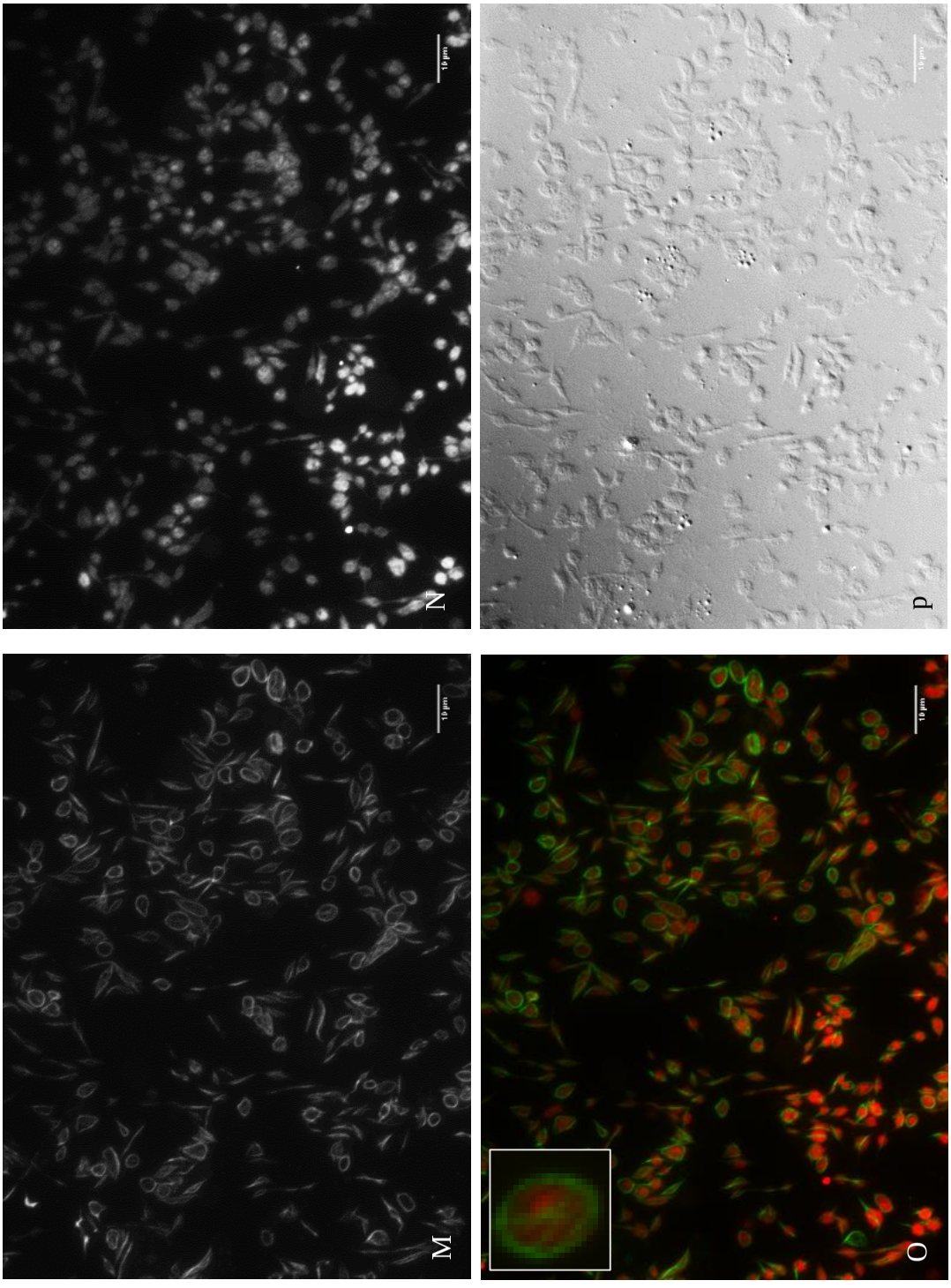
APPENDIX A

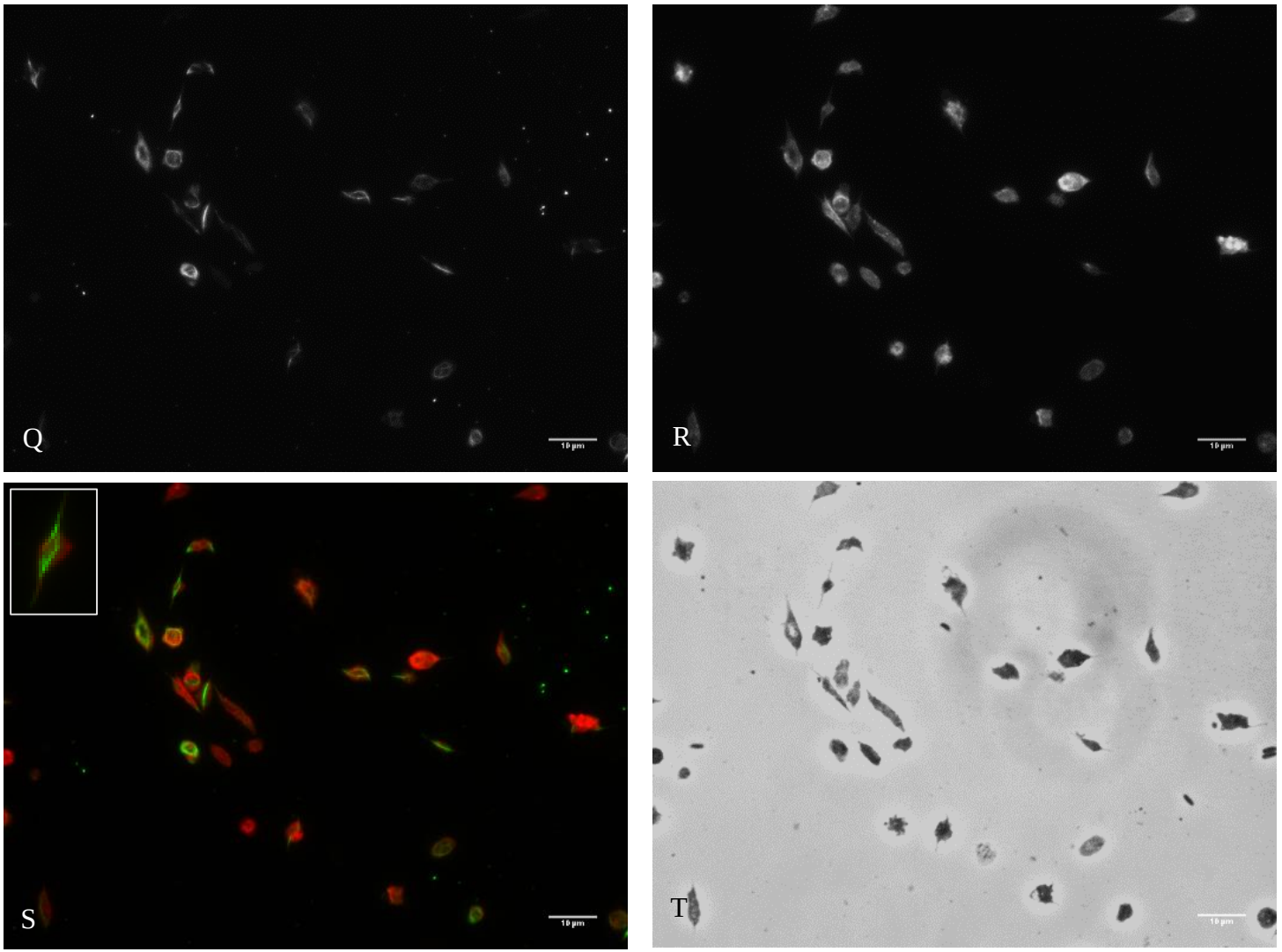
VISUALIZATION OF CONTROL PLATELET AND MICROTUNULE SHAPE CHANGES UNDER DIFFERENT TEMPERATURES

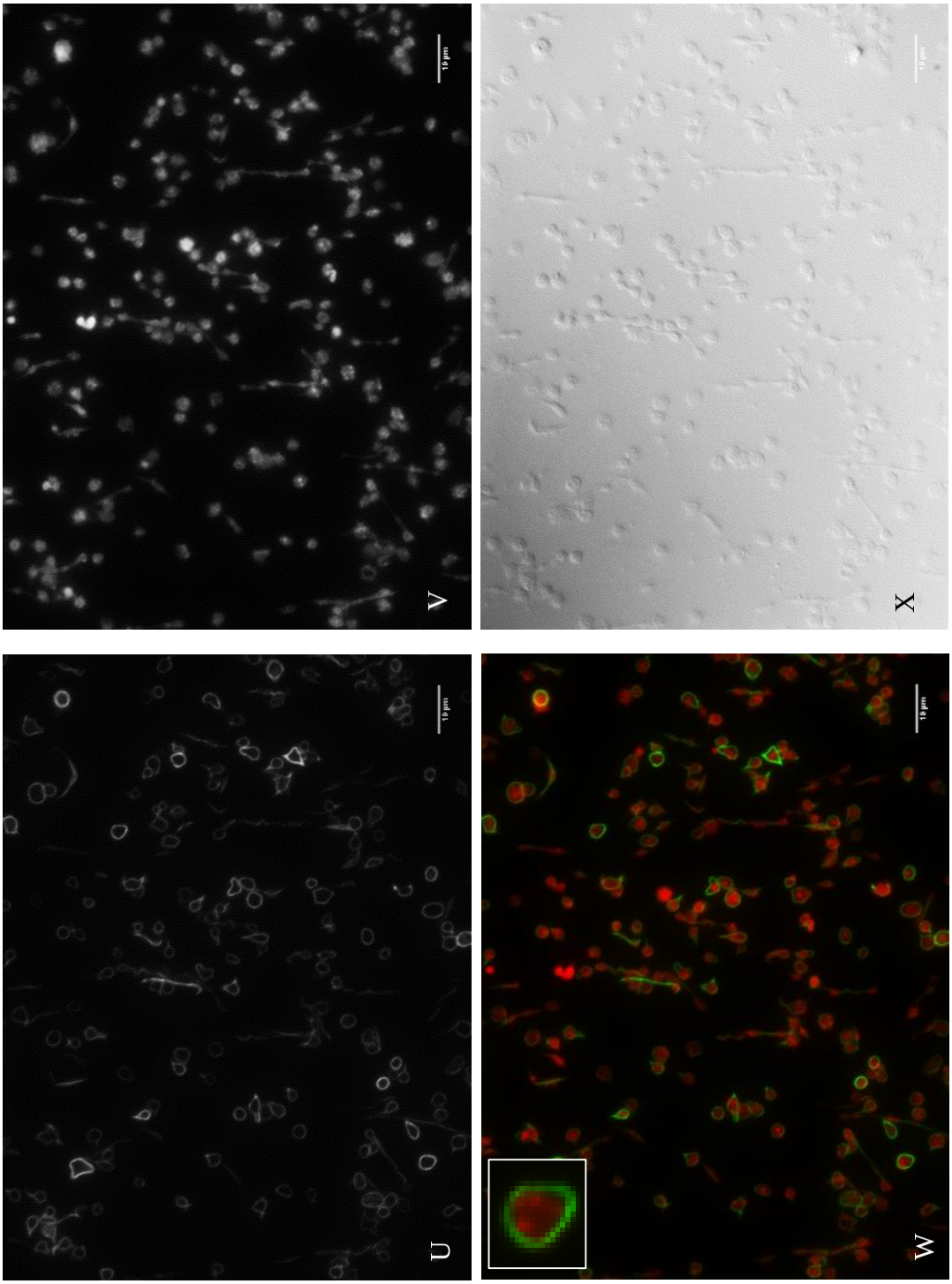

A
B
C
D
10 µm


G
E
H
F

I
J
K
L


M
N
O
P
10 µm

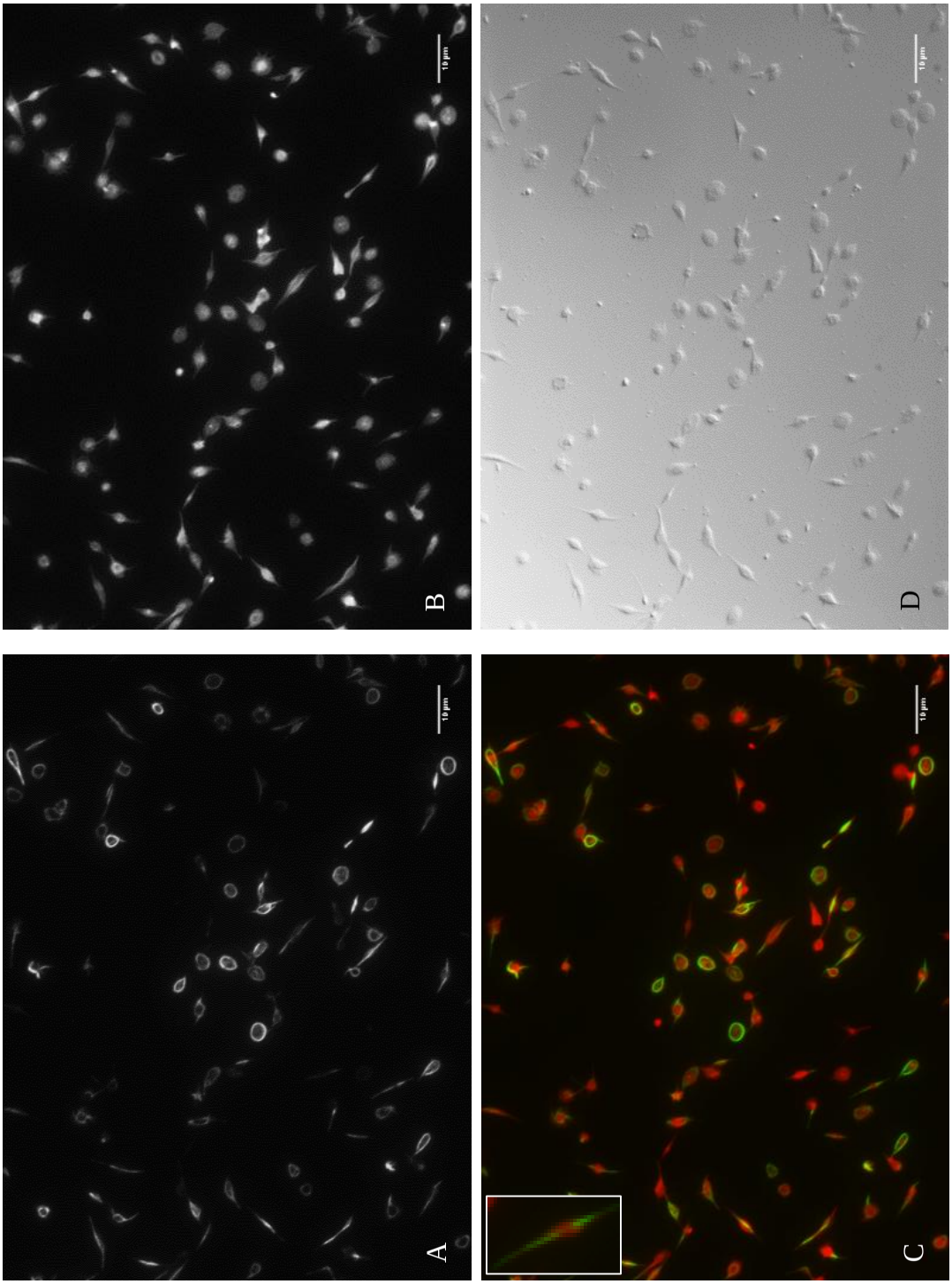

Q
R
S
T

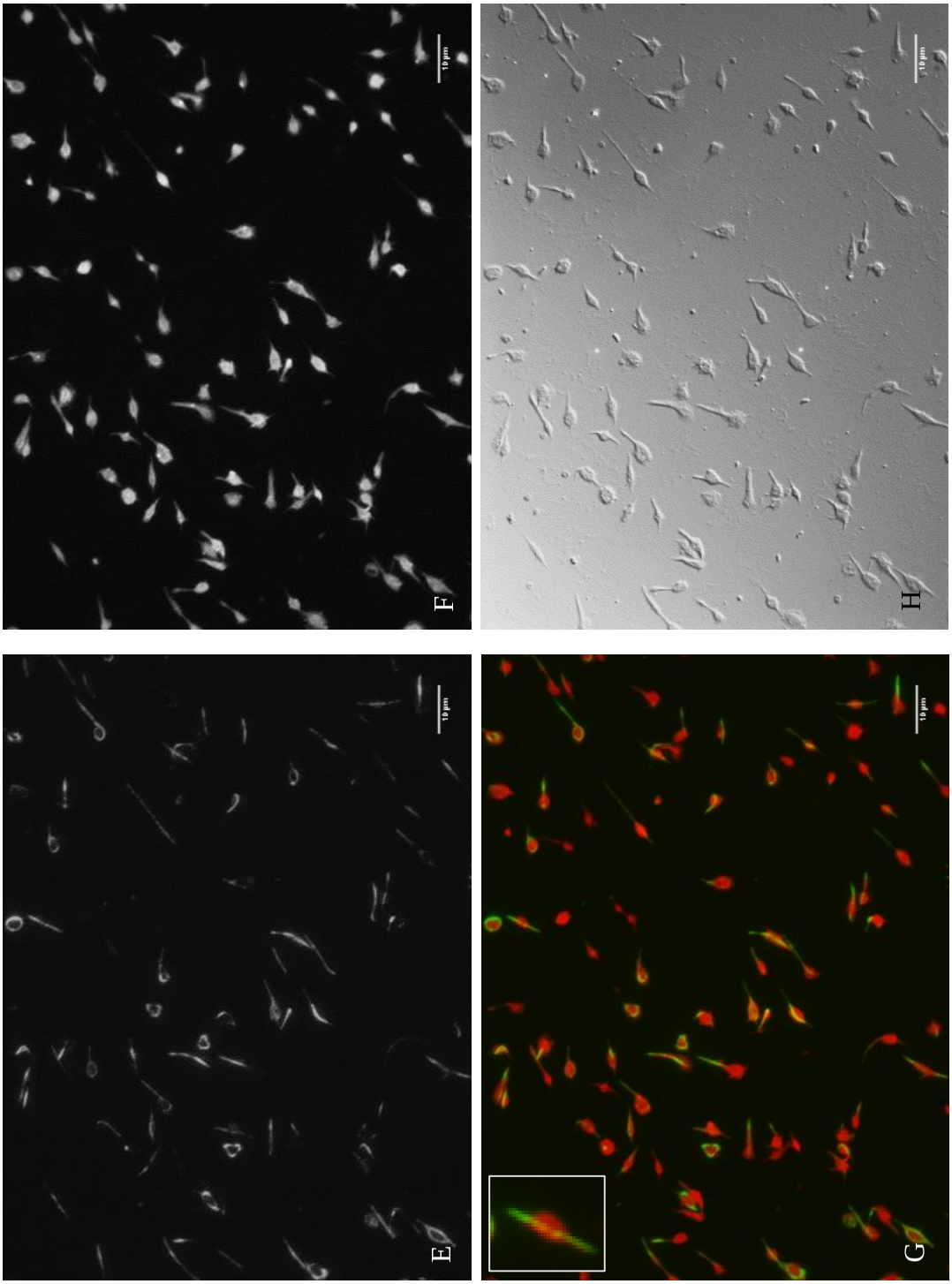

U
V
W
X
10 µm

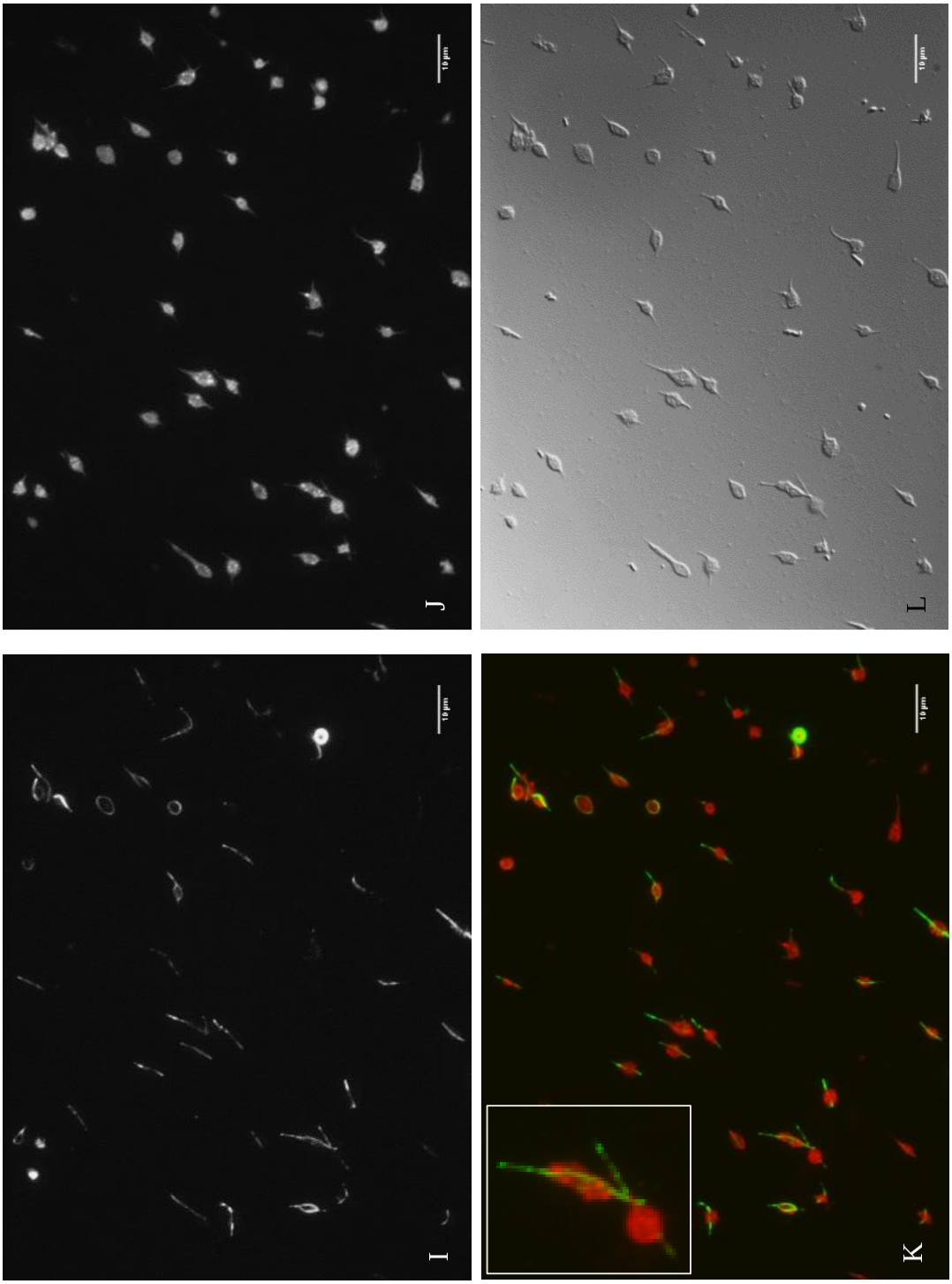
Visualization of Microtubule Shape Changes in Control Platelet Were Incubated under Different Temperatures. After 1h pre-heat at 37℃, platelets were heated to 37℃ for 0h (A-D); at 37℃ for 2h (E-H); at 37℃ 4h (I-L); at 4℃ 2h (M-P); at 4℃ 4h (Q-T); after 2 h chilled at 4℃ rewarm to 37℃ for 2h (U-X). Images of anti-beta tubulin (A, E, I, M, Q, U) and actin (B, F, J, N, R, V) were displayed separately. Merged image (C, G, K, O, S, W) of actin (red) and anti-beta tubulin (green) were presented after individual images. In C, G, K, O, S, and W, the white frames are enlarged platelet image. Platelets were also showed in phase contrast (D, H, L, T) and DIC (P, X). Bar, 10μm.

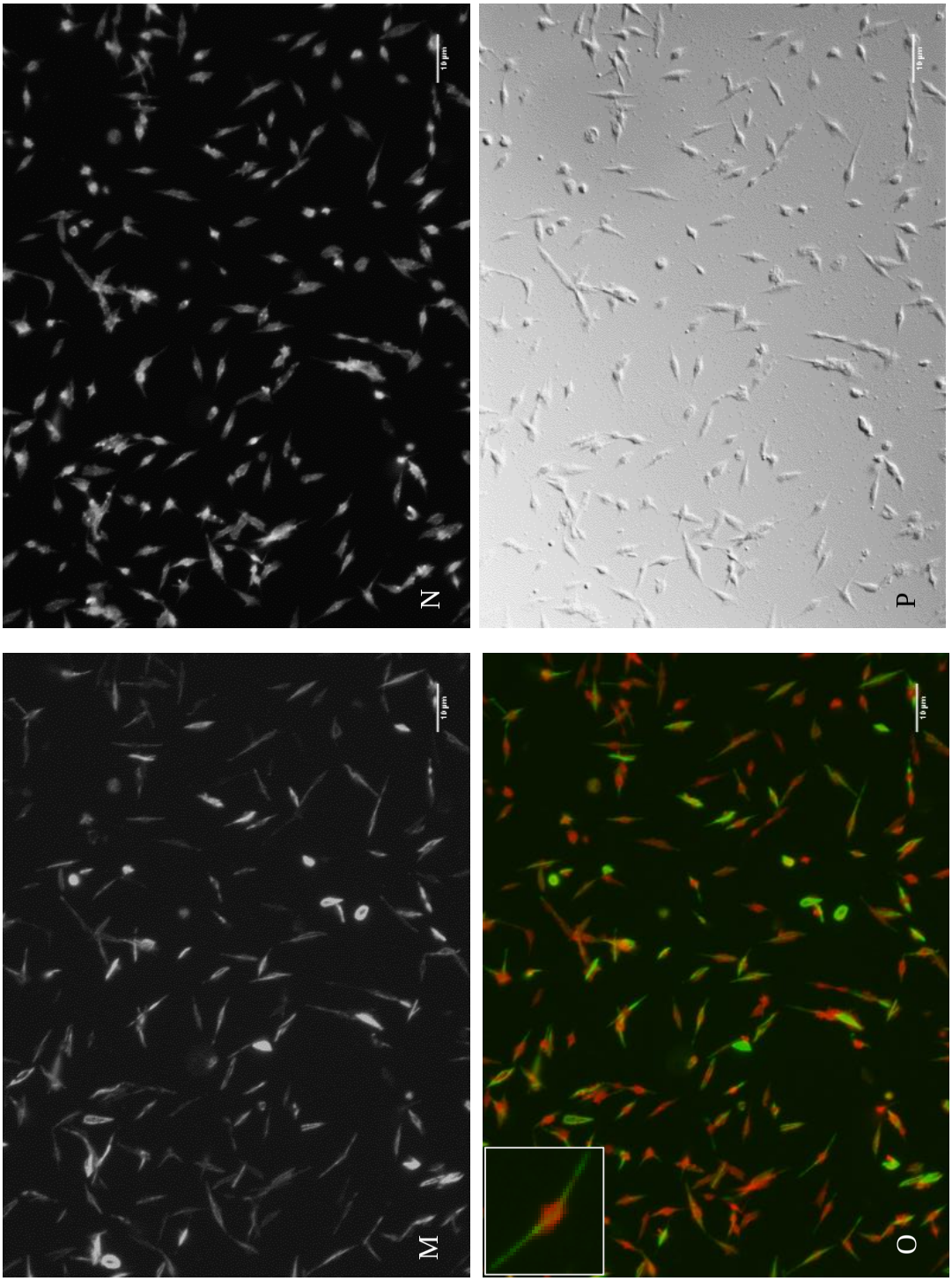
APPENDIX B

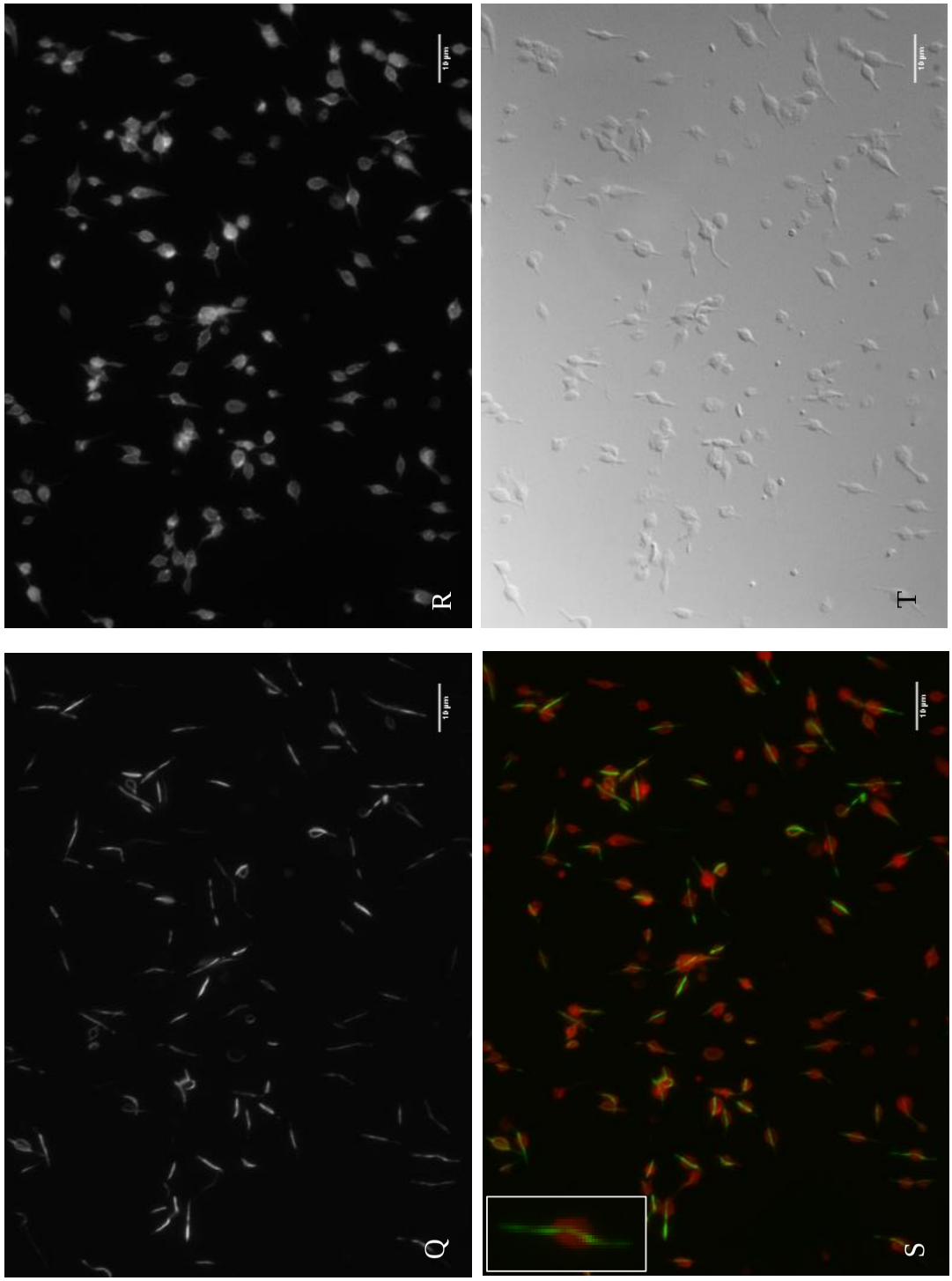
VISUALIZATION OF TAXOL TREATED-PLATELET AND MICROTUBULE SHAPE CHANGES UNDER DIFFERENT TEMPERATURES

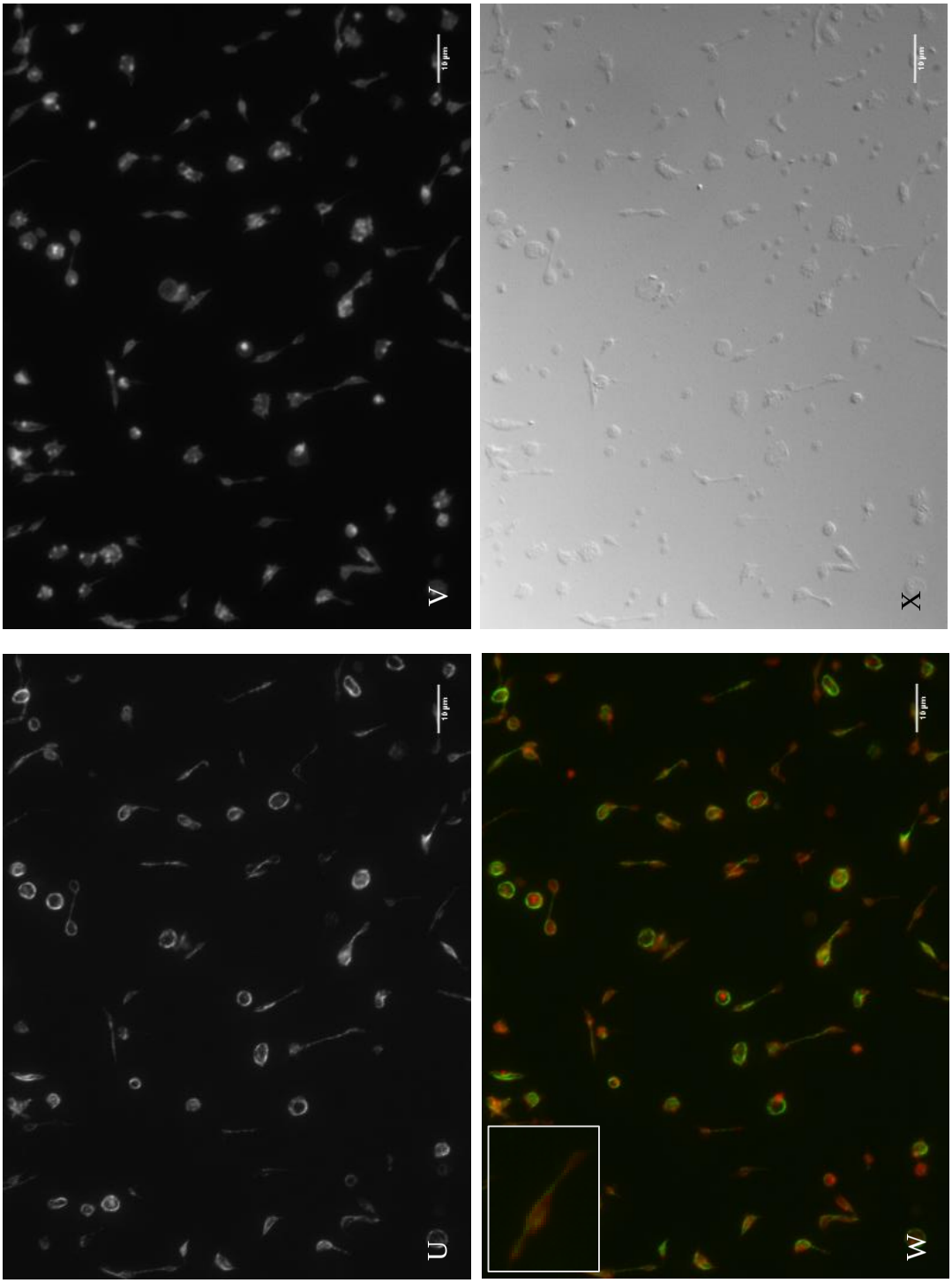

A
B
C
D
10 µm


F
H
E
G


I
J
K
L
10 µm


N
P
M
O
10 µm

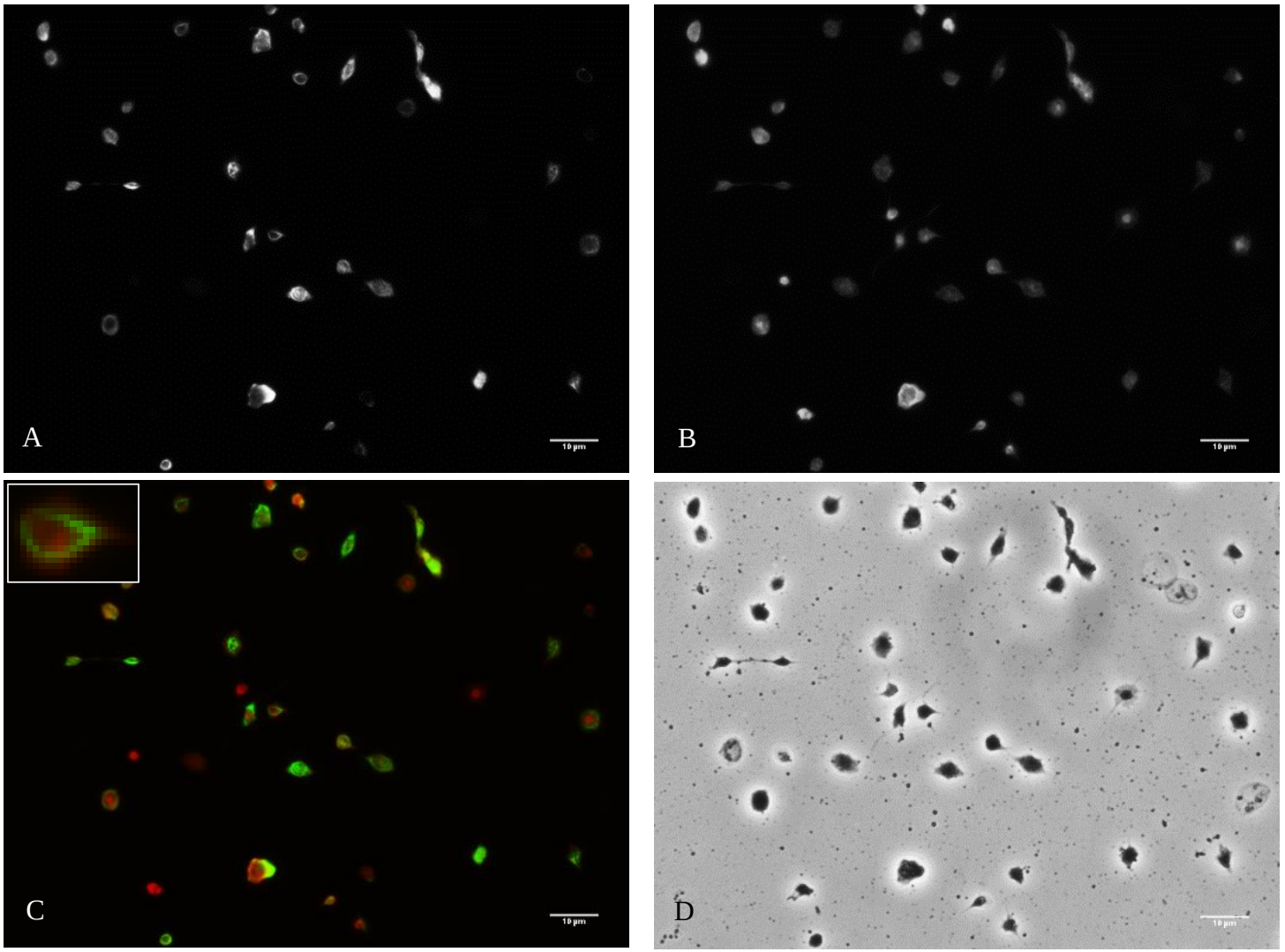

Q
R
S
T
10 µm

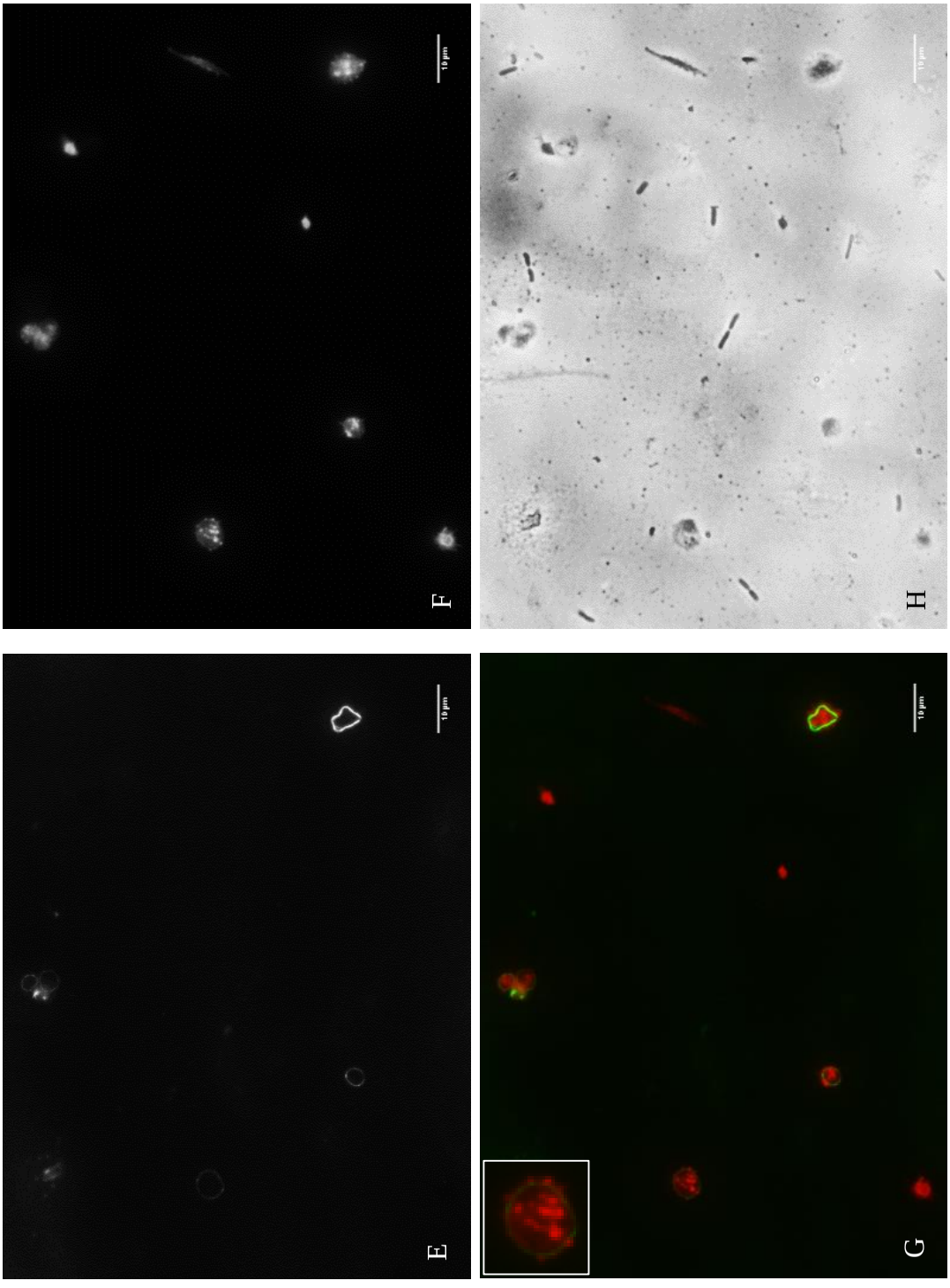

U
V
W
X
10 µm

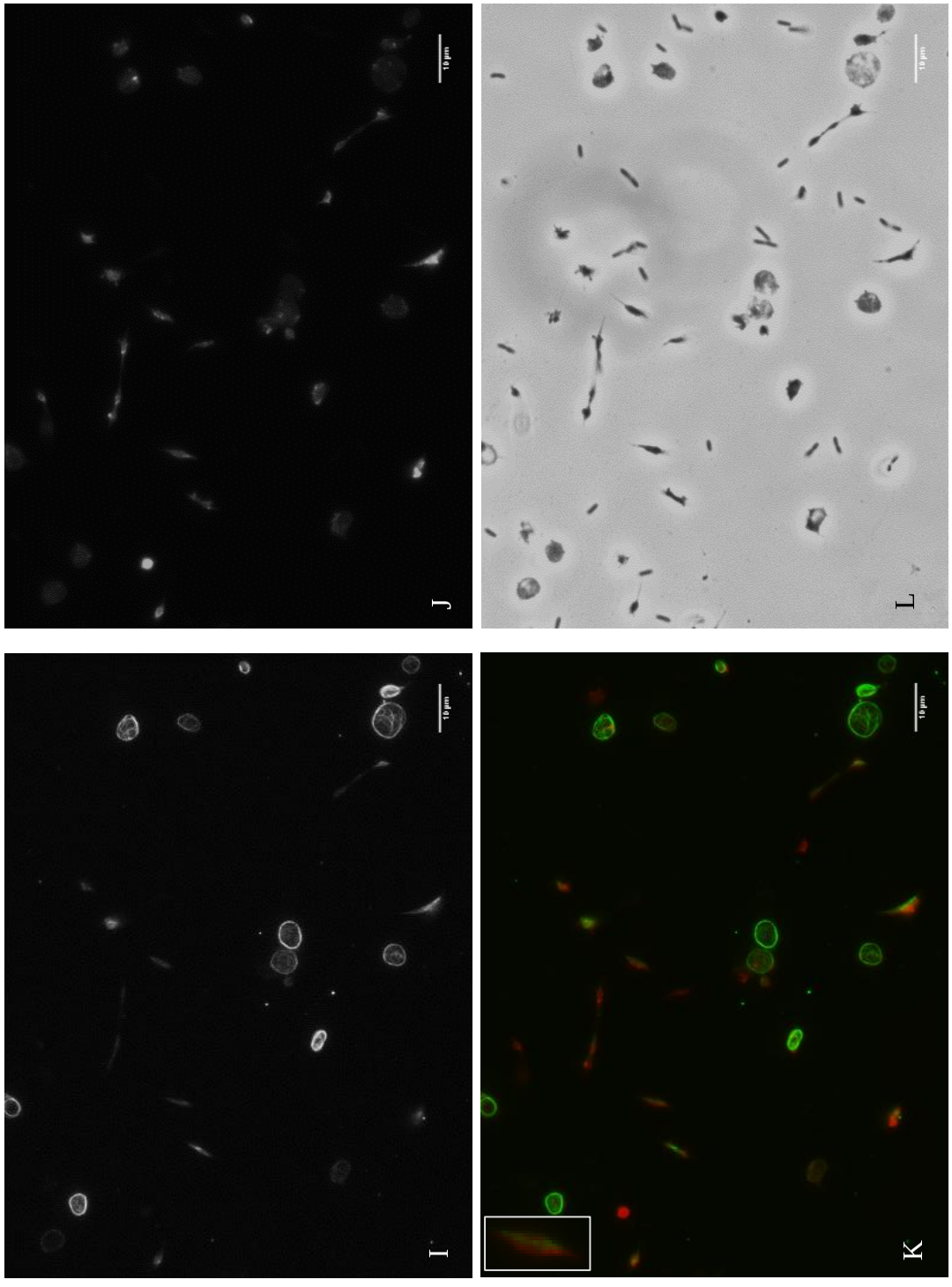
Visualization of Microtubule Shape Changes in Taxol Treated Platelets Were Treated with Different Temperatures. After 1h pre-heat at 37℃ following by 20min taxol treatment, platelets heated to 37℃ for 0h (A-D); at 37℃ for 2h (E-H); at 37℃ 4h (I-L); at 4℃ 2h (M-P); at 4℃ 4h (Q-T); after 2 h chilled at 4℃ rewarm to 37℃ for 2h (U-X). Images of anti-beta tubulin (A, E, I, M, Q, U) and actin (B, F, J, N, R, V) were showed separately. Merge image of (C, G, K, O, S, W) actin (red) and anti-beta tubulin (green) were presented after the individual images. In C, G, K, O, S, and W, the white frames are enlarged platelet image. Platelets were also displayed in DIC (D, H, L, P, T, X). Bar, 10μm.

APPENDIX C

VISUALIZATION OF NOCODAZOLE-TREATED PLATELET AND MICROTUBULE SHAPE CHANGES UNDER DIFFERENT TEMPERATURES


A
B
C
D


E
F
G
H
10 µm


I
J
K
L
10 µm

M
N
O
P

Q
R
S
T

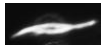
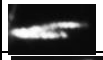
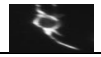
U
V
W
X

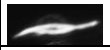
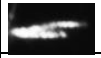
Visualization of Microtubule Shape Changes in Nocodazole Treated Platelet Were Incubated under Different Temperatures. After 1h pre-heat at 37℃ following by 1h 250nM nocodazole treatment, platelets were heated to 37℃ for 0h (A-D); at 37℃ for 2h (E-H); at 37℃ 4h (I-L); at 4℃ 2h (M-P); at 4℃ 4h (Q-T); after 2 h chilled at 4℃ rewarm to 37℃ for 2h (U-X). Images of anti-beta tubulin (A, E, I, M, Q, U) and actin (B, F, J, N, R, V) were showed individually. Merged images (C, G, K, O, S, W) of actin (red) and anti-beta tubulin (green) were presented after individual images. In C, G, K, O, S, and W, the white frames are enlarged platelet image. Platelets were also displayed in phase contrast (D, H, L, P, T, X). Bar, 10μm.

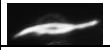
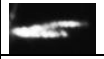
APPENDIX D

RAW DATA OF TAXOL TREATMENT

Raw Data of Taxol Treated Ground Squirrel Platelets. I, II, and III are datas from three individual experiements. MT is short for microtubule. Platelet ID number A-N represents the platelet shapes from Figure 6. n=6 aminals.

I / ID	MT shapes	37C 0h control	37C 0h taxol	4C 2h control	4C 2h taxol	37C 2h control	37C 2h taxol	37C 4h control	37C 4h taxol	4-37C 2h control	4-37C 2 taxol
A		14	53	25	61	12	57	6	50	11	70
B		2	8	3	23	3	1	0	0	2	1
C		2	6	1	7	4	1	2	1	1	1
D		3	13	1	27	2	1	4	1	2	1
E		1	4	0	1	0	0	0	0	0	0
F		4	6	0	1	1	1	0	1	1	0
G		13	5	0	0	3	1	0	0	0	0
H		0	1	0	0	0	0	0	0	0	0
I		4	3	0	0	1	0	1	0	0	0
J		4	2	1	0	1	2	1	2	0	0
K		4	6	5	0	5	5	1	2	14	0
L		16	30	5	2	18	18	15	10	15	0
M		10	20	5	1	29	10	54	1	25	0
N		39	13	12	3	45	2	7	1	24	0
Total		116	170	58	126	124	99	91	69	95	73

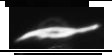
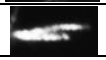
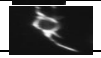
II / ID	MT shapes	37C 0h control	37C 0h taxol	4C 2h control	4C 2h taxol	37C 2h control	37C 2h taxol	37C 4h control	37C 4h taxol	4-37C 2h control	4-37C 2 taxol
A		15	54	32	60	14	56	7	49	10	71
B		1	12	2	23	3	0	0	1	2	1
C		2	6	1	7	3	0	2	2	0	0
D		2	15	0	27	2	2	3	1	3	0
E		3	4	0	1	0	0	0	0	0	1
F		4	5	1	0	0	2	0	1	1	0
G		14	5	0	0	2	2	0	0	1	0
H		0	1	0	0	1	0	0	0	0	0
I		3	2	0	0	0	0	1	0	0	0
J		5	2	0	0	0	2	0	2	0	0
K		4	6	13	1	4	5	1	1	14	0
L		15	26	8	2	18	18	14	9	15	0
M		11	19	2	0	27	9	54	2	24	0
N		41	12	15	2	43	2	6	0	25	0
Total		120	169	74	123	117	98	88	68	95	73

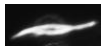
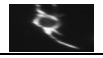
III / ID	Microtubule shapes	37C 0h control	37C 0h taxol	4C 2h control	4C 2h taxol	37C 2h control	37C 2h taxol	37C 4h control	37C 4h taxol	4-37C 2h control	4-37C 2 taxol
A		14	52	49	61	12	57	8	50	9	72
B		1	10	4	24	4	1	0	1	2	1
C		1	7	0	8	4	0	2	1	0	1
D		3	13	0	26	1	1	4	1	2	0
E		1	4	1	1	0	0	0	1	0	0
F		3	5	1	0	1	2	0	1	1	0
G		13	6	0	0	3	1	0	0	0	0
H		0	1	1	0	0	0	0	1	1	0
I		2	2	0	0	0	0	1	0	0	0
J		6	2	0	0	1	1	0	2	0	0
K		5	7	13	0	5	6	1	2	15	0
L		16	24	7	3	19	19	15	9	16	0
M		11	18	6	1	27	10	56	2	25	0
N		43	11	16	2	41	2	7	0	26	0
Total		119	162	98	126	118	100	94	71	97	74

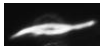
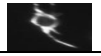
APPENDIX E

RAW DATA OF 250nM NOCODAZOLE TREATMENT

Raw Data of 250nM Nocodazole Treated Ground Squirrel Platelets. I, II, and III are datas from three individual experiements. MT is short for microtubule. Platelet ID number A-N represents the platelet shapes from Figure 6. n=6 aminals.

I / ID	MT shapes	37℃ 0h control	37℃ 0h noco	4℃ 2h control	4℃ 2h noco	37℃ 2h control	37℃ 2h noco	37℃ 4h control	37℃ 4h noco	4℃ 4h control	4℃ 4h noco	4-37℃ 2h control	4-37℃ 2h noco
A		44	15	238	135	30	28	29	27	171	73	43	40
B		4	0	20	0	9	0	0	0	0	3	1	0
C		6	1	6	9	6	1	6	1	3	12	2	1
D		6	6	3	19	4	4	10	6	1	29	8	8
E		3	0	1	1	0	0	0	1	2	0	3	2
F		9	3	4	2	3	1	0	2	0	1	3	4
G		44	0	0	1	6	0	0	0	0	0	0	1
H		0	1	0	2	1	0	0	0	0	0	1	0
I		14	1	1	0	1	0	5	0	2	1	1	1
J		18	2	3	2	1	1	1	2	2	1	1	1
K		14	9	66	38	7	3	4	3	26	8	11	19
L		52	28	43	60	46	12	67	11	33	10	31	5
M		35	37	26	50	86	62	244	54	37	17	64	123
N		132	66	93	41	121	16	28	42	33	26	17	65
total		381	169	504	360	321	128	394	149	310	181	186	270

II / ID	MT shapes	37℃ 0h control	37℃ 0h noco	4℃ 2h control	4℃ 2h noco	37℃ 2h control	37℃ 2h noco	37℃ 4h control	37℃ 4h noco	4℃ 4h control	4℃ 4h noco	4-37℃ 2h control	4-37℃ 2h noco
A		51	14	239	142	32	28	30	29	176	75	44	42
B		5	1	20	0	8	0	0	0	1	3	1	0
C		5	1	5	11	12	1	9	1	4	13	2	0
D		13	6	2	19	5	3	20	6	1	32	8	10
E		9	1	2	1	0	0	0	1	2	1	3	2
F		15	3	4	3	2	2	0	2	0	0	4	3
G		38	0	0	0	8	0	0	0	0	0	1	0
H		0	0	1	1	1	0	0	0	0	0	0	1
I		6	0	2	0	1	0	4	0	4	0	2	1
J		13	3	2	2	2	0	1	1	2	1	0	0
K		15	10	68	39	17	3	4	4	29	8	11	19
L		50	27	44	61	57	11	64	10	34	12	30	6
M		34	39	30	53	80	64	243	57	36	16	65	128
N		131	68	96	32	127	15	32	44	34	24	17	62
total		385	173	515	364	352	127	407	155	323	185	188	274

III / ID	MT shapes	37℃ 0h control	37℃ 0h noco	4℃ 2h control	4℃ 2h noco	37℃ 2h control	37℃ 2h noco	37℃ 4h control	37℃ 4h noco	4℃ 4h control	4℃ 4h noco	4-37℃ 2h control	4-37℃ 2h noco
A		44	13	242	150	50	30	34	32	180	71	47	45
B		4	1	21	0	12	0	0	0	1	3	2	0
C		5	1	6	13	14	1	11	1	2	15	2	1
D		7	7	3	21	5	4	18	7	1	35	7	12
E		4	0	1	1	0	0	0	1	3	0	3	1
F		11	3	3	2	1	1	0	3	0	0	2	4
G		46	0	0	0	10	0	0	0	0	0	0	0
H		0	0	0	1	1	0	0	0	1	0	0	0
I		9	1	1	1	1	0	4	0	3	1	3	0
J		17	2	3	3	3	1	2	1	1	0	1	1
K		13	11	72	42	17	4	5	4	32	7	13	20
L		49	26	46	63	59	10	64	11	36	14	30	7
M		34	43	34	57	79	66	244	60	34	13	68	135
N		132	72	100	34	130	16	28	46	37	22	19	60
total		375	180	532	388	382	133	410	166	331	181	197	286