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Purification of Mammalian Vimentin via the Reassembly of BJ5TAa IF Method

Elizabeth Puck

A Thesis in the Field of Bioengineering and Nanotechnology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2023

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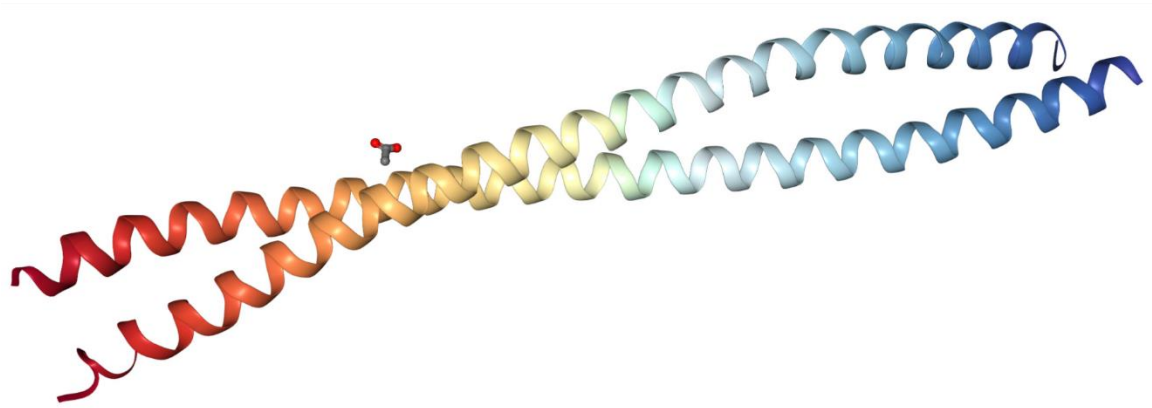
Abstract

The protein vimentin is an intermediate filament and is part of the cytoskeleton in cells and tissues. The cytoskeleton is comprised of three total protein-based components that undergo liquid-liquid phase separation (LLPS). Liquid-liquid phase separation occurs when proteins or nucleic acids (macromolecules) condense into what resembles liquid droplets. A major factor concerning whether a solution will undergo LLPS is the concentration and identities of the macromolecules and the solution. Vimentin is associated with multiple human diseases including cancer, Crohn's disease, HIV, and cataracts. One area that has not been studied fully is the purification of mammalian vimentin, which is an intermediate filament. Intermediate filaments, along with microfilaments and microtubules, are the basis of the cytoskeleton in eukaryotic cells. These entities are interconnected, helping to give the cell its shape while also regulating mechanical, spatial, and biochemical properties of the cell. The name intermediate filament was first established when it was discovered that IF were localized between actin and myosin in muscle cells. The Weitz Lab has reported the formation of a reconstituted network containing physiologically relevant concentrations of all three cytoskeletal proteins: F-actin, microtubules, and vimentin intermediate filaments (VIFs). They have characterized the structure of the network using scanning and transmission electron microscopy as well as confocal fluorescence microscopy. In this work, multiple purification methods for vimentin were considered; ultimately, the method that was selected for the vimentin purification was reassembly of BJ5TAa IF in MEF cells.

Frontispiece



Vimentin protein structure. (2022). Sino Biological. Retrieved from https://www.sinobiological.com/resource/vimentin/proteins

Author's Biographical Sketch

Elizabeth Puck is a bioprocess engineer working in the biopharmaceutical industry for the past eight years in a variety of roles, with the most recent role being a process development engineer in a protein purification lab. The majority of proteins purified were fusion proteins via column chromatography. For this thesis, manual methods were explored for the extraction of vimentin, utilizing skills and knowledge from past work.

Dedication

To my family, who has always supported me in my endeavors.

Acknowledgments

Many thanks to David Weitz for supporting this thesis and Arka Basu in the Weitz Lab for collaborating with me.

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Chapter I.

Introduction

The cytoskeleton of the cell is made of three protein-based components that form filamentous structures. One of them, the intermediate filaments, can undergo liquid-liquid phase separation (LLPS). Liquid-liquid phase separation occurs when proteins or nucleic acids (macromolecules) condense into what resembles liquid droplets. Protein solutions separate into protein-dense and protein-light phases. A major factor concerning whether or not a solution will undergo LLPS is the concentration and identities of the macromolecules and the solution.

LLPS is critical in the formation of organelles with no membrane. Condensed phases that undergo LLPS are typically reversible; however, they can undergo further transitions such as gel-transitions. Further, assemblies can transition to hydrogels, which typically form irreversibly. The actomyosin cytoskeleton is often used as an example of viscoelasticity, in which the liquid has properties of shear elasticity similar to a solid over short times and viscosity and flow behavior over longer times. When actomyosin gel is deformed, it responds with elastic behavior. If kept under force, it will change shape and lose memory of its previous shape. Therefore, actomyosin gel and the actomyosin cytoskeleton have solid properties at short times and liquid properties at long times. Thus, the cytoskeleton acts as a complex fluid.

The cytoskeleton of a cell is made up of microtubules, actin filaments, and intermediate filaments. *In vitro* studies have shown a role for LLPS of proteins in cytoskeleton assembly. However, the exact physiological contribution is unclear. Many

proteins have been shown to go through LLPS *in vitro*, and the roles of LLPS in cells is still being discovered. The cytoskeleton of eukaryotic cells is comprised of entangled filamentous proteins that provide structural support and mechanical stability, enabling cells to resist deformation.

Study of the behavior of the three major cytoskeletal components reconstituted together to form all three networks is required to form a more full understanding of the mechanical properties of cells. Unfortunately, the chemical buffer conditions typically used to reconstitute each individual component are incompatible, precluding the formulation of mixed component interpenetrating cytoskeletal networks. Consequently, the structure and mechanical behavior of such networks have never been explored and the effects of interaction among the three components forming such an interpenetrating network are not known.

The materials required to study this are microtubules, actin filaments, and intermediate filaments. It is required to perform experiments in a laboratory to test combining each of the components and demonstrating reproducibility. Current methods for combining the protein substituents have been unsuccessful and exploration of a method to do so will be done in the future. Testing will need to be done on the cytoskeletons to confirm they are structurally viable, fulfill criteria, and meet certain requirements. Analytical methods as previously decided upon will be used to analyze the structures. The requirements for success will be clearly defined in order to determine successful versus unsuccessful trials. Statistical analysis will need to be used in experimentation to analyze the comparability between trials. A software such as JMP will be required complete the analysis, dependent upon software availability.

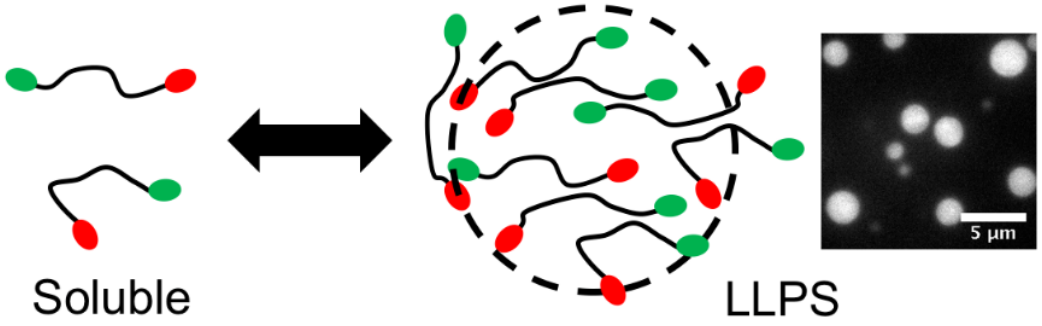
Liquid-liquid phase separation occurs when proteins or nucleic acids (macromolecules) condense into what resembles liquid droplets, which is a dense phase. This dense phase exists with a dilute phase. Thus, a protein solution separates into protein-dense and protein-light phases. LLPS involves the exchange of macromolecule/water interactions for macromolecule/macromolecule and water/water interactions while the process is thermodynamically and entropically favorable. One major factor concerning whether or not a solution will undergo LLPS is the concentration and identities of the macromolecules and the solution. Other factors are the environmental conditions such as salt type and concentration, temperature, co-solutes, and pH.

LLPS is critical in the formation of organelles with no membrane. Currently, the kinetics, thermodynamics, and molecular mechanisms of LLPS are being studied in cell biology, requiring reproducible methods for studying often aggregation-prone proteins. LLPS can produce a variety of assembled material states in cells. Molecules undergoing LLPS can adopt many material properties. If the assembly resulting from LLPS is liquid, long-range molecular disorder and short-range order occurs within the first few layers of molecules. Due to surface tension, the liquid assemblies can fuse, coalesce, and drip. If the dense phase has liquid-like properties, the polymer molecules are typically mobile within the dense phase and between the dense and light phases. Condensed phases that undergo LLPS are typically reversible; however, they can undergo further transitions such as gel-transitions. Further, assemblies can transition to hydrogels, which typically form irreversibly.

One interesting property of liquids is viscoelasticity, in which the liquid has properties of shear elasticity similar to a solid over short times and viscosity and flow behavior over longer times. The actomyosin cytoskeleton is often used as an example of viscoelasticity. When actomyosin gel is deformed, it responds with elastic behavior. If kept under force, it will change shape and lose memory of its previous shape. Therefore, actomyosin gel and the actomyosin cytoskeleton have solid properties at short times and liquid properties at long times. Thus, the cytoskeleton acts as a complex fluid.

The cytoskeleton of a cell is made up of microtubules, actin filaments, and intermediate filaments. These structures give the cell its shape, help organize the cell's parts, and provide a basis for movement and cell division. The microtubule cytoskeleton arranges the inside of the cell and segregates chromosomes. *In vitro* studies have shown a role for LLPS of proteins in cytoskeleton assembly. However, the exact physiological contribution is unclear. Many proteins have been shown to go through LLPS *in vitro*, and the roles of LLPS in cells is still being discovered. To give a size reference for protein subunits that make up the cytoskeleton, cytoskeleton structures typically reach from one end of the cell to the other, roughly tens or even hundreds of micrometers. The individual protein molecules making up the cytoskeleton are typically only a few nanometers in length. Due to the fact that these protein subunits are small, they can diffuse rapidly within the cytoplasm, while the assembled filaments cannot. The cytoskeleton of eukaryotic cells is comprised of entangled filamentous proteins that provide structural support and mechanical stability, enabling cells to resist deformation.

Figure 1. LLPS Illustration.



UBQLN2 undergoes liquid-liquid phase separation. (2023). Castaneda Lab. Retrieved from https://cacastan.expressions.syr.edu/liquid-liquid-phase-separation-in-protein-quality-control/.

All three types of cytoskeleton structures are comprised of helical assemblies of subunits. Intermediate filaments are made of smaller subunits and are elongated and fibrous. Actin filaments and microtubules are made of subunits that are compact and globular. These helical assemblies of subunits self-associate via attractive forces. There is a difference in stability and mechanical properties of each filament. Each of the three types of cytoskeleton polymers are held together by weak noncovalent interactions, meaning assembly and disassembly occurs quickly. Many cytoskeleton proteins in the cell regulate the spatial distribution and the dynamic behavior of the filaments. Accessory proteins allow the cytoskeleton structure to be under control of extracellular and intracellular signals; these accessory proteins also result in a highly organized internal structure in the cell.

Protein synthesis occurs on ribosomes, and the proteins are released into the cell prior to diffusing within the cell and assembling into the cytoskeleton or other structures. This model is followed for tubulin and actin. Actin filaments are cross linked by actin-

binding proteins to form bundles or three-dimensional networks. Intermediate filaments are encoded by 70 different genes and are the most diverse of the three major cytoskeletal filaments. There are five types of proteins just for intermediate filaments, indicating that it is quite complex to assemble the cytoskeleton with proteins. Assembling the cytoskeleton with proteins will expand on previous attempts to do this successfully.

Study of the behavior of the three major cytoskeletal components reconstituted together to form all three networks is required to form a more full understanding of the mechanical properties of cells. Unfortunately, in past work, the chemical buffer conditions typically used to reconstitute each individual component are incompatible, precluding the formulation of mixed component interpenetrating cytoskeletal networks. Consequently, the structure and mechanical behavior of such networks have never been explored and the effects of interaction among the three components forming such an interpenetrating network are not known.

The Weitz Lab has reported the formation of a reconstituted network containing physiologically relevant concentrations of all three cytoskeletal proteins: F-actin, microtubules, and vimentin intermediate filaments (VIFs). They have characterized the structure of the network using scanning and transmission electron microscopy as well as confocal fluorescence microscopy. The mechanical network has been probed with two-point microrheology using micrometer-sized particles embedded within the reconstituted networks.

One area that has not been studied fully is the purification of mammalian vimentin, which is an intermediate filament. Thus, the thesis will focus on proving a method for purification of vimentin. It will be required to perform experiments in the

laboratory to test the purification method and demonstrate reproducibility. This thesis will explore and research multiple methods to purify vimentin; ultimately, a method will be selected and utilized. Cell culturing will need to take place and testing will need to be done on the protein to confirm it is structurally viable, fulfill criteria, and meet certain requirements. The requirements for success will be clearly defined in order to determine successful versus unsuccessful trials.

Vimentin, an intermediate filament, is present in many organisms within the cells and tissues. Even though the context of vimentin was previously limited when it comes to physiological and pathophysiological applications, more recent work has discovered vimentin has multiple roles in cells and tissues and is also associated with many human diseases; among these diseases are cataracts, cancer, Crohn's disease, and HIV.

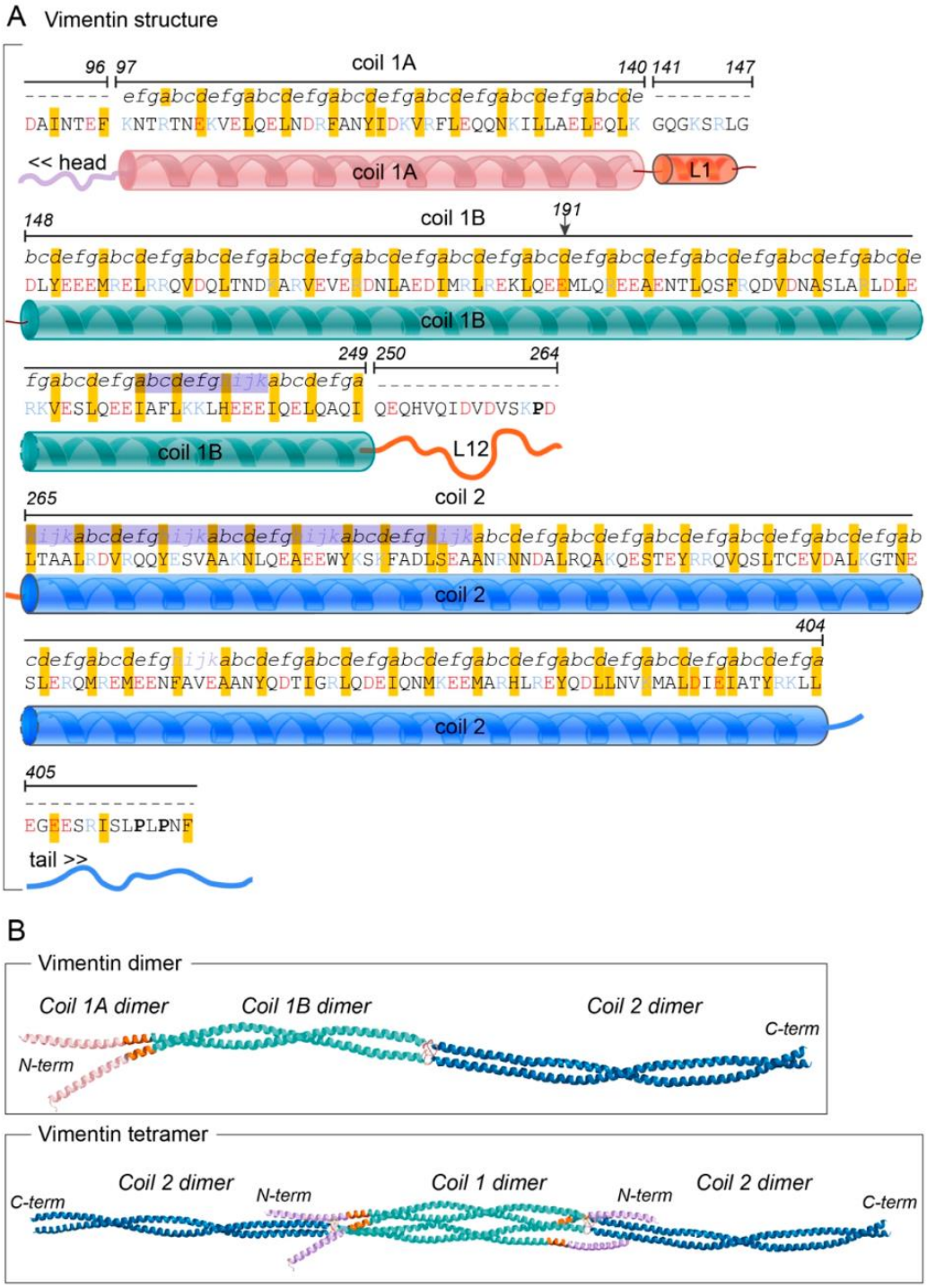
Intermediate filaments, along with microfilaments and microtubules, are the basis of the cytoskeleton in eukaryotic cells. These entities are interconnected, giving the cell its shape while also regulating mechanical, spatial, and biochemical properties of the cell. The name intermediate filament was first established when it was discovered that IF were localized between actin and myosin in muscle cells. Vimentin was previously referred to both the names desmin and skeletin. As can be easily deduced, intermediate filament diameters are in between that of actin and microtubules. Thus, intermediate filaments are not assumed to be positioned in a certain way, they are named based on size.

Proteins that exist as intermediate filaments are distinguished in six categories, types I to VI. Vimentin are type III IFs, those being vimentin, desmin, glial fibrillary acidic protein, and peripherin. Vimentin was the first type III to be cloned in 1983 in hamsters; cloning of the vimentin gene was also performed in chicken and mouse models.

Ten transcripts of the human gene have been discovered. Four are protein coding and two translate into 57 kDa, 466 amino acid, vimentin protein.

The domain of vimentin protein is a 310 amino acid long alpha-helical rod, containing 70 acidic and 46 basic amino acids. The N-terminus is a coil 1 motif, with sequences of seven amino acid residues. The C-terminus has a coil 2 motif with hydrophobic pattern and 11 residue long repeats and a hydrophobic core. There are 12 arginine residues in the 102 amino acid long sequence located N-terminally to the rod domain. See Figure 2A for the amino acid sequence of vimentin.

Figure 2. Vimentin Structure.

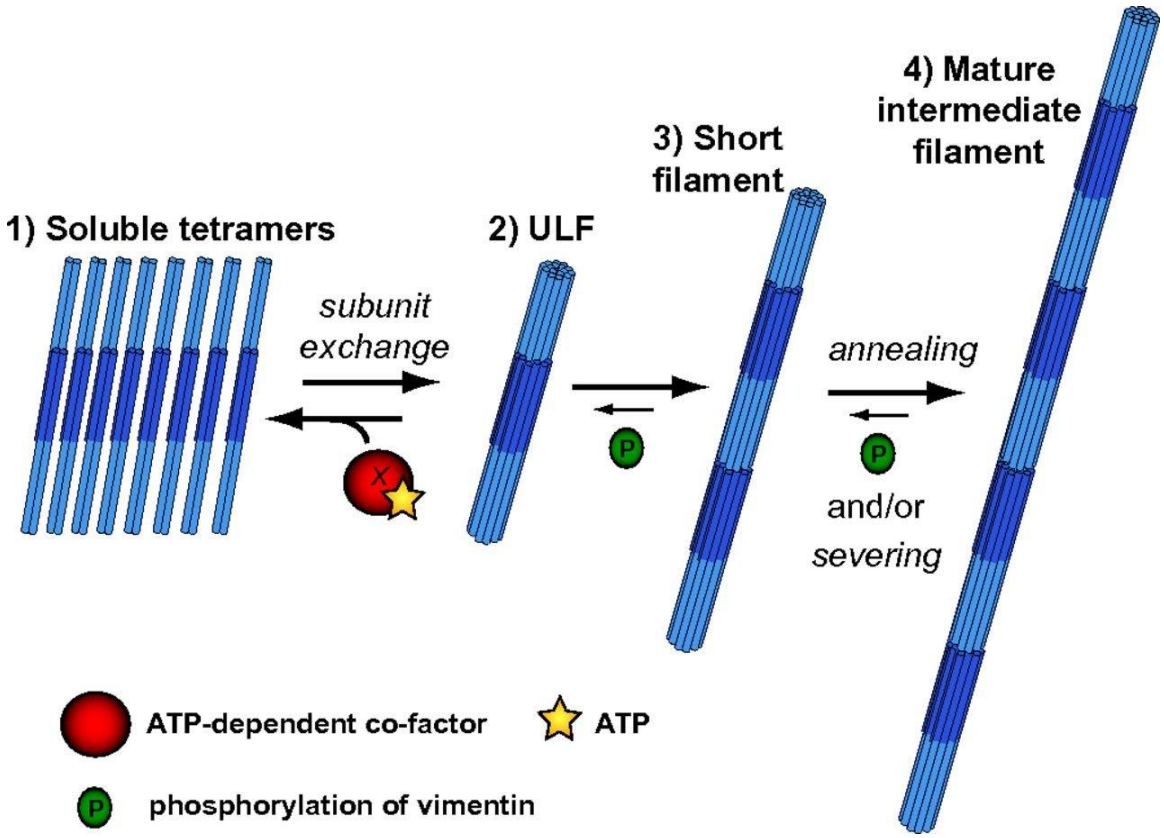


Vimentin structure. (2018). MDPI. Retrieved from https://www.mdpi.com/2073-4409/7/10/147.

Vimentin dimers are formed in a left-handed coiled-coil configuration in the central rod domain. There is a shift in the formation, as seen in Figure 2B, where coil 1A (the N-terminus of coil 1) is followed by the alpha-helical linker L1 structure. The next section is the C-terminus of coil 1, also defined as coil 1B. Coil 1B is connected to a short beta-strand linker, which is a hinge to connect 1B to coil 2. N-terminus and C-terminus areas of coil 2 repeats are conserved between different IF proteins. The N-terminus is capped by the Pro263 residue where the N-terminal region forms parallel alpha-helical bundles. Coil 2 is a regular left-handed structure, which is an important property for the mechanical strength of vimentin.

Two dimers of vimentin can interact in an anti-parallel manner, which associate laterally into a soluble, non-polar tetramer. As seen in Figure 3, eight tetramers are assembled laterally which makes the unit-length filament (ULF). As elongation takes place, ULFs can anneal longitudinally via the coil 2 domains, resulting in short filaments. Short filaments are two, three, or more ULFs. Lastly, filaments assemble through radial compaction to form long compacted filaments of vimentin (mature intermediate filaments).

Figure 3. Vimentin Assembly.



Model of Vif dynamics in cell. (2015). PNAS. Retrieved from https://www.pnas.org/doi/10.1073/pnas.1505303112.

Chapter II.

Materials and Methods

Method 1: Drip Column Method

The first technique considered was a manual drip column protein purification method where one packs a column, equilibrates the column, loads with the protein solution, does a wash three or four times, and finally the elution. The purification utilizes the following buffers/solutions corresponding to the appropriate step:

Table 1. Drip Column Buffer/Solution Detail.

Step	**Buffer/Solution**	**Volume**
Resin packing	Indigo Ni-Agarose	1 mL
Equilibration	50mM NaH_2PO_4, 300mM NaCl, 10mM $C_3N_2H_4$	2.5mL
Add Centrifuged Protein Lysate; incubate @4C for 1 hour	50mM NaH_2PO_4, 300mM NaCl, 10mM $C_3N_2H_4$	10mL lysis buffer resuspended
Wash (repeat 3-4x)	50mM NaH_2PO_4, 300mM NaCl, 20mM $C_3N_2H_4$	100mL/time
Elution (repeat once)	50mM NaH_2PO_4, 300mM NaCl, 500mM $C_3N_2H_4$	2mL

For the drip column process, the steps are as follows:

1. Pipette 1 mL Indigo Ni-Agarose slurry into drip column
    a. Let it run through to remove the storage buffer
2. Pour the binding buffer onto the drip column
3. Wait for the binding buffer to drip through the column
4. Seal the drip column
5. Add centrifuged protein lysate
6. Let mixture incubate at 4 degrees Celsius for 1 hour
7. Remove the seal from the column and allow the lysate to drip through
    a. Keep the lysate at 4 degrees Celsius at all times
8. Add 100 mL wash buffer and let it drip through
    a. Repeat washing process for purer result
    b. Process should be repeated until excess liquid is clear
9. Seal the drip column
10. Add 2 mL of elution buffer
11. Shake or vortex mixture. Let settle for 5 minutes. Repeat once.
12. Let final product drip into Eppendorf tube
    a. Repeat until all tagged protein is eluted

Method 2: Purification of Vimentin Kinase II

The second technique considered was a purification of vimentin kinase II utilizing BHK-21 cells. For the cell culture, BHK-21 cells would be grown in Dulbecco's Modified Eagle's (DME) medium with the addition of 10% calf serum, 10% tryptose phosphate broth, 50 U/mL penicillin, and 50 µg/mL streptomycin in cell culture dishes. Cells would be plated in flasks and 0.4 µg/mL nocodazole would be added 15 hours later. So-called "mitotically arrested cells" would accumulate for 8 hours in the presence of the drug.

Cells would be detached by mechanical shock and centrifuged at 500xg at ambient temperature. Cells would be rinsed with cold PBS (6 mM Na^+-K^+ phosphate buffer (pH 7.4), 171 mM NaCl, 3 mM KCl) with 5 mM EDTA and 0.4 µg/mL nocodazole. This would be pelleted at 500xg and lysed in a buffer containing 10 mM potassium phosphate (pH 7.0), 5 mM EDTA, 20 mM β-glycerolphosphate, 2 mM dithiothreitol (DTT), 0.5% Nonidet P-40, and a cocktail of protease inhibitors (20 µg/mL each of leupeptin, aprotinin, and pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Then, one would centrifuge at 200,000xG at 4 degrees Celsius for 30 minutes and the pellet would be frozen at -80 degrees Celsius. Table 2 indicates the purification technique followed subsequently.

Table 2. Purification of Vimentin Kinase II.

Step	**Details**

1	Frozen pellets from 15 mL of packed mitotic cells homogenized in hydroxyapatite column buffer (10 mM potassium phosphate (pH 7.0), 500 mM KCl, 1 mM DTT, 20 mM β-glycerolphosphate)
2	Homogenate subject to brief microprobe sonication
3	Sonicate centrifuged at 200,000xg for 30 minutes at 4 degrees Celsius and supernatant loaded onto a hydroxylapatite column (2 x 15 cm)
4	Adsorbed proteins eluted on a 10-250 mM potassium phosphate gradient in the column buffer
5	Fractions assayed for vimentin and histone H1 kinase activity. Kinase I would be detected in the flowthrough fractions and Kinase II would be found in the retained fractions. Fractions having vimentin and histone H1 kinase activities (Kinase II) pooled and subject to further purification
6	Pooled fractions brought to 45% saturation with NH_4SO_4
7	Precipitated proteins centrifuged at 20,000g for 10 minutes
8	Pellet solubilized in 10 mM potassium phosphate buffer (pH 7.0), 10 mM β-glycerolphosphate, 500 mM KCl, 1 mM DTT and 1 mM EDTA
9	Solution applied to a Sephacryl S-300 column (1.6 x 93 cm) that was already equilibrated with buffer
10	Kinase-containing fractions pooled and loaded onto a phenyl-Sepharose column (2 mL) that was equilibrated
11	Column rinsed with gradient of KCl from 500 mM to 0 mM in 10 mM potassium phosphate (pH 7.0) and 1 mM DTT
12	Kinase activity eluted with 0.5% Nonidet P040 in 10 mM potassium phosphate (pH 7.0) and 1 mM DTT

13	Pooled fractions from phenyl-Sepharose column dialyzed against 10 mM potassium phosphate (pH 7.0), 50 mM KCl, 1 mM DTT and 1 mM EDTA
14	Solution loaded on a DEAE-cellulose column (2 mL) that was equilibrated with the same buffer
15	Adsorbed materials were eluted with 50-400 mM KCl gradient in the column buffer
16	Purified vimentin kinase dialyzed against 50% glycerol, aliquoted, and frozen at -80 degrees Celsius

Method 3: Purification of Vimentin from Ehrlich Ascites Tumor Cells

Ehrlich ascites tumor (EAT) cells would be grown *in vitro* where vimentin is first incorporated into residual cell structures by extracting EAT cells with Triton X-100 in the presence of Mg^{2+} and solubilized with low ionic strength buffer in the absence of Mg^{2+}.

The expected density of cells is 1.5 x 10^6 cells/mL for this purification, and the steps in Table 3 were followed.

Table 3. Purification of Vimentin via EAT Cells

Step	**Details**
1	Wash cells with 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl (Tris-saline); Centrifuge at 600xg for 5 minutes at 2 degrees Celsius; cells frozen in liquid nitrogen and stored at -80 degrees Celsius
2	To prepare vimentin, a culture (6.6 x 10^5 cells/mL) is harvested by centrifugation at 600xg for 5 minutes at 37 degrees Celsius
3	Cells would be washed twice with warm MEM (without amino acids, but supplemented with tryptophan, cysteine, and methionine, and 5% fetal calf serum which is dialyzed against 0.15 M NaCl)
4	Resuspend cells in 450 mL of MEM and incubate for 4 hours with 9 mCi of H-amino acid mixture. During the incubation, 900 mL of MEM is to be added in 150 mL portions.
5	At the end of the incubation, 150 mL portions of the cell culture are to be diluted with 850 mL of the original medium (MEM containing all amino acids and 5% fetal calf serum)
6	Cells would be harvested by centrifugation, washed in Tris-saline, frozen in liquid nitrogen, and stored at -80 degrees Celsius

7	Thaw 60 g of frozen EAT at 4 degrees Celsius in 10 mM Tris-acetate, pH 7.6, 1 mM EGTA, 4 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 0.5% (w/v) Triton X-100
8	Extract four times in a volume of 1/20th of the volume of the original cell culture by 10 strokes in an all glass Dounce homogenizer
9	Pellet cell structures by centrifugation at 1,500xg for 5 minutes
10	Combine supernatants and centrifuge at 2,000xg for 10 minutes
11	Combine pellet with main fraction of residual cell structures
12	Extract five times with 10 Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol
13	Using a Dounce homogenizer, apply 10 strokes with interval centrifugation at 50,000xg for 10 minutes
14	Combine supernatants and recentrifuged at 50,000xg for 5 minutes
15	Make 23% saturated with solid $(NH_4)_2SO_4$ by stirring for 1 hour at 0 degrees Celsius
16	Precipitated vimentin to be collected by centrifugation at 50,000xg for 10 minutes
17	Redissolve pellet in 30 mL of 10 Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol by sonication at 2 degrees Celsius
18	Vimentin to be reprecipitated with 23% saturated $(NH_4)_2SO_4$ by stirring for 1 hour at 0 degrees Celsius
19	Following centrifugation at 50,000xg for 10 minutes, the pellet is to be dissolved in 10 Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol and reprecipitated as indicated above

20	Lastly, the precipitate is to be dissolved in 30 mL of 10 Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol by sonication, dialyzed overnight, and lyophilized
21	Lyophilized powder extracted five times with 30 mL portions of chloroform: methanol (2:1) by sonication at 2 degrees Celsius for 5 minutes
22	Between extractions, vimentin is to be pelleted by centrifugation at 4,000xg for 5 minutes
23	Remaining traces of chloroform/methanol are to be removed under vacuum produced by an aspirator
24	Vimentin is then dissolved in 20 mL of 6 M Urea, 10 Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol by sonication
25	Solution is applied to a column (2x20cm) of DEAE-Sepharose, equilibrated with 6 M Urea, 10 Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol at a flow rate of 55 mL/hour
26	Elute proteins with a linear (24 hour) gradient of 0 to 500 mM NaCl in 6 M Urea, 10 Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol
27	Monitor eluate with UV monitor at a wavelength of 280 nm and NaCl concentration of each fraction determined with a digitalmeter
28	Use polyacrylamide gel electrophoresis on each of the 6 mL fractions eluted in 6 M Urea, 6% acetic acid
29	Peak fractions to be combined, diluted 1-fold 6 M Urea, 10 Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol at a flow rate of 50 mL/hour
30	When the load was applied, the flow rate is to be reduced to 20 mL/hour and vimentin is eluted with 6 M Urea, 10 Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol, and 500 mM NaCl
31	Peak protein fractions (approximately 10 mL) dialyzed overnight against 6 M Urea, 10 Tris-acetate, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol, divided into aliquots and frozen in liquid nitrogen

Method 4: Reassembly of BJ5TAa IF Method

The method that was selected for the vimentin purification was reassembly of BJ5TAa IF. This method has been successfully executed on other intermediate filaments. MEF (Mouse Embryonic Fibroblast) cells were cultured and passaged according to the protocol in Table 4.

Table 4. MEF Cell Passaging Technique.

Step	**Details**
1	In biosafety hood, siphon off liquid in dish
2	Wash dishes with 3 mL of PBS; shake; wipe off
3	Add 1 mL of trypsin to each dish to chop off cells from dish
4	Incubate 3-5 minutes
5	Inside hood, add double the volume of media as trypsin added (2 mL); mix gently in pipette
6	Centrifuge tubes with counterweight for 6 minutes @ 1.8xg

7	Pellets formed at bottom of tube(s); siphon off supernatant
8	Dissolve pellet in 1 mL of fresh media (DMEM + 10% FBS, 1%penicillin/strep.)
9	Seed new dishes using fresh media: 50-60 µL seed with 3 mL media for small dish 100 µL seed with 25 mL media for larger dish

Passaging the MEF cells for the experiments proved to be challenging in that the process was a new technique for the experimenter. At times, cells were not seen in subsequent passaging, indicating that the cells had not been passaged appropriately. There was also contamination of cells, which happens often in the process of cell culture. Culturing cells was by far the most time-consuming activity to prepare for the experiment.

MEF cells are produced from mouse embryo and are a type of fibroblast. They are widely used in the life science industry and are prepared by killing a pregnant female mouse. The stomach is incised, and the embryo is detached from the placenta in a biohazard hood. Remains are digested by enzymes to produce the isolated cells to be cultured.

MEF cells were grown, and IF were isolated via the following buffers and the protocol indicated in Table 5.

- Lysis Buffer:
    - PBS / 0.6 M KCl / 10 mM $MgCl_2$ / 1% TX-100 / 1 mM PMSF / protease inhibitor tablets
    - DNAse 1
- Wash Buffer:
    - PBS / 5 mM EDTA / 0.2 mM PMSF
- Urea Disassembly Buffer:
    - 8 M Urea / 5 mM $NaPO_4$ pH 7.2 / 1 mM PMSF / 0.2 % BME / protease inhibitor tablets
- IF Assembly Buffer
    - PBS / 0.1 mM BME / 0.1 mM PMSF
- Poly-1-lysine coated coverslips

Table 5. Reassembly of BJ5TAa IF Method.

Step	**Details**

1	Remove medium from the dishes and wash x3 with PBS
2	Add 2mL Lysis Buffer per dish. Allow to lyse the cells 5-10 minutes.
3	Scrape into Dounce homogenizer and give it 3-5 strokes without too much frothing.
4	Add DNAse 1 at concentration of 0.5-1 mg per mL and when the viscosity has dropped spin at 1600xg/15mins/4deg in Beckman tabletop centrifuge
5	Wash pellet x3 with Wash Buffer using a thin spatula to disperse the pellet.
6	Suspend the pellet in approximately 5 mL Disassembly Buffer and stir at RT about 45 minutes
7	Spin in TL-100 75,000xg/39mins/20deg to clarify
8	Dialyze the supernatant against a large volume of Assembly Buffer at RT overnight.
9	Remove dialysate to a tube. Place a drop onto coated coverslips.
10	Drain after about 5 minutes and add 3% paraformaldehyde to c/s 5-10 minutes
11	Blot off and dunk into PBS
12	Stain with vimentin antibody.

Table description style, legend, brief citation etc.

First, medium was removed from the dishes and washed three times with phosphate buffered saline. The lysing solution consists of 0.6 M KCl, 1% Triton X-100, 10 mM $MgCl_2$, 1 mM $PhMeSO_2F$ (phenylmethylsulfonyl fluoride), and protease inhibitor tablets in Ca^{2+}/Mg^{2+}-free PBS (phosphate buffered saline). Cells were lysed for 10 minutes. The cell remains were disrupted with three strokes of a pestle and outer piece glass homogenizer. DNase I was added at a concentration of 0.5 mg/mL and let rest until the viscosity dropped. The solution was centrifuged at 1600xg for 15 minutes at 4 degrees Celsius. The pellet was washed three times in Ca^{2+}/Mg^{2+}-free PBS containing 5 mM EDTA and 0.2 mM $PhMeSO_2F$ to disperse the pellet. The pellet was suspended in 5 mL disassembly buffer (8 M Urea, 5 mM $NaPO_4$ pH 7.2, 1 mM $PhMeSO_2F$, 0.2% β-mercaptoethanol, protease inhibitor tablets) and stirred at room temperature for 45 minutes. The solution was ultracentrifuged at 75,000xg for 30 minutes at 20 degrees Celsius to clarify. The supernatant was then dialyzed against a large volume of IF assembly buffer (PBS, 0.1 mM β-mercaptoethanol, and 0.1 mM $PhMeSO_2F$) for 12-18 hours. The dialysate was removed and added to a tube; a drop was placed onto coated coverslips. Subsequently, they were drained after about 5 minutes and 3% paraformaldehyde was added to c/s 5-10 minutes. It was blotted off and dunked into PBS. Finally, it was stained with vimentin antibody.

Chapter III.

Results

Method 1: Drip Column Method

This method seems straightforward and simple; however, screening appropriate resins for use could make it complicated and time-consuming. Another thing to consider is the temperature control required for this method. It is also not specific for vimentin and has not been tried on other intermediate filaments; this is the most simple method presented in this thesis but it was decided not to attempt it.

Method 2: Purification of Vimentin Kinase II

Due to the complex nature of this purification technique, it was decided against for processing. The main reason this technique was not used is that the column chromatography equipment capabilities were not present in the lab for the thesis.

Method 3: Purification of Vimentin from Ehrlich Ascites Tumor Cells

This third method was considered and decided against since it is extremely complex and requires use of column chromatography, which was determined not feasible. Additionally, this cell type (EAT cells) is not available as the experiment will need to be done with MEF cells.

Method 4: Reassembly of BJ5TAa IF Method

As with any major experiment, it took multiple trials before a successful run was seen. The steps are quite sensitive and multiple pieces of equipment are required to perform the experiment. The lab centrifuges were capable of performing the first centrifugation step, but it required reaching out to a different lab that had an ultracentrifuge for use. Making the buffers for the experiment took a few trials since the number of protease inhibitor tablets was not included in the experiment steps. Other techniques such as dialysis were also new to the experimenter. One issue seen in the first set of experiments was that no pellet was present after the first centrifuge step, so it was required to start from the beginning with cell culture.

Chapter IV.

Discussion

Much work has already been completed concerning vimentin intermediate filaments as well as actin and microtubules as the cytoskeleton is formed. There are many experiments that can be executed now that a purification method has been selected.

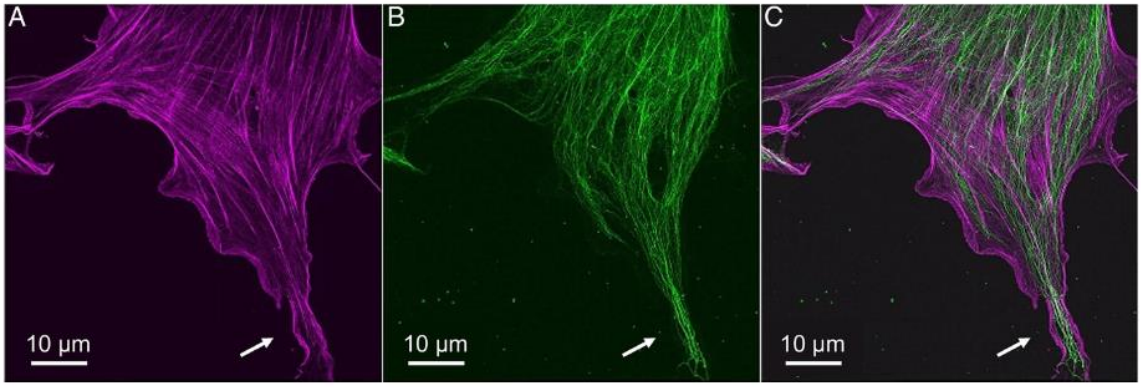
Vimentin/Actin *In Vitro* Model

Vimentin has been modeled and characterized by researchers in the past, laying a foundation for work that is yet to be done. Vimentin intermediate filaments and actin form an interpenetrating network within the cytoskeleton. Imaging was done by a group of researchers to examine this interpenetrating network that is formed. They combined cryo-electron tomography (cryo-ET) and high-resolution structured illumination microscopy (SIM) to image MEFs and observe the interaction between actin and vimentin intermediate filaments.

This work was done in part partly to contradict the thought that actin and vimentin intermediate filaments are segregated. Vimentin intermediate filaments and actin interaction occurs undoubtedly within the cell. There are also contractile forces that exist in this interaction. It was discovered that the vimentin network can influence the behavior of actin in the interpenetrating network. The images of MEF intercellular

networks show actin is defined in a location within the cell; see Figure 4A. Vimentin intermediate filaments are present closer to the core of the cell, as seen in Figure 4B. When vimentin intermediate filaments and actin are imaged at the same time, bundles or stress fibers are seen (Figure 4C).

Figure 4. Vimentin and Actin in MEF Cells.

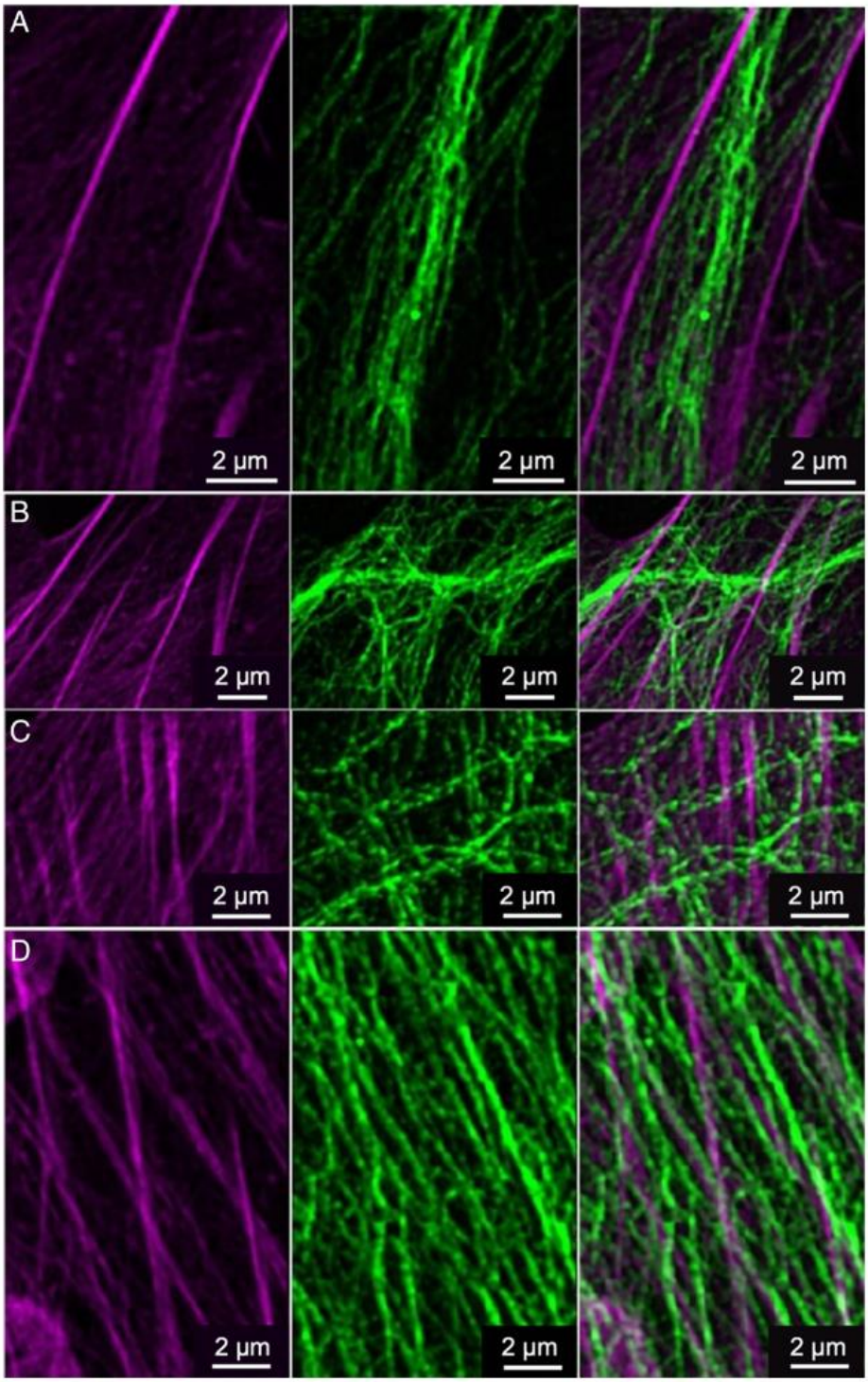


MEF cells showing actin-rich periphery and cortex and cytoplasmic core containing concentrated VIFs. (2022). PNAS. Retrieved from https://www.pnas.org/doi/epdf/10.1073/pnas.2115217119.

The same research group analyzed the relationship between actin and vimentin intermediate filaments by first looking at the actin stress fibers as they form in the cell. There were patterns seen pointing to an interpenetrating network. As seen in Figure 5A, some regions have noticeable stress fibers and vimentin intermediate filaments. They are parallel and fairly closely spaced. The actin stress fibers are mostly in a pattern and in mostly straight tracks; however, vimentin intermediate filaments fill the space between the stress fibers but are must less consistent. Some vimentin intermediate filaments are

thicker and run perpendicular to the actin stress fibers, suggesting the ability to form a bridge-like structure of the two interacting entities; see Figure 5B. It is also seen in MEF cells that the vimentin intermediate filaments are much more sparse. This can be seen in Figure 5C. As seen in this figure, vimentin intermediate filaments are shown to be cross-hatched and forming a mesh with actin filaments. In Figure 5D, it is seen that the filaments of both actin and vimentin intermediate filaments are long and entangled, resembling an interpenetrating network of polymers. Different functions are therefore seen for vimentin intermediate filaments and actin.

Figure 5. Vimentin and Actin Interaction.



F-actin containing stress fibers and VIFs in close proximity to cell surface. (2022). PNAS. Retrieved from https://www.pnas.org/doi/epdf/10.1073/pnas.2115217119.

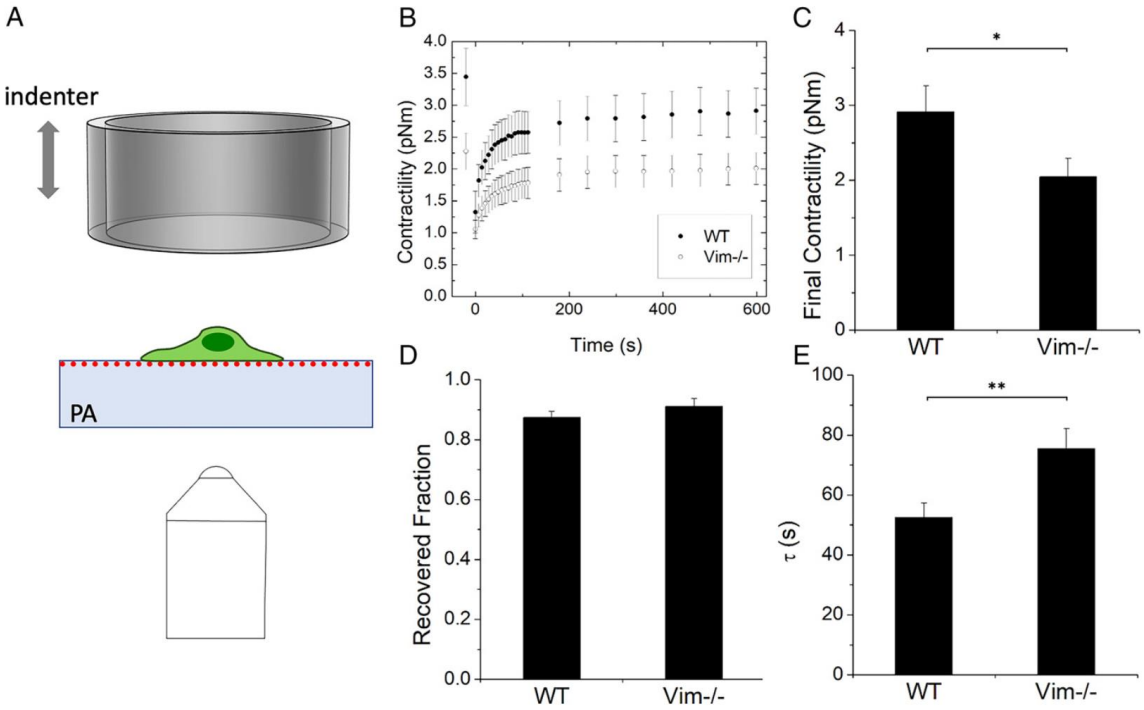
The distance between actin filaments and vimentin intermediate filaments was measured on three different MEF cells. These measurements were made where vimentin intermediate filaments are close to a parallel actin filament, which is very short, approximately 11.3 +/- 5.3 nm. In the studies done, it was found that vimentin intermediate filaments are often seen weaving in and out of actin or wrapping around them, and other areas are abundant in actin only.

To study the impact of vimentin intermediate filaments on actin in cell contractility, mechanical interactions were analyzed *in vitro*. Traction force microscopy (TFM) is used, and vimentin knockout and WT MEFs were utilized for comparison. Figure 6A shows the setup of the stretching that was performed in this experiment. The MEF cells were grown on soft polyacrylamide with beads. Cells are stretched by a large cylindrical indenter centered on the cell. TFM is used to study the dynamics of recovery by tracking the contractility of the cells for the duration of 10 minutes. Actin appears to fluidize, causing a drop in cell contractility but it eventually recovers. See Figure 6B for a graph of WT MEFs and knockout vimentin.

WT cells appeared to remain more contractile than the knockout vimentin as shown in Figure 6C. The research team concluded that decreased contractility is a characteristic of the knockout vimentin MEFs. Cells appear to plateau at a similar fraction of the initial contractility (see Figure 6D). As seen in Figure 6E, a recovery curve fitted with an exponential function shows that knockout MEFs take longer to reach a steady-state contractility, suggesting that these cells build their actomyosin system more slowly. Thus, the results demonstrated in Figure 6 show that the assembly of actin

contractile networks can be regulated by vimentin intermediate filaments in mesenchymal cells including fibroblasts.

Figure 6. Vimentin and Actin Cell Contractility.

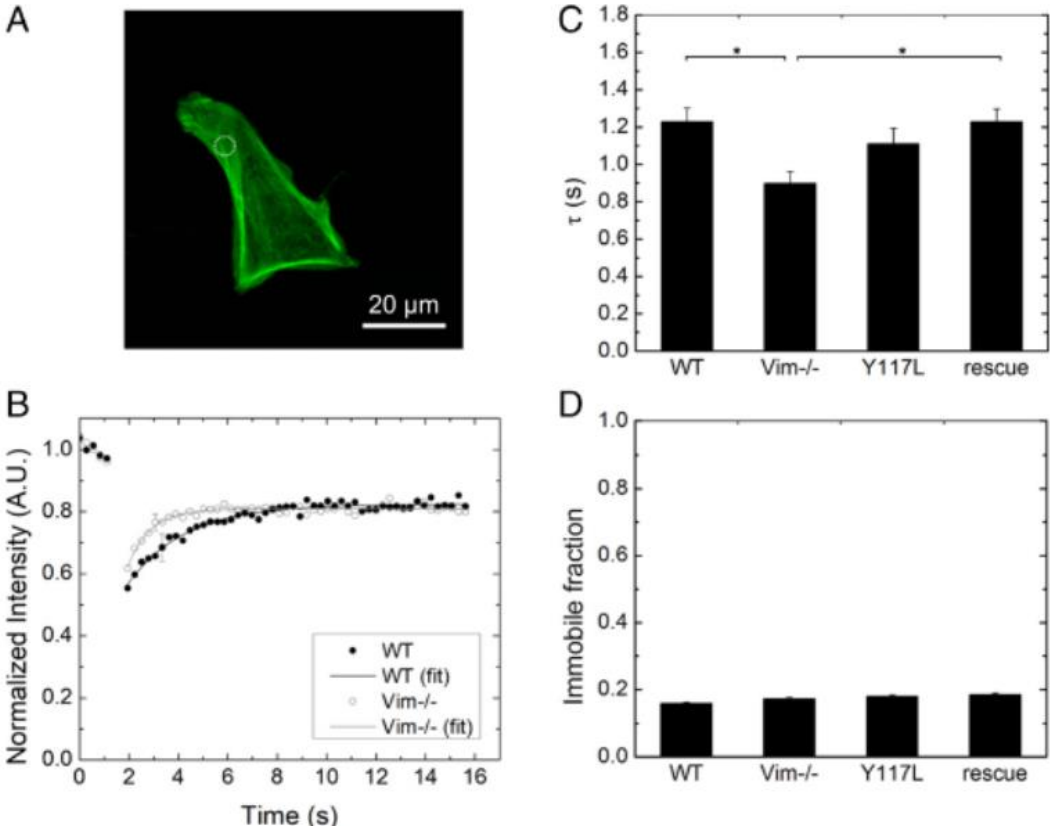


Transiently stretching of cells fluidizing F-actin cytoskeleton; cell contractility recovers within several minutes. (2022). PNAS. Retrieved from https://www.pnas.org/doi/epdf/10.1073/pnas.2115217119.

Fluorescence recovery after photobleaching (FRAP) was used to analyze the effect of vimentin intermediate filaments on the diffusive behavior of G-actin, where F-actin was used previously. WT and knockout vimentin MEF cells were transfected with EGFP-actin to confirm stress fibers appear normal; in order to make FRAP measurements, a small circular region is illuminated that contains fluorescent stress fibers

with an intense laser to locally photobleach actin and measure the recovery of the fluorescence over time (see Figure 7A). In Figure 7B, you can see representative recovery curves for WT and knockout vimentin MEFs. The time dependence of the intensity is normalized to an initial value; fluorescence intensity drops by approximately 40% during bleaching for WT and knockout vimentin MEFs and recovery plateaus at approximately 80%. The curves shown are fitted to an exponential function. WT <EFs recover fluorescence roughly 33% slower than knockout vimentin cells as seen in Figure 7C, which intimates that vimentin intermediate filaments inhibit the motion of actin monomers. No apparent difference is seen in the immobile fractions of the two cell types, suggesting that the fraction of actin is not significantly affected by the presence of vimentin (Figure 7D).

Figure 7 Effect of Vimentin IFs on Diffusive Behavior of G-actin.

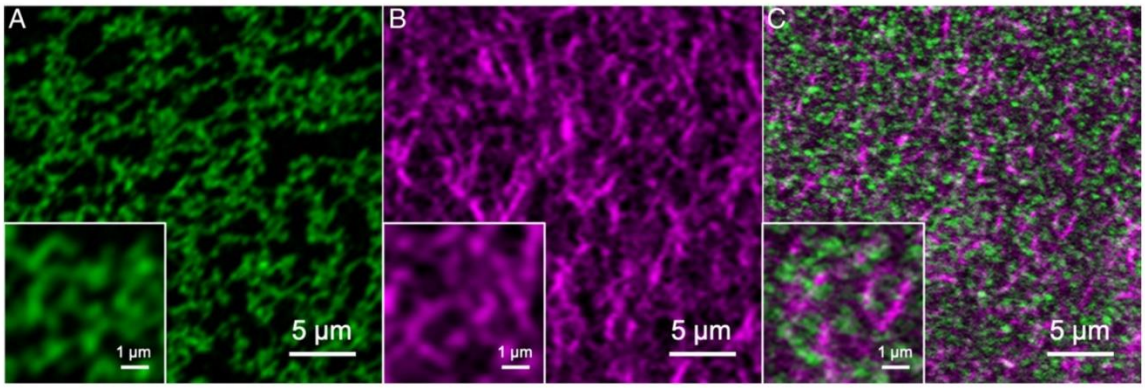


FRAP using EGFP-tagged actin. (2022). PNAS. Retrieved from https://www.pnas.org/doi/epdf/10.1073/pnas.2115217119.

Ultimately, this research group aimed to show that *in vitro* reconstituted networks for an interpenetrating network in the absence of other cellular components. It is highly unlikely that mixed complexes would be formed due to the fact that they are both highly negatively charged; thus, it is highly unlikely that they would assemble *in vitro*. However, when a buffer is applied to the purified proteins, they assemble *in vitro*. Images were taken with a confocal microscope. Figure 8A shows vimentin, where Figure 8B shows actin in the same buffer. When the two proteins are polymerized, randomly oriented filaments are seen and no large-scale phase separation is seen. Actin seems

more rigid and vimentin intermediate filaments fill the pore space. Most importantly, the results show that actin and vimentin intermediate filaments can form interpenetrating networks in the absence of other cell components.

Figure 8 Confocal Microscope Images of Vimentin and Actin.



Confocal fluorescence images of reconstituted networks. (2022). PNAS. Retrieved from https://www.pnas.org/doi/epdf/10.1073/pnas.2115217119.

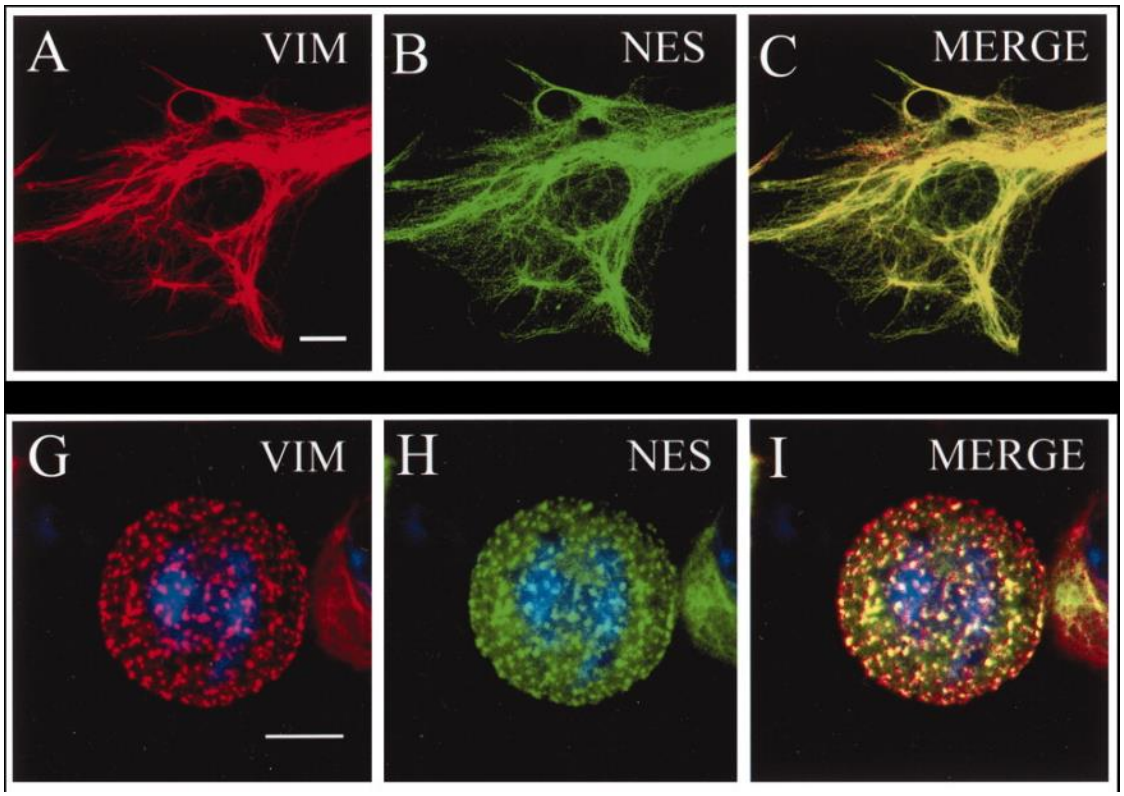
The findings of the research group were expansive, including that vimentin intermediate filaments exist significantly it the cell cortex. There is a strong interpenetrating network formed with actin, specifically the stress fibers. This discovery is novel since it was previously thought that vimentin fibers were localized to the bulk cytoplasm and the cell cortex was defined by actin proteins. Vimentin intermediate filaments are critical components of the stress fibers, and it is suggested that the vimentin intermediate filaments become introduced when stress fibers are forming and not as secondary structures. Actin and vimentin intermediate filaments also have important consequences for cell contractility, and the presence of vimentin enhances this contractility. Importantly, when vimentin intermediate filaments and actin form the

interpenetrating network, MEF cells are more motile and are tougher, correlated with cell stiffness. Thus, vimentin intermediate filaments are more important in cellular dynamics than previously thought. Vimentin intermediate filaments and actin are both critical when it comes to mechanics of the cell.

Future Vimentin Studies

Vimentin appears to form puncta *in vitro*, which has been seen in some research. During mitosis, vimentin forms puncta which are again recycled into filaments in the daughter cells. Something that is not known is what these puncta are and how they are formed. They are dynamic and may be phase separated droplets. They are difficult to study and therefore it is necessary to find a cell line where they are stabilized. See Figure 9.

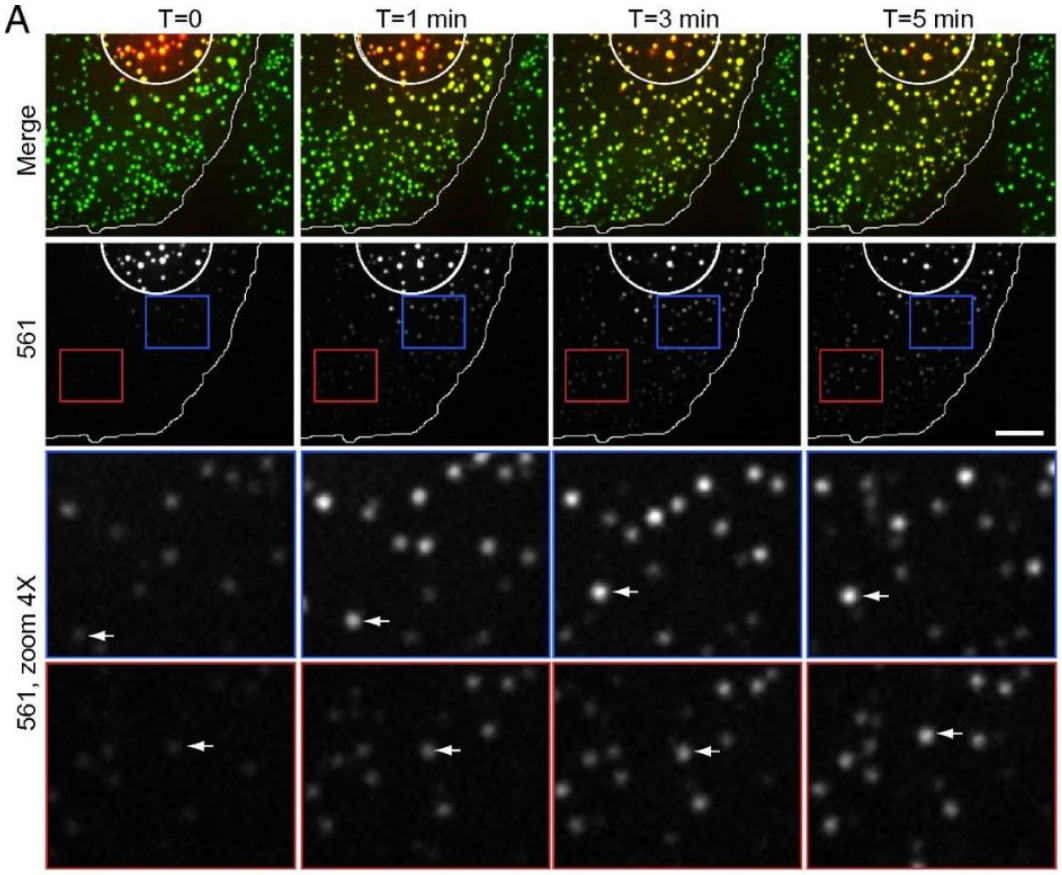
Figure 9 Vimentin and Nestin Expression.



Nestin-specific siRNA prevents the disassembly of vimentin Ifs in mitotic BHK-21 cells. (2003). Mol Biol Cell. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC153115/

Other studies done show that the Y117L mutation inhibits the formation of longer filaments from the ULF stage. It was found that droplet-like structures exist in these cells. However, these drops appear to be situated along some filamentous structure; vimentin in these cells is incapable of forming filaments. In experiments done on MEF Vimentin-Y117L cells, a rapid exchange of droplets was seen with the soluble vimentin pool in the cytoplasm, indicating the liquid-like nature of the condensates; see Figure 10.

Figure 10 Vimentin Puncta *In Vitro*.



ULFs exchange vimentin subunits. (2015). PNAS. Retrieved from https://www.pnas.org/doi/full/10.1073/pnas.1505303112

Therefore, vimentin Y117L forms droplet-like structures throughout the cell, and some of these droplets are attached to actin stress fibers. It is thought that these droplets are phase separated condensates that are formed through non-specific interactions. Drug treatment dissolves the droplets. The actin-vimentin interaction is specific to those droplets partially surviving drug treatment, and the droplets recover after drug withdrawal.

In the future, the group wants to study what these puncta are and how they rearrange into filaments. Specifically, it will be studied what effects the phase properties of the puncta; the group is interested in other proteins that may be at play and what are the conditions under which puncta form. Further, the assembly of vimentin overall *in vitro* will be studied. The group is interested in what adding other biochemical entities does to the puncta and which step it effects. Some specific work could be identifying other proteins or mRNA that might be involved in the formation of these droplets. One way to study this would be isolating the droplets from the cells and analyzing them.

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