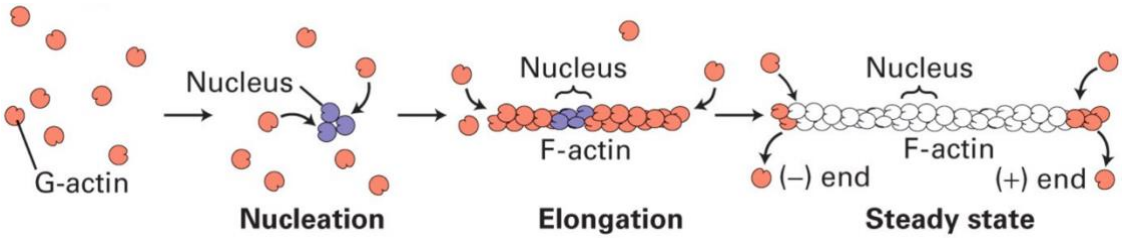
Cytoskeleton

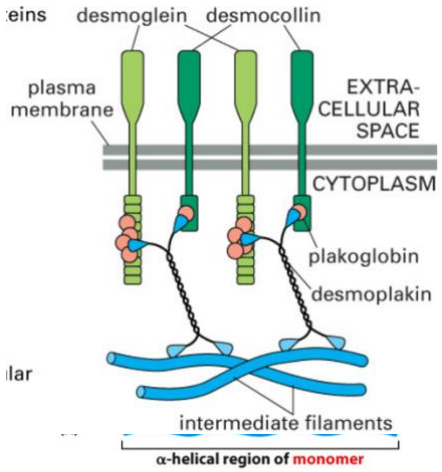
Actin filaments

- **Actin filaments** (F-actin) are helical polymers of globular actin subunits (G-actin)
- Actin is the most abundant protein in eukaryotic cells (makes up 5%, 20% of muscle)
- Actin exists as a dynamic equilibrium of 2 states: G-actin (monomeric globular actin) and F-actin (polymeric fibrous actin)
- G-actin can polymerise to form filaments (filamentous actin/F-actin) which are a linear chain of G-actin subunits
- 3 phases of G-actin polymerisation
- The % of actin subunits in filaments increases until it reaches a steady state where concentration of G-actin and F-actin are in equilibrium (trembling)

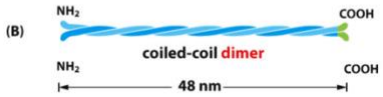


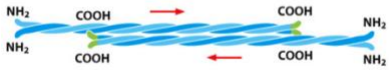
- Treadmilling occurs in actin filaments
- Treadmilling is associated with the rate of growth at the positive end and rate of disassembly at the negative end of the fibre
- Polymer formation involves nucleoside triphosphate hydrolysis which changes the critical concentration at the 2 ends of the polymer
- Ratio of k^{D}_{off}/k^{T}_{on} must not be the same at both ends of the polymer, so that C_c(minus end) > C_c(plus end)
- If both ends of the polymer are exposed, polymerisation proceeds until the concentration of free monomers reaches a value that is above C_c (plus end) but below C_c (minus end)
- At this steady state, subunits undergo a net assembly at the plus end and a net disassembly at the minus end at an equal rate
- The polymer maintains a constant length as subunits are added and removed from the polymer (known as **treadmilling**)
- Actin filaments are flexible and their organisation into linear bundles, 2D networks and 3D gels is controlled by accessory proteins
- Nucleation factors such as the ARP2/3 complex and formins promote formation of branched and parallel filaments
- Dispersed throughout the cell but highly concentrated in the cortex below the plasma membrane
- Aid in cell shape, strength (muscle contraction) and cell movement
- 2 major actins: actins (α-actins) and non-muscle actins (β- and γ- actins)
- Drugs that interfere with actin polymerisation are **Phalloidin** (Amanita toxin - the death cap toadstool) which inhibits actin depolymerisation and blocks cell migration and **Cytochalasin B** (a fungal toxin) which inhibits cell motility and phagocytosis by inhibiting polymerisation

Intermediate filaments



- **Intermediate filaments** are rope-like fibres made of intermediate filament proteins
- 8-10 nm diameter
- Forms a cobweb structure across the cytoplasm, giving cells and tissue strength
- Intermediate filaments do not depolarise, they're stable
- Abnormal filaments can cause diseases
- The filament grows with the addition of 8 tetramers each time
- 8 tetramer structure twists/rolls to form a rope like filament
- Intermediate filaments are the strongest, followed by microtubules and then actin filaments





Functions

- Distribution of tissue tensile stress in epithelia by anchor junctions through desmosomes (cell-cell junction) and hemidesmosomes (cell-matrix junction)
- Position organelles (organelles are linked to intermediate filaments)
- Compartmentalisation: nuclear lamins form the nuclear lamina which is a felt-like structure that lines the inside of the nuclear envelope
- Provide mechanical strength to cell layers at cell junctions

Nucleus

- The nuclear lamina is made up of intermediate filaments which line the inner face of the nuclear envelope
- The nuclear lamina undergoes depolymerisation when the nucleus is reorganised during cell division
- Lamins represent the precursor of all the other types of intermediary filaments which occur only in multicellular organisms

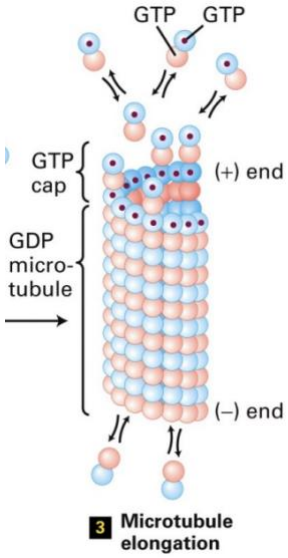
Cancer

- In tumour cells, intermediate filaments lose their normal appearance and their origin is difficult to identify
- Tumour cells still retain the differentiated properties of the cells that they are derived from including expression of IF proteins
- Use fluorescent tagged antibodies specific to IF proteins can determine whether tumour originated in epithelial, mesenchyma, or neuronal tissue
- Epithelial cancers and mesenchymal cancers are sensitive to different treatments

Microtubules

- **Microtubules** are long, straight, hollow cylinders made of tubulin (a protein)
- More rigid than actin filaments
- One end is usually attached to a microtubule organising centre (MTOC) known as the centrosome

- Cilia and flagella are made up of microtubules
- Each protofilament formed from 2 components, α-tubulin and ß-tubulin, combine to form a heterodimer (microtubule subunit)
- These heterodimers then combine to form a microtubule
- 13 heterodimers per circle in helical array
- ß-tubulin can bind either GTP or GDP whilst α-tubulin can only bind GTP
- At the growing/plus end, α-tubulin and ß-tubulin are both bound to GTP (this is known as the **GTP cap**)



Functions

- Microtubules are involved in transporting and mitosis (spindle fibres pull chromatin to either end of the cell)
- MTOC/centrosome is for organising cell polarity
- Microtubule dynamics are responsible for chromosome movements and microtubule assembly
- Kinesin motors are involved in (+) end directed vesicle and chromosome transport
- Dynein motors are involved in (–) end directed vesicle transport and spindle assembly

Centrosome

- Centrosome is located close to the nucleus
- It has 2 centrioles which the microtubules form from
- Microtubules are nucleated by a γ-tubulin complex at the centrosome
- γ-tubulin interacts with accessory proteins (γ-TuSC) to form an arrangement that α-tubulin and ß-tubulin can build onto to form the microtubule seam

Dynamic Instability

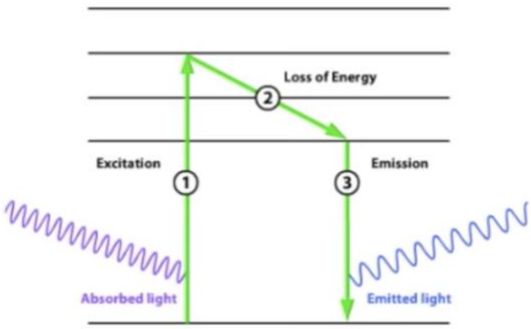
- Microtubules depolymerise 100x faster from an end containing GDP-tubulin than from one containing GTP-tubulin
- GTP cap favours growth, when it is lost, depolymerisation occurs
- Microtubules have slow growth and rapid disassembly which is known as **dynamic instability**

Diseases

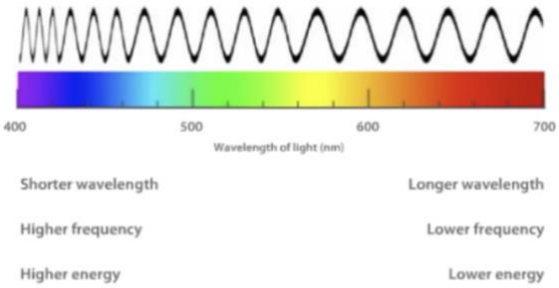
- Some of the most efficient cancer drugs target microtubules (eg. taxol drugs: beta-Tubulin isotypes)
- Dementia/Alzheimers Diseases result from abnormal Tau protein which causes aggregates in neurons leading to dementia states
- Ciliopathies are where cells have varied numbers of cilia
- Many hereditary diseases cause defects in cilia such as Joubert Syndrome, and Meckel Syndrome
- Parkinson's Disease, some caused by defective microtubule regulation

Fluorescence

- **Fluorescence** is the emission of light by a **fluorophore** (that absorbs light) at a particular wavelength which we detect as particular colours
- In its ground state, a fluorophore is of low energy, is stable and does not fluoresce
- When a fluorescent molecule absorbs a photon from an external light source of the correct energy (wavelength) it excites an electron from a lower energy level (S0) to a higher energy level (S1)



- Molecules stay in a transient excited state for a very short time, then the electron falls back to the S0 level and the excess energy is released and emitted as light from a different photon (fluorescence)
- Some energy is lost through molecular vibrations (dissipates as heat) during the transient excited lifetime/state, so the emitted light (emission) is always of lower energy and longer wavelength than the absorbed light (excitation)



- Colour of light emitted is different from the colour of the light absorbed
- The fluorophore's structural instability during the excited state makes it susceptible to degradation – high intensity illumination can result in a structural change in the fluorophore so that it can no longer fluoresce (**photo bleaching**)
- Fluorescent dyes/molecules have an excitation range, however some wavelengths within the range are more effective at exciting the fluorophores than others
- Light is emitted at a range of wavelengths and a molecule may emit at a different wavelength with each excitation event due to degradation/changes that occur in the excited state

Green Fluorescent Protein (GFP)

- GFP is a genetically encoded fluorophore that functions in live cells and can be visualised using fluorescence microscopy
- The gene for GFP can be introduced into any cell where it can then be transcribed and translated to produce GFP
- GFP is found in the bioluminescence jellyfish, Aequorea Victoria
- Aequorin is a protein which when bound to calcium, becomes excited and releases blue light of the wavelength 470nm
- This blue light excites GFP which releases green light of the wavelength 508nm
- GFP can function independently from aequorin (manually excite GFP using a wavelength of 470nm)
- GFP emits green light when exposed to blue light
- GFP is a compact, barrel-like protein, with the light-producing system (chromophore) in the centre
- The $Ser^{65}Tyr^{66}Gly^{67}$ residues in the centre of the a-helix make up the chromophore/fluorophore of GFP
- These residues must be modified by cyclisation and oxidation to produce the mature chromophore

Advantages

- GFP is highly resistant to denaturation by chemicals and proteases
- GFP folds and its chromophore matures without needing cofactors
- Can be directed to any cell, organelle or subcellular structure by fusion to the appropriate gene/protein
- GFP transgene can be inherited by daughter cells
- Non-toxic, non-invasive (most of the time) and can be used in conjunction with other fluorophores
- GFP has been used to develop biosensors that are sensitive to pH, calcium concentration or proteases

GFP as a Reporter Molecule

Gene Expression

- Attach GFP coding sequence to a gene promoter of interest to generate a new transcriptional unit
- Introduce construct into cells (infection, transfection, microinjection)
- GFP will be synthesized if/when promoter is active
- Use fluorescence microscopy to detect GFP fluorescence in live or fixed cells

Protein Localisation & function (fusion proteins)

- Attach GFP coding sequence in frame to either end of gene of interest to generate a hybrid gene encoding a fusion protein
- Clone into expression vector that provides appropriate promoter, and transcriptional terminator
- Introduce vector into cells (infection, transfection, microinjection)
- Fusion protein will be synthesized and should travel to normal location of protein of interest
- Use fluorescence microscopy to detect GFP fluorescence in live or fixed cells, and report on location of fusion protein

Derivatives of GFP (mutants or hybrids)

- Photo-activation – these proteins show a large increase in fluorescence intensity in response to irradiation with specific light. This property can be used to ‘switch on’ a fluorescent signal to specifically track the movement of illuminated cells, organelles or individual proteins
- To measure pH (fluorescence brightness/spectrum of GFP is pH sensitive)
- Calcium measurement
- Protease activity measurement
- Investigation of protein-protein interactions using FRET

Fluorescence Resonance Energy Transfer (FRET)

- FRET occurs if 2 fluorophores are in very close proximity (1 – 10 nm)
- Excitation of one fluorophore results in emission from the other fluorophore
- Loss or gain of FRET can be used to monitor protein:protein interactions using GFP derivatives

Antibodies in fluorescence

- **Antibodies (immunoglobins)** are proteins that can bind antigens (protein, carbohydrate, nucleic acid)
- **Polyclonal antibodies** are mixtures of different antibody molecules, each recognizing a different part/epitope of a particular antigen
- **Monoclonal antibodies** recognize a single epitope of a particular antigen
- Antibodies can be coupled to fluorescent dyes (eg. fluorescein isothiocyanate (FITC))

Direct Immunofluorescence

- An antibody is produced against the target of interest
- The antibody is coupled to a fluorophore
- Antibodies are incubated with fixed tissue or cells to bind to target
- If the target protein is intracellular, cells must be permeabilized to allow entry of antibody
- Cells are examined by fluorescence microscopy to determine presence and/or position of protein

Indirect Immunofluorescence

- **Indirect immunofluorescence** is where a cell is incubated with a primary antibody that reacts with a specific cellular antigen (eg. transferrin receptor). It can be detected by staining with a fluorophore-conjugated secondary antibody that binds specifically to the primary antibody
- An antibody is produced against the target of interest (the primary antibody)
- A separate secondary antibody is produced that binds to any antibody (immunoglobulin) from the species used to make the primary antibody
- A fluorophore is coupled to the secondary antibody
- Primary antibodies are incubated with fixed tissue or cells to bind to target
- Secondary antibodies are added later
- Secondary antibodies must be labelled with different fluorophores
- If the target protein is intracellular, cells must be permeabilized to allow entry of antibody
- Cells are examined by fluorescence microscopy to determine presence and/or position of protein
- Indirect immunofluorescence provides increased fluorescence amplification when compared to direct
- Different antibodies with different fluorophores can be used to label the same cell
- Usually use 2 primary antibodies generated in 2 separate species, and 2 secondary antibodies (each labelled with a different colour and recognizing only one of the primary antibodies)

  eg. primary antibody raised in mouse, secondary antibody raised in sheep injected with mouse antibodies. The other primary antibody raised in rabbit and secondary antibody raised in sheep injected with rabbit immunoglobins. Secondary antibody will only bind to the corresponding primary antibody

Fluorescence Activated Cell Sorting (FACS)

- Cells are analysed by fluorescence and light scattering (indicates size and shape)
- Several fluorescent dyes can be used simultaneously

- Allows identification and analysis of specific cell types in complex mixtures (eg. blood); isolation of pure cell populations for subsequent culture; analysis of cell growth and death; analysis of gene expression
- Cells pass in single file through the detector, information on every cell is then analysed
- Machine coats each cell with negative charge in proportion to its fluorescence intensity
- Cells pass through an electric field, which allows differently-charged populations to be separately collected