



Biological Screening Workshop

Semen

Semen Composition

- **Semen is a fluid of complex composition, produced by the male sex organs**
- **There is a cellular component, spermatozoa, and a fluid component, seminal plasma**

Seminal Plasma

- **Composed of salts, sugars, lipids, enzymes (Acid Phosphatase), nutrients, proteins (p30, Prostate-Specific Antigen), hormones, basic amines (spermine), flavins (source of fluorescence under alternate light)**
- **The components originate from several sources, including seminal vesicles and the prostate gland**
 - **The prostate is the source of the enzyme acid phosphatase and the protein Prostate-Specific Antigen (PSA), or p30 protein**

Sperm Cells

- **Sperm are the male reproductive cells**
- **Develop in the seminiferous tubules, which are in the testes**
- **Development process is called Spermatogenesis**

Factors that effect Spermatogenesis

- **Genetic abnormalities**
- **Disease**
- **Injury**
- **Chemicals – chemotherapeutic agents**
- **Drugs**
- **Alcohol**
- **Age**

Sperm Cell Morphology

- **Composed of a head, midpiece and tail**
- **Head**
 - **Acrosome cap – aids sperm entry to egg**
 - **Houses nuclear material (DNA)**
 - **About 4.5 μm long and 2.5 μm wide**

Sperm Cell Morphology

- **Midpiece**
 - Houses mitochondria – energy for sperm cell
- **Tail**
 - Used for mobility, made of protein
 - Long and fragile – first part to breakdown and easily detached
 - About 40 μm long

Analysis of Semen

- **Semen stains fluoresce under Alternate Light Sources (ALS)**
 - **It is common practice to visually assess items of evidence under alternate light sources to locate possible semen stains**
 - **The intensity of the fluorescence can be affected by the substrate, concentration of the stain, other body fluids and the light source**

Analysis of Semen – Presumptive Tests

- **Acid Phosphatase Detection**
 - **Human semen contains high concentrations of acid phosphatase (AP), which can therefore be the basis of a screening test**
 - **While AP is detected in high concentrations in semen, it can also be detected in other body fluids**

Analysis of Semen – Confirmatory Tests

- **p30 Identification**
 - Found in semen
- **Microscopy**
 - Identification of sperm cells, usually done using a staining method

Analysis of Semen

- **Acid Phosphatase degrades at a much faster rate than sperm cells**
 - **While presumptive tests for acid phosphatase can be helpful; a negative result does not necessarily mean semen is not present**
 - **Many laboratories conduct microscopic examinations on items that have negative AP test results – based on the circumstances of the case and evaluation of the item**

Survival Times

- **Dried Stain**
 - **Sperm, Acid Phosphatase, Prostate-Specific Antigen**
p30 – years if proper storage
- **Vagina / Cervix**
 - **Sperm, Acid Phosphatase = about three days (sperm possibly longer)**
 - **Prostate-Specific Antigen p30 = about one day**

Survival Times

- **Mouth**
 - Sperm about six hours
 - Acid Phosphatase / Prostate Specific Antigen p30 – less due to water solubility
- **Anal cavity**
 - Sperm about nine to 20 hours
 - Acid Phosphatase / Prostate Specific Antigen p30 – less

Survival Times

- **Why do sperm survive longer?**
 - **Sperm heads, very strong**
 - **Designed to last to penetrate egg**
 - **Proteins are water soluble, breakdown quicker**
 - **Sperm tails are lost first**
 - **Tails are fragile and break off**
 - **Made of proteins, bacteria attack first**

Time Lapsed Sex Kit #1 Results Table

Time	Acid Phosphatase	Microscopic Exam
0 hour	+ immediate	Many sperm, easy to find, five to 10 per field, intact sperm found
1 hour	+ 6 seconds	Many sperm, easy to find, three to eight per field, intact sperm found
3 hours	+ 6 seconds	Many, easy to find, one to three in most fields, intact sperm found
8 hours	+ 11 seconds	One to three in some fields, sperm w/ broken tails, one intact sperm
12 hours	+ 14 seconds	Seven sperm on slide, none intact
24 hours	+ 30 seconds	Two sperm on slide, none intact
37 hours	- Reaction at 56 seconds	Negative
48 hours	- No reaction at 1 minute	Negative
72 hours	- No reaction at 1 minute	Negative

Time Lapsed Sex Kit #2 Results Table

Time	Microscopic Exam
0 hour	<i>4+ Many sperm, intact</i>
2 hours	<i>One or more in most fields, intact sperm present</i>
12 hours	<i>Hard to find, none intact</i>
24 hours	<i>Hard to find, none intact</i>
48 hours	<i>One possible sperm on slide</i>

Time Lapsed Sex Kit #3 Results Table

Time	Acid Phosphatase	Microscopic Exam
0 hour	<i>+ immediate < 5 seconds</i>	<i>Neg for sperm, epi cells present</i>
1 hour	<i>+ 8 seconds</i>	
3 hours	<i>+ 9 seconds</i>	
8 hours	<i>+ 8 seconds</i>	
12 hours	<i>+ 25 seconds</i>	
24 hours	<i>+ 30 seconds</i>	
36 hours	<i>+ 30 seconds</i>	
48 hours	<i>- > 30 seconds</i>	
72 hours	<i>- No reaction at 1 minute</i>	

Acid Phosphatase

- **Secreted from the prostate gland**
- **Cleaves phosphate group from molecules**
 - **Phosphoryl choline → choline**
 - **Choline is important in cellular membrane composition and repair**

Acid Phosphatase – AP Spot Test – Reaction

- **Acid Phosphatase cleaves the phosphate from α -naphthyl phosphate to form naphthol**
- **Naphthol and Brentamine Fast Blue B forms a purple azo dye**

Acid Phosphatase – AP Spot Test – How to Make

- **Buffer**
 - **Glacial acetic acid 5 ml**
 - **Sodium acetate, anhydrous (0.24M) 10 g**
 - **Distilled Water 500 ml**
- **Solution A**
 - **Buffer 250 ml**
 - **Sodium a-naphthyl phosphate, 0.25% (w/v) 0.63 g**

Acid Phosphatase – AP Spot Test – How to Make

- **Solution B**
 - Buffer 250 ml
 - Naphthyl diazo blue B, 0.5% (w/v) 1.25 g (Fast Blue B)
 - Both solutions can be combined for working solution in spot test or used separately

Acid Phosphatase – AP Spot Test – How to Store

- **If purchased from a commercial supplier, follow product insert regarding storage requirements**
- **Solution A and Solution B can be stored frozen for months**
- **Solution B should be stored in an amber bottle**
- **When solution A and B are combined, the reagent is not as stable as when stored separately**

Acid Phosphatase – AP Spot Test – How to Perform

- 1. Take a small cutting of the questioned sample and place on filter paper/absorbent pad or swab the questioned evidence stain.**
 - Use a known semen stain as a positive control and an unstained swab or filter paper as a negative control**

Acid Phosphatase – AP Spot Test – How to Perform

- 2. Add one drop of AP Spot Test reagent if both solutions combined or one drop each solution; A followed by B.**
 - POSITIVE if purple color within one minute**
 - NEGATIVE if no color change, pink color change or color change after one minute**

Acid Phosphatase – False Positives

- **Vaginal Acid Phosphatase**
- **Fecal Material**
- **Plant matter**
- **Spermicides**
- **Some feminine hygiene products**

AP 1-Step
AP 2-Step

AP General Swabbing

- **Used on large items of evidence to screen for AP activity**
- **Use a slightly moistened swab to test zones or sections of a large item**
 - **Follow the AP spot test or BCIP procedure**
 - **The technique allow the analyst to screen large items quickly**

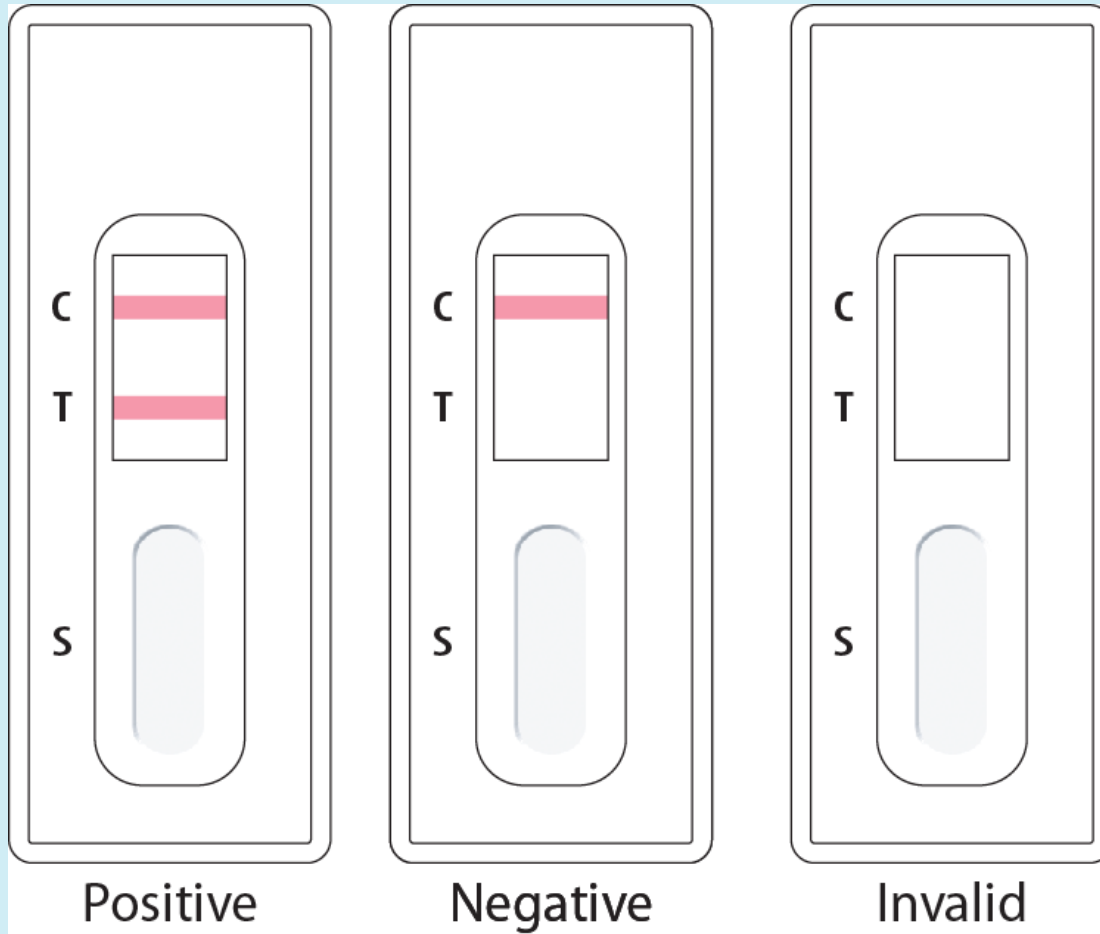
Prostate Specific Antigen (PSA) p30

- **Antigen made in the prostate gland**
- **Weighs 30,000 kD**
- **Liquefies semen and is instrumental in dissolving the cervical mucous cap for sperm entry**

Commercial p30 Tests

- **Ouchterlony, electrophoresis and ELISA methods are labor intensive – this lead to the development of faster methods**
 - **ABAcad® p30**
 - **Seratec® PSA Semiquant**
 - **Semi-quantitative**
 - **Slight differences between the two methods**

ABAcard[®] p30



ABAcad[®] p30 – Reaction

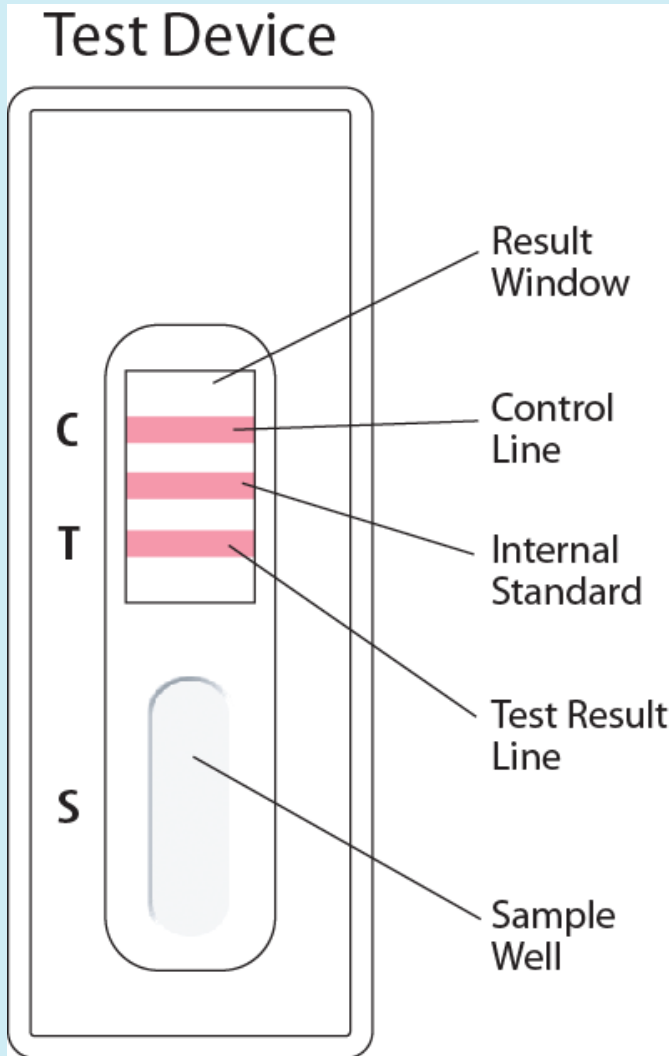
- **“S” area – mobile monoclonal antihuman Prostate-Specific Antigen p30 antibodies**
- **Prostate-Specific Antigen p30 antibodies bind to Prostate-Specific Antigen p30 antigens present in sample**
- **Form Antibody-Antigen complex**
- **Travel through card towards the “T” area**

ABAcad[®] p30 – Reaction

- **“T” area – stationary monoclonal antihuman Prostate-Specific Antigen p30 antibodies**
- **Stationary antibodies in the “T” area catch the mobile Antigen-Antibody complex**
- **Forms Antigen-Antibody – Antibody complex**
- **Antibodies labeled with a pink dye**
- **When antibodies aggregate – form a pink line in the “T” area**

p30 Video

Seratec[®] PSA Semiquant



High Dose Hook Effect

- **Each method has noted a high dose hook effect**
 - **Occurs with excess prostate specific antigen p30 (high concentration of semen) which binds to the stationary antihuman Antibody in the “T” area**
 - **This prevents the mobile Antibody-Antigen complex from binding**
 - **Results in no pink line = FALSE NEGATIVE**
 - **Can be overcome by diluting the sample and reanalysis**

ABAcard[®] p30 Test – How to Make

- **Phosphate buffered saline (PBS) pH 7.4**
- **ABAcard[®] p30 kit**

ABAcad® p30 Test – How to Store

- **Follow product inserts for proper storage**
- **PBS can be purchased**
- **If made in house, follow laboratory procedures**

ABAcard[®] p30 Test – How to Perform

- **Some laboratories quality control check each kit with a 4 ng/ml and a 10 ng/ml dilution of a p30**
- **It is common practice to include a known semen standard (positive control) and a PBS (negative control) with each run set**

ABAcad[®] p30 Test – How to Perform

- 1. Place a small piece of sample material (5mm x 5mm) in a 1.5 ml microcentrifuge tube.**
- 2. Add 250 μ l of 1X PBS to the tube.**
- 3. Incubate at room temperature for two hours with gentle agitation on a shaker.**
- 4. Pipette 200 μ l of extract to the sample well “S” of the test strip.**

ABAcad® p30 – How to Perform

- 5. Read results at 10 minutes.**
 - A positive result is indicated by the presence of one pink line in the test area “T”, at or before the 10 minute reading**

ABAcad® p30 – How to Perform

- 5. Read results at 10 minutes.**
 - A negative result is indicated by the absence of one pink line in the test area**
 - This may result from either the absence of PSA in the sample or it may be due to “high dose hook effect”, which may give false negatives in the presence of high concentrations of PSA**

ABAcad[®] p30 – How to Perform

5. Read results at 10 minutes.

- If a high concentration of PSA is suspected, a 1:100 dilution of the sample may be made to and re-run to confirm the negative result**
- Results are invalid if no pink line is present in the control area “C”**
- In this case the test should be repeated**

Microscopic Examination

- **Sperm cells**
 - **Spermatozoa = plural**
 - **Spermatozoon = singular**
 - **Male reproductive cells**
 - **Haploid – one copy of each homologous chromosome**
 - **Confirmation of the presence of semen by microscopically identifying sperm cells**
 - **The morphology and dimensions of the human spermatozoon are unique**

Microscopic Examination

- **Sperm cells**
- **Detection is simplified by histopathological staining**
 - **The most common stain is known as the Christmas tree stain because of the bright colors (kernechtrot picroindigocarmine stain (KPIC))**
 - **It utilizes nuclear fast red that differentially stains the DNA containing head bright **crimson**, and a counter stain of picric acid – indigocarmine (PIC) that stains the tails **green-blue-gray****

Microscopic Analysis Using KPIC – How to Make

- **Solution #1:**

1. **Dissolve 5g aluminum sulfate in 100ml of hot water.**
2. **Add 0.1g nuclear fast red dye.**
3. **Cool and filter.**

- **Solution #2:**

1. **Dissolve 1g of indigocarmine dye in 300ml of saturated picric acid solution (indigocarmine dye = 5,5-indigo-sulfonic acid, disodium salt).**

Microscopic Analysis Using KPIC – How to Store

- **Solutions are relatively stable when stored at 4°C**
- **If purchased commercially, follow product insert instructions**

Microscopic Analysis Using KPIC – How to Perform

- 1. Fix a smear or stain extract to the slide by gentle heating.**
 - A small cutting can be extracted in PBS or water (stain extract) or a smear can be made directly on the slide using a small cutting**
- 2. Add Solution #1 (nuclear fast red) to the smear or stain extract on the slide and allow to stand five to ten minutes.**
- 3. Rinse slide with water.**

Microscopic Analysis Using KPIC – How to Perform

- 4. Add Solution #2 (picro-indigocarmine dye) to slide and allow to stand three seconds.**
- 5. Rinse slide with ethanol or methanol.**
- 6. Many procedures include the application of a cover slip using mounting media – for example, Permout™.**

Microscopic Analysis Using KPIC – How to Perform

- **All nuclear material will stain a red or red-purple color**
- **Background materials will be stained blue or green**
- **A prepared semen slide should be available for comparison purposes**

Microscopic Analysis Using KPIC

- **Spermatozoa will appear as differently-stained red bodies, somewhat oval in shape with a slight pink cast**
- **The acrosomal cap will be stained less intensely red than the nuclear portion of the sperm head**
- **If present, the midpiece and tail sections will be stained green or blue-green**

Microscopic Analysis Using KPIC

- **It is common practice to grade the abundance of spermatozoa on the smear from 1+ to 4+ at 200x magnification which aids in future DNA analysis**
- **Some procedures require that spermatozoa be verified at 1000x magnification**

Kind Classification

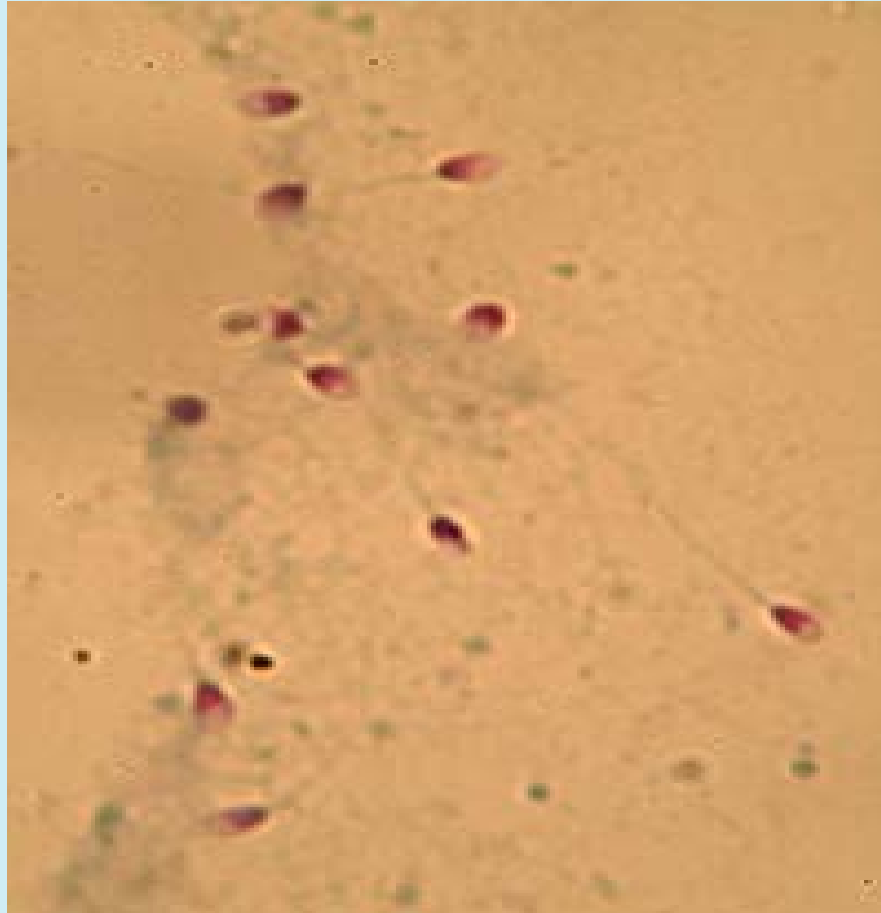
- **Method of classification originally published in 1965 by S.S. Kind**
 - **Rare = < 10 cells on slide**
 - **1+ = Few; difficult to locate**
 - **2+ = Some in some fields**
 - **3+ = Some in many fields; easy to locate**
 - **4+ = Many in most fields**
- **Some laboratories have made slight modifications to this rating scale**

Microscopic Analysis Using KPIC

- **Head of the sperm is red**
- **Tail is green**
- **Acrosome remains clear or faintly colored**



Digital Image of KPIC Stained Sperm Cells



Microscopic Analysis Using KPIC

- **Yeast, bacteria, free nuclei, white blood cells, and dog sperm are stained with this method, and in some instances can interfere with the identification of sperm cells**

Microscopic Analysis Using KPIC

- **Always look for:**
 - **Differential staining – acrosomal cap**
 - **Tail, if attached**
 - **Size**
 - **Larger than yeast or bacteria, but smaller than free nuclei or dog sperm**
 - **Should have prepared human semen, dog semen, yeast, and bacteria slides stained with KPIC available for comparison purposes**

Christmas Tree Stain Video

Yeast

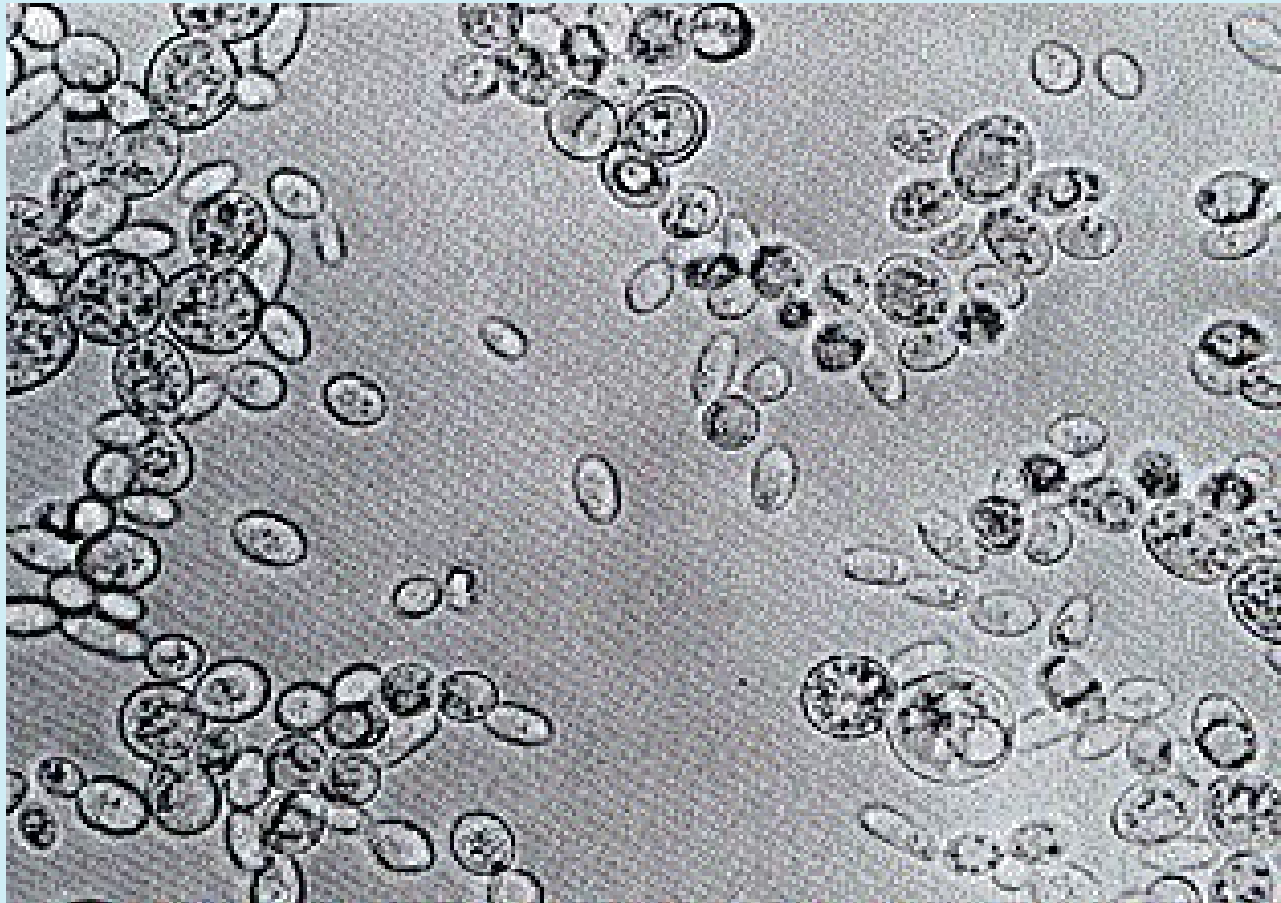


Image courtesy Kimball's Biology Pages (<http://biology-pages.info>)

Biological Screening
Workshop

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Other Stains

- **Hematoxylin and Eosin staining**
 - **Hematoxylin**
 - **Affinity for nuclear material in the presence of a metal**
 - **Stains nuclear material purple**
 - **Eosin is a counterstain**
 - **Non-nuclear material stains pink**

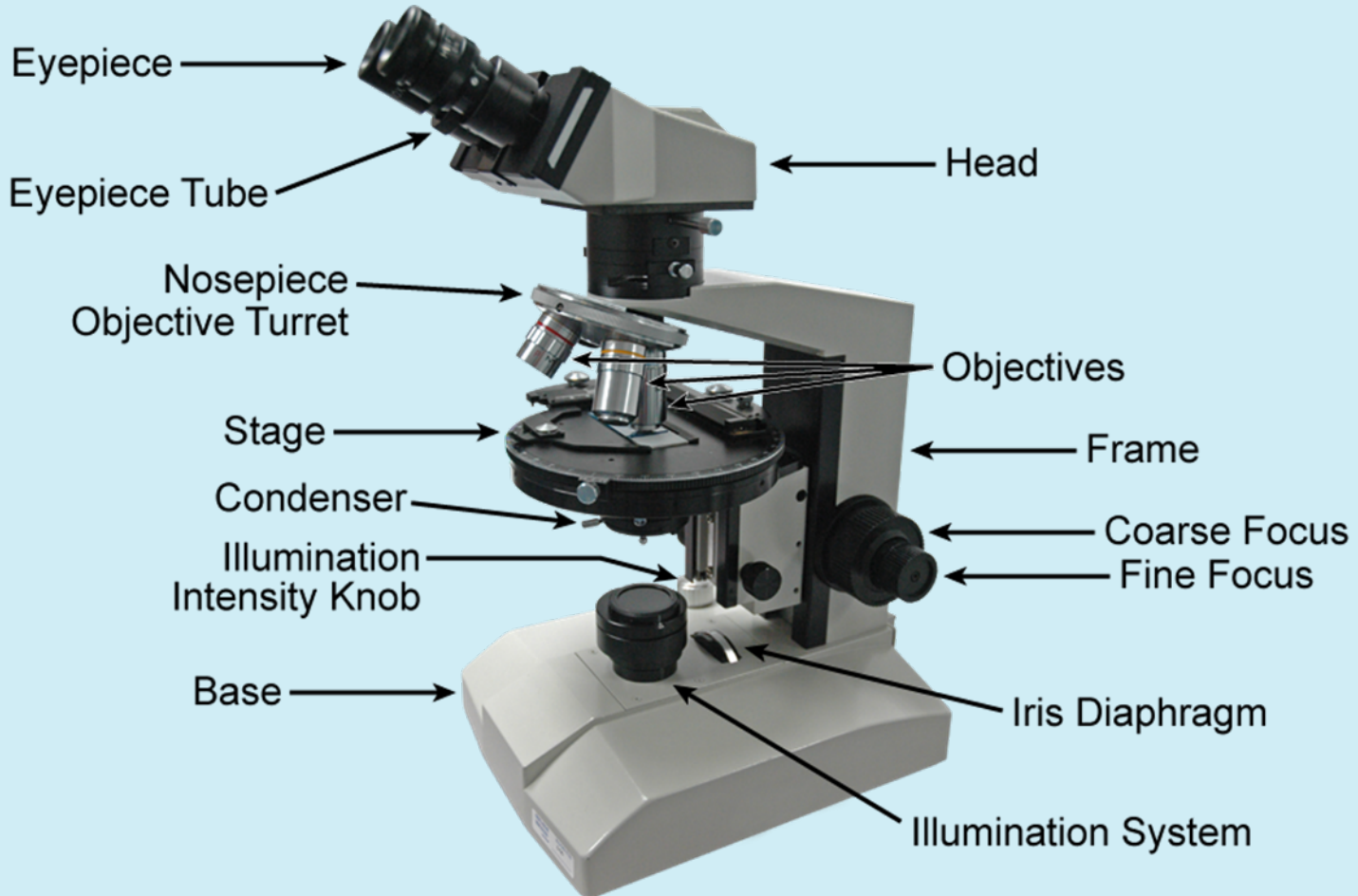
Other Stains

- **Fluorescent staining**
 - **Example: SpermPaints – fluorescent dye mixed with monoclonal antibodies which bind to the sperm head and tail antigens**
 - **Clear, bright, selective**
 - **Will not stain other tissue types**
- **Giemsa staining**
 - **Nuclei stain red**

Cleaning Microscopes

- **Use lens paper and lens cleaner (isopropyl alcohol solution) for ocular lenses and glass surface (over the light source)**
- **Use cotton swabs for smaller objective lenses**

Microscopes



Questions?