New York State Histotechnological Society

On Stage

Volume 29, Issue 4 Fall 2010

TROUBLESHOOTING THE GMS STAIN*

By Peggy A. Wenk, HTL(ASCP)SLS

The Grocott Methenamine Silver (GMS) stain, used to demonstrate fungus, yeast and pneumocystis, is probably one of the *least* capricious of the silver stains. It usually works every time. The fungi, when stained correctly, should be dark black on the outside and light gray on the inside. This will allow the pathologist to observe characteristics of the fungi necessary to diagnose which type of fungus it is. The pathologist needs to see:

- yeast (round) or hyphae (lengths)
- size, shape, width, length
- budding, size, location
- thickness of the wall and/or septates
- length of the segments of the hyphae
- thickness of the septate that divide the hyphae
- branching, if any, how often and at what angle
- any endospores

If the fungus is overstained or understained, these characteristics cannot be easily demonstrated. In addition, overstaining the fungi also tends to stain the background, particularly the connective tissue. This makes it difficult to locate the fungi or yeast in the tissue, particularly if they are few in number or of a small size. If asked what can cause over- or understaining, most histotechs would say the time in the staining solution was incorrect, or the temperature was incorrect. Both of these are true, but there are other reasons why the GMS may not stain correctly. Let's explore some of these other reasons.

FUNGUS

Fungus is a eukaryotic organism, just like most plants and animals, unlike bacteria, which are prokaryotic (more primitive). Attached is a table that delineates the differences between bacteria and fungus, and helps to explain why the GMS doesn't work very well in demonstrating bacteria but works very well for fungus. The most commonly seen fungi in the U.S. are:

- Candida – causing thrush or vaginitis. Is a yeast 2-6 um in diameter in normal flora, but seen as pseudohyphae in infections with sparse septate and is non-branching.

- Aspergillus – Found in the soil worldwide and in immunosuppressed patients as a tangled mass of mycelia. The hyphae are a branching Y shape and 3-6 um wide.

- Tinea – Found in the keratin layer of skin. The cause of athlete's foot and ring worm. Hyphae that are about 2-4 um in diameter. Depending upon which tinea, they have different shapes.

(Continued on page 4)

OnStage is published quarterly by the New York State Histotechnological Society for its membership. Contributions, suggestions and advertisements are welcome. Please visit the NYSHS website for submission information and guidance. Permission to reprint is granted as long as source and author are acknowledged and a copy of the reprint is sent to the editors. Articles without bylines are written by the editors. Please submit manuscripts to the editor-in –chief:

Amy Farnan pita444@yahoo.com

Deadlines for Submission are:

December 1 – Winter

March 1 - Spring

June 1 - Summer

September 1 - Fall

On Stage Editorial Staff

NYSHS Officers

NYSHS Board of Directors

Membership to NYSHS, includes a subscription to *On Stage*. The annual membership fee is $20.00 per year. Please direct membership inquiries to: NYSHSmembership@yahoo.com

Inside this issue:

Troubleshooting The GMS Stain 1

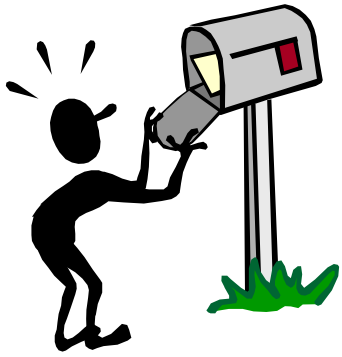
Presidents Letter 3

Election Results 6

NSH Quick Facts 10

Licensure Question 12

Fall 2010
Letter from your President



Dear Members

It is an honor and a privilege to now represent you as president of the NYSHS. My first order of business is to thank all of you for being members and for supporting the society. Without you, this wonderful society would not exist and we would not be able to host educational meetings, lobby on your behalf or provide a professional organization that represents the talented and hardworking histologist in New York State. Thank You!

Second, I want to thank the outgoing BOD members. Christine Miller, whose ability to follow and record our board meetings amazes me. Cindy Kosuda, who has worn many hats and has always, brought a smile and a laugh to our meetings. Sandy Mendel, whose years of experience and dedication as a board member, officer and teacher are an inspiration to us all, and finally, a special thanks to Mary Georger. To the best of my knowledge, no one has worked longer (with the exception of Sandy) on the local, regional and national level for all of you than her. She deserves special thanks from all of us. I hope that I can fill the "big shoes" that she is leaving behind for me. I also want to welcome and thank our new BOD members Emily Ash, Laurie Marien and Diana Scott as well as our returning "cast of characters": Linda Chen, Amy Farnan, Angela Fogg, Michelle Fuller, and Sarah Mack.

As the leaves begin to fall and the season changes I am reminded of how this is a time of transformation, not only for the society and its new members, but also in our profession. The last few years have seen some dramatic changes in our field: licensure, managed healthcare, diminishing resources and personnel, the economic crisis; the push to do more with less. Never in the history of this discipline has the need for a unified and strong professional society been greater. How can you help? Ask those colleagues that are not members to join the society and to participate. Make sure you and your colleagues are informed, join the NYSHS message board and get the latest news in your industry and stay abreast of any regulatory changes that might be happening. The society is also changing to adapt to the times. We are trying out a new 1 day meeting format this coming spring. We hope to improve upon our website and make it more interactive and provide a better value to our members. We are revitalizing our efforts to recruit students into the field by offering another career day at our annual meeting. We are working with academic programs around the state to create a support structure for exiting students as well as to support the academic programs and students entering the pipeline. We are hopeful that we can achieve all of these goals but I would like to remind all of you that the Officers and Board members are volunteers; we all have regular jobs and busy lives. Nonetheless, we all feel it is important that we help to further the Society and our profession by volunteering our time. While we will all work hard as Officers and Board members, we cannot do this alone. We do need your help and we hope that some of you will volunteer some time by sitting on a committee, helping out at a meeting or just promoting the society. It is up to all of us to make this society successful.

Lastly, please feel free to contact me and or any of the officers or board members (Board and officers contact directory at the end of this issue) if any of you have any questions, concerns, comments or suggestions. We are here to help and serve our membership. Best regards for a wonder fall season!
Luis Chiriboga

• Cryptococcus – Yeast of varying sizes (2-20 um in diameter) with a mucin capsule around it. Found in areas with high bird population, such as pigeons, causes lung infections in humans, but can also be found in other organs, such as brain.

• Blastomyces – Thick walled yeast, 8-15 um in diameter, often with a single bud. Found in soil and causes lung infections.
• Coccidiomyces – Spherule with endospores (looks like a large ball (20-200 um in diameter) with smaller balls inside (2-5 um in diameter)). Found in desert/arid soil and causes lung infections.

• Histoplasmosis – Spherical yeasts that are small (2-4 um in diameter), and usually found inside macrophages. Found in the soil around roosting areas of birds and causes lung infections.

• *Pneumocystis jiroveci* (formerly *P. carinii*) is genomically a fungus, but somewhat resembles some parasites, in that it has a cyst wall about 5 um in diameter. Causes lung infections. In addition, there is the family of Actinomycetes that look like fungi but are actually bacteria. The rod-shaped bacteria lie end to end, and appear as frequently branching filaments, similar to hyphae. In addition to staining variably with a Gramtype stain, they will also stain with GSM, but not with PAS.

• Actinomyces – Normally found in the tonsil and dental caries, but abscesses can originate from these sources and be found in the lung, liver, GI or bone. Have sulfur clubs at their ends.

• Nocardia – Found in the soil, but can be inhaled to cause lung abscesses that can spread to the brain and kidney.

CHARACTERISTIC	PROKARYOTIC (e.g., bacteria)	EUKARYOTIC (most plants and animals, including fungus)
Outside Membrane	Contains muramic acid	*Contains carbohydrates*
Location of DNA	In cytoplasm	In nucleus
DNA shape	One long strand	Chromosomes
Duplication of DNA	Binary fission	Mitosis
Energy makers	No mitochondria or chloroplasts	Mitochondria (animal) Chloroplasts (plant)
How food gets around inside cells	Diffusion	Cytoplasmic streaming
How food gets into cell	Diffusion, Osmosis	Phagocytosis

CHEMISTRY OF STAIN
In order to troubleshoot a stain that *isn't* working, it is helpful to understand the chemistry of how the stain is *supposed* to work.

Oxidation: The carbohydrates in the fungal walls need to be converted to aldehydes. To do this, an oxidizer such as chromic acid (chromium trioxide) or periodic acid is used. If chromic acid is used, the tissue can pick up a slight orangish tinge, which can be removed with a chemical such as sodium bisulfite. Chromic acid is usually used as the oxidizer, as it is stronger than periodic acid, and will overoxidize background carbohydrates to carboxylic acid, which will not stain with silver ions. Therefore, the background reticulin, basement membrane and glycogen will not be stained black.

Silver Solution: Silver nitrate is dissolved in water, and hexamethylenetetramine (hexamine or methenamine) is added. Methenamine is a chemical made from ammonia and formaldehyde. The ammonia combines with the silver nitrate to form an ammoniacal silver group, which readily combines with aldehydes. Fungus aldehydes will be

(Continued on page 6)

Decalcifying Solutions

Cat#	Description
s169K	Buffered Formic Acid
s170	Decalcifying Solution 5% Nitric Acid
s172	Decalcifying Fixative (HCl-Formic Acid in Formalin)
s171	Decalcifying Solution (Formic-HCl in Distilled Water)
s172D	EDTA Disodium Salt Formalin
s2593	Formic Acid-Formalin
s2516	Decalcifying EDTA pH 7.2 - 7.6

stained a reddish-brown when properly done.
Gold Chloride: Used to tone the sections, changing the fungus to a gray-black color, and the background more clear.

Sodium Thiosulfate: Also known as "hypo", this chemical removes the unreduced silver ions.

Counterstain: Most commonly used is Light Green, but others, such as H&E, can also be used. These will highlight the background.

TROUBLESHOOTING
Well, this step seems fairly straight forward. However, if done incorrectly, these steps can lead to false-negative staining, as well as over- or understained fungi, or overstained background. Let's go through each step, and find out what some of the problems could be.

Underoxidation: If not enough carbohydrates on the fungus are converted to aldehydes, then there will not be enough binding sites on the fungus for the silver ions. What will be seen is pale to no staining of fungus. Also, the background carbohydrates (retic, glycogen, etc.) will not be overoxidized, so will convert just to aldehyde and will continue to pick up silver and turn black.
Periodic acid is a weak oxidizer. If used in a 0.5% solution for 5-15 minutes, the fungal carbohydrates will convert to aldehydes. However, the background carbohydrates will *not* be overoxidized, and will also stain with the silver. Thus, the fungus is stained gray to black, and so is the reticulin, basement membranes and glycogen. It is very difficult to see the fungus against this black connective tissue and glycogen background. Chromic acid is usually reused. With continuous reuse, it becomes weaker. This can be seen by the increase of background carbohydrate staining, similar to what was just described for the periodic acid oxidation. Also, with time, chromic acid starts to break down, as evidence by the orange color turning brownish. When this happens, there is underoxidation, resulting in pale staining of the fungus and increased staining of the background. If you are consistently seen connective tissue or glycogen staining black and you know that you have not kept the tissue in the solution too long, or had the temperature too hot, it might be time to change the chromic acid. When using chromic acid, make certain to apply it in the correct concentration for the correct time, and make certain it is still orange in color.

Overoxidation: With overoxidation, the fungal carbohydrates are oxidized to aldehydes, but then continue to oxidize to carboxylic acid, which will not stain with silver ions. Therefore, one would expect decreased to no staining of fungus when overoxidized. If slides remain in chromic acid too long, or if the concentration is too high, then the fungal carbohydrates will be overoxidized, and light to no staining will be seen. One of the advantages of using periodic acid is that it is very difficult to overoxidize the fungal carbohydrates. However, this advantage is offset by the fact that the background carbohydrates are not overoxidized, so will continue to stain.

Methenamine: Given time, powdered methenamine and the methenamine solutions will breakdown into ammonia and formaldehyde. When this happens, the formaldehyde will reduce the silver ions in the solution, *before* they get a chance to bind to the aldehydes in the tissue. This will cause a precipitation of silver all over the slide and tissue. To prevent this, store the methenamine powder in a cool, dry location, and store the methenamine solutions in the refrigerator in a dark bottle. Discard if a change of color or texture is seen.

Borax (Sodium borate): Is used in the solution to help raise the pH. This stain works best at a more alkaline pH. I have, on a couple of occasions, forgotten to add the borax to the methenamine silver solution. The fungus never started to turn brown-red, even after doubling and tripling the time. On these epidodes, after I realized what I had done, I simply added the borax to the staining solution the slides were in. At that point, the stain proceeded like is always does. Fungus now stained great.

Silver Nitrate: There are different grades of silver nitrate. The more pure the silver nitrate, the more expensive it is, because there are fewer contaminates for the silver to bind to and precipitate out, all over the slides. When I've used the cheaper, less pure silver nitrate, we had to change the solution halfway through each stain. When

2010 Election Results are In!

By Kathleen Caleri
Roswell Park Cancer Institute
Nominations Chairperson

It was great to see so many candidates interested in volunteering their time for the society, thank you to everyone that ran !! We have some great new members elected to the board this year. Congratulations are in order for the newly elected members to the following positions in the NYSHS! Luis Chiriboga was elected as your new President, newcomer Emily Ash as Corresponding Secretary, and Amy Farnan, as Membership Secretary. Also newcomers Diana Scott and Laurie Marien were elected to the Board of Directors. Angela Fogg was unanimously appointed to the office of vice president and Linda Chen, who voluntarily left the position as NYSHS membership secretary was also unanimously re-appointed to the board by the current board of directors.

this happened, all I could do was pour off the precipitated solution, rinse the slides in d. water, make up new silver-

methenamine solution, and continue staining the slides in this. Even though the purer silver nitrates are a little more expensive, it is still a lot cheaper than having to use twice as much cheaper/less pure silver solution due to contaminating precipitate. The purer silver nitrate also saves tech time, disposal costs of silver, and speeds up turnaround time.

Gold Chloride: This is another chemical that can be reused. But reusing a chemical means that, each time it is used, there will be fewer chemicals available. If the fungal walls are not changing from reddish-brown to black, and the background is not changing from gray to clear, then it may be time to make new gold chloride. If it is taking a lot longer than usual, possibly all that needs to be done is to "spike" the solution, by adding some fresh. If the gold chloride is a very pale yellow and/or has brownish precipitate on the bottom, this is a good indication that it has been overused. Either start with fresh, or filter and spike.

Sodium Thiosulfate: Forgetting this step, or having a weak solution, is not readily apparent on newly stained slides. Sometime later, such as months later, any unreduced silver that was not removed will turn black due to exposure to light over time. So if the slides looked OK when they were done, but when pulled from archive now have black precipitate on them, look into this step as the culprit.

Counterstain: If the counterstain is too dark, it may mask the fungus, particularly the small fungi, or in the cases where there are only a few fungi in the tissue. Keep the color very pale. The pathologist just wants to see *where* in the tissue the fungus is. They have already seen the H&E, so they know what type of tissue it is, and know a lot of other things about the tissue, such as the type of inflammatory cells there are. The pale counterstain helps iden-

(Continued on page 9)

tify some landmarks, so they know if the fungus is, say, inside a blood vessel. Pale counterstains can also aid the pathologist by giving them a size references – is the fungus larger or smaller than the diameter of a red blood cells (7 um), or of a nucleus? Tissue: Maybe the problem isn't with the stain, but rather it is with the tissue. What if the control is looking good, but the patient's tissue has a lot of background staining? Possibly, the patient has a lot more background carbohydrates than the control does.

- Is it liver? There might be a lot of glycogen.
- Is the tissue GI? Then it might be mucin.
- Is it a plant? Then it has cellulose and starch.
- Is it a parasite or insect? It might have chitin.

All of these are carbohydrates. If your control has "X" amount of background carbohydrates, and your oxidation step eliminates "X" amount, then when faced with a patient's tissue with double, triple or quadruple times the amount of "X" carbohydrates, then the usual amount of time in the oxidizer may not be enough to eliminate the patient's background carbohydrate. Cut another slide, add a fungal control, and restain, increasing the time by a few minutes in the chromic acid.
Maybe the tissue is skin, and it has a lot of melanin and/or keratin. Both of these components like to pick up the silver stains, making it difficult to see tinea in the epidermis. This is the reason why many dermatopathologists choose to order a PAS or PAS digestion to demonstrate tinea. There are very few fungi that are found in the epidermis, beside tinea. So there is no need for the GMS to demonstrate fungal size, shape, wall thickness, etc. If it's fungus and it's in the epidermis keratin – then it's tinea. And PAS does a good job of demonstrating this.

Fungal Size: The size of the fungus in the patient's tissue should match the size of the fungus in the control. Silver gets deposited on the outside of the fungus. The longer the slide remains in the silver solution, the more layers are deposited and reduced to color. The more layers there are deposited; the fungus becomes larger in size. Also, the darker it becomes. Plus, the background begins to pick up silver, too. Let's assume a pneumocystis control is used for a patient tissue with candida or aspergillus. When enough layers of silver have been deposited on the 5 um pneumocystises so we can see it, the larger fungus and yeast have too much silver on them, and will appear totally black. Let's do the reverse scenario – candida, aspergillus or blasto are used as the control for a patient with either pneumocystis or histoplasmosis. When enough time as gone by that enough silver has been deposited on the

(Continued on page 11)

KEEP THIS IN YOUR SITE

Histology World

– By Sarah Bellham

Play all kinds of histology related games, check out all the histology crossword puzzles !

http://www.histology-world.com/crossworddirectory/crossword1.htm

NSH Quick Facts: Contact Hour Certificates

- NSH reconciles and maintains contact hours for NSH members AND non-members for each approved meeting (state, regional and national)

- NSH does not authorize or assign credit until the meeting sign-in sheets are returned to the NSH office

- NSH members will receive an email notifying them that their contact hour certificates are available online for immediate download through their *My NSH* account

- Non-NSH member attendees of the meeting will be mailed a certificate within approximately four weeks of receipt of completed meeting paperwork

- If an attendee loses their certificate:
  - Members may reprint from their *My NSH* account at any time
  - Non-members can request a new copy from NSH but there is a transcript fee

- Questions? contact NSH by email at histo@NSH.org or call 443- 535-4060

larger fungus control, we can see them. However, the patient's small fungus cannot be seen yet at the same time interval. If we stop staining at this point, the patient's tissue with pneumocystis or histoplasmosis will be a false-negative. It is best therefore to have at least two different control slides, one with larger fungus/yeast such as candida, aspergillus or blastomycosis, and one with a small fungus/yeast such as pneumocystis or histoplasmosis. Then pull the control slide that best corresponds to the size of the suspected fungus/yeast in the patient. When the control is the correct color, then we can be fairly certain that the patient's fungus will be stained correctly, also.

Other factors: Cleanliness, purity. If the coplin jar is dirty, such as still containing a little bit of previous stain in it, the silver will precipitate out. If the coplin jar still contains soap residue, the silver will precipitate out. If the coplin jar was rinsed in tap water instead of deionized water, the silver will precipitate out. If the silver solution was made in tap water instead of deionized water, the silver will precipitate out. If the water rinses in the stain were with tap water instead of deionized water, the silver will precipitate out. Getting a pattern? Keep everything clean, and use only deionized or distilled water.

Think About It

Next time you are doing a GMS, stop and marvel at all the chemistry you are performing on that patient's tissue. And when the stain doesn't go exactly right, remember, the problem may not be due to the time in the silver, or the temperature of the silver solution. It could be a little more complex than that, such as related to the chemistry of the stain or the biochemistry of the tissue.

References:

Kiernan, JA: *Histological & Histochemical Methods – Theory and Practice*. 3rd edition, 1999, Butterworth.

Grizzle WE: Theory and Practice of Silver Staining in Histopathology. *Journal of Histotechnology*. 19(3):183-195, 1996.

Churukian CJ: Consistent Silver Methods for Demonstrating Basement Membrane, Reticulum and Fungus. *Journal of Histotechnology*, 19(3):211-217, 1996.

*Reprinted with permission. Originally published in the August 2006 issue of *Mikro-Graf*, the newsletter for the Michigan Society for Histotechnologist

Licensure Questions

By Amy Farnan Albany Memorial Hospital
Licensure Task Force Chairperson

It has been two years since the histology license was enacted and many of us have renewed or are up for renewal so many of you may think, "What is there left to talk about?." Well, I actually still receive email questions regarding the licensing procedure. I thought since one person is asking there are probably many more asking the same questions. I decided to start posting the questions and answers in the newsletter as it may help you, a colleague, or an out of state friend. Here is the question we received:

Q: *I just moved to NY and need to get my NYS license to practice histology. I have a histotechnology degree from a NAACLS accredited program and my HTL (ASCP) certification that I received in October 2009. I have been on the NYS website but haven't seen a clear cut licensing route for someone who is already ASCP certified. I did find info on the ASCP website that made it seem like I only needed to send in verification of certification to NYS without filling out NYS forms 1-3 but I am thinking this may be too good to be true. Any clarification would be helpful. Thank you for your help.*

A: The licensing pathways can be viewed on the SED website, located at:

www.op.nysed.gov/prof/clt/clp-histologicaloptions.htm

Standard Pathway – Method 5 : Licensure for Histological Technician
The applicant will have to submit an application, fee, documentation of his/her education, including an associate's degree or higher, and a form from California or Florida providing information on his/her licensure or certification status in that state, and including any disciplinary actions taken against the licensee.

There is only one route for grand parenting that remains, and that would be if he/she were to have been employed by a laboratory licensed by the NYS Department of Health (they license labs all over the world) and had been employed as a histological technician between January 1, 2004 and December 31, 2007 for a minimum of six months and where the director would attest to both employment and competence. If that is the case, he/she would have to submit the application and fee and have the experience Form 4C submitted by the clinical director of the laboratory in which the work experience was obtained.

Kudos to Our Members!!
Regional and State Appointments

Angela Fogg has been re-elected as NSH Region 1 director for another two year term . Congratulations Angela !

Carolyn Demarinis of Saratoga Hospital has been appointed to the New York State Education Department Clinical Laboratory Licensing Board as a representative for the profession of histology. We have representation !!! Congratulations Carolyn, the NYSHS supports your efforts !

We are proud of our state members. If you would like to recognize a member for a professional achievement please contact the newsletter editor at pita444@yahoo.com

Join The NYSHS Message Board

Do you have questions? Do you want quick answers? Are you looking for a job or have a job opening? Do you want the latest news and info from the histotechnology field? Join the New York State Histotechnology Society Message Board. It's easy; just visit the message board website at http://tech.groups.yahoo.com/group/NYSHS1972/

Don't Miss our *NEXT* issue coming out this winter……

NYSHS 2011 Preliminary Program

NYS Election Information

Call for NYSHS 2011 Awards Nominees

New York State Histotechnological Society

Officers

President	Luis Chiriboga	NYU School of Medicine	Litepath2000@yahoo.com
Vice President	Angela Fogg	Columbia Memorial Hospital	angelafogg@aol.com
Corresponding Secretary	Emily Ash	Roswell Park Cancer Institute	emily.ash@roswellpark.org
Membership Secretary	Amy Farnan	Albany Memorial Hospital	pita444@yahoo.com
Treasurer	Michelle Fuller	University of Rochester Medical Center	michelle_fuller@urmc.rochester.edu
Past President	Mary Georger	University of Rochester Medical Center	mary.georger@rochester.edu

Directors

Linda Chen	Weil Cornell Medical Center	kooki55@hotmail.com
Sarah Mack	University of Rochester Medical Center	sarah_mack@urmc.rochester.edu
Laurie Marien	Department of Veterans Affairs	lmarien@roadrunner.com
Diana Scott	University of Rochester Medical Center	diana_scott@urmc.rochester.edu
Student Representative	Vacant	

Committee Chairs

Membership	Amy Farnan
Nominations	Kate Caleri
Awards	Sarah Mack
Legislative	Amy Farnan
Website	*Vacant*

Coordinators

News Letter Advertising	Stephanie Krauter
Career Day	Laurie Marien
Education	Luis Chiriboga
Meeting/Symposium	Amy Farnan
Newsletter Editor-in-chief	Amy Farnan
Meeting Registration	Michelle Fuller
Vendor Coordinator	Linda Chen