REVISED NATIONAL TUBERCULOSIS CONTROL PROGRAMME

Manual for Sputum Smear Fluorescence Microscopy

Central TB Division
Directorate General of Health Services
Ministry of Health and Family Welfare, Nirman Bhavan,
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SPUTUM SMEAR FLUORESCENCE MICROSCOPY

PURPOSE

The most important tool in the diagnosis of tuberculosis is direct microscopic examination of appropriately stained sputum specimens for acid-fast bacilli. The technique is simple and inexpensive, and detects those cases of tuberculosis, which are infectious. Sputum microscopy is also useful to assess the response to treatment, and to establish cure or failure at the end of treatment.

BACKGROUND INFORMATION

Fluorescent microscopes are provided to the state designated Intermediate Reference Laboratories (IRLs) under Revised National Tuberculosis Control programme(RNTCP) and at present, the use of fluorescence microscopy is linked to the culture and Drug Sensitivity Testing (DST) activities of the IRLs.

Fluorescence staining utilizes basically the same approach as Z-N staining, but carbol fuchsin is replaced by a fluorescent dye (auramine-O, rhodamine, auramine-rhodamine, acridine orange etc), the acid for decolorisation is milder and the counter stain, though not essential, is useful to quench background fluorescence. Both sensitivity and specificity of fluorescence microscopy are comparable to the characteristics of the Z-N technique. The most important advantage of the fluorescence technique is that slides can be examined at a lower magnification, thus allowing the examination of a much larger area per unit of time. In fluorescence microscopy, the same area that needs examination for 10 minutes with a light microscope can be examined in 2 minutes.

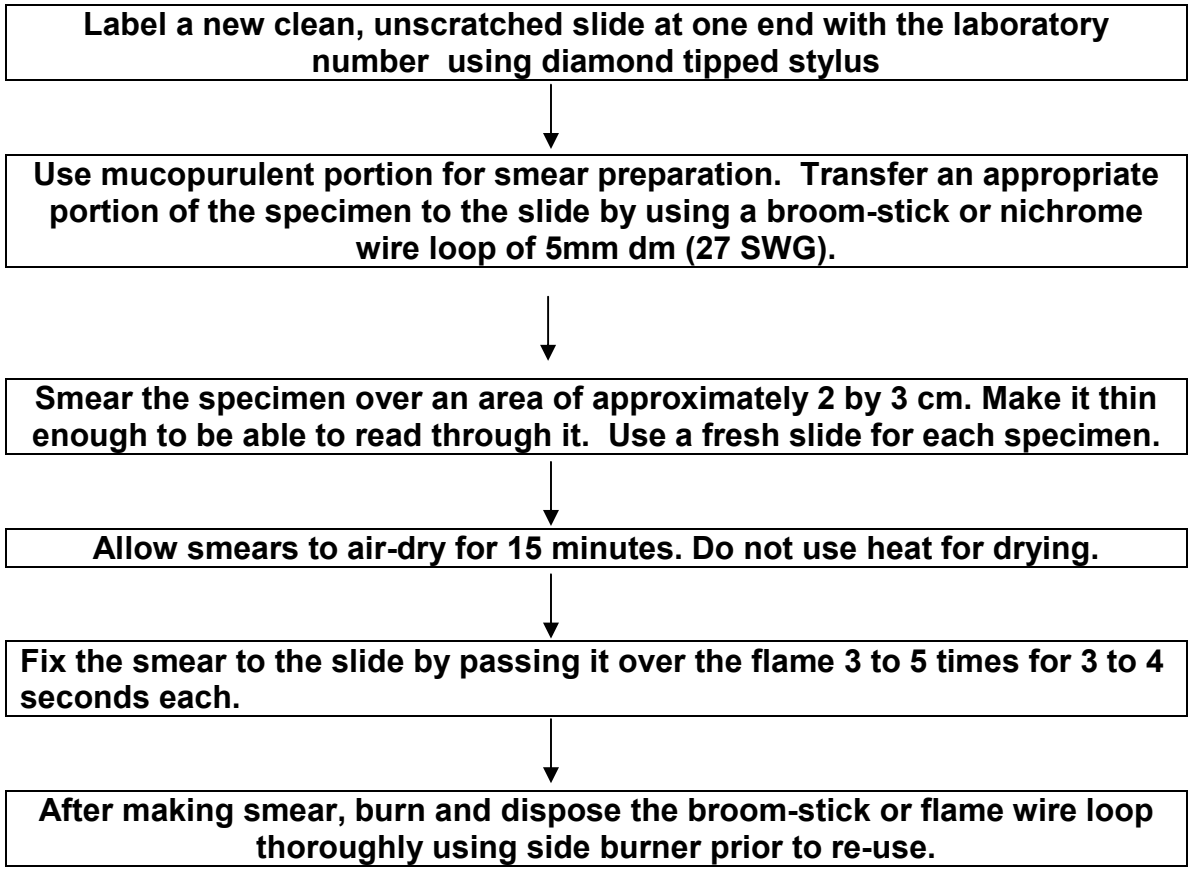
PRINCIPLE

Mycobacteria retain the primary stain even after exposure to decolorizing with acid-alcohol, hence the term “acid-fast”. A counter-stain is employed to highlight the stained organisms for easier recognition. Potassium permanganate is used as counter-stain and it helps prevent non-specific fluorescence. With auramine staining, the bacilli appear as

slender bright yellow luminous rods, standing out clearly against a dark background. The identification of the mycobacteria with auramine O is due to the affinity of the mycolic acid in the cell walls for the fluorochromes. In fluorescent microscopy, light rays of shorter wave length pass through smear stained by a fluorescent dye, such as auramine O, which have the property of absorbing light rays of shorter wave length and emitting light rays of longer wave length. A mercury vapour lamp is used as a source of light and by means of suitable filter only light rays of shorter wave lengths are allowed to emerge and these rays are used for microscopy. The condenser of the microscope is made of quartz which will not absorb ultra-violet rays.

SPUTUM SMEAR PREPARATION

The procedure for smear preparation is described below: Sputum smear should be prepared nearer to the flame (spirit lamp/Bunsen burner).



MATERIALS REQUIRED FOR STAINING

Auramine-Phenol solution
1% Acid alcohol
0.1% Potassium permanganate solution

PREPARATION OF STAINS AND REAGENTS (AURAMINE TECHNIQUE)

a. 3 % Stock solution of phenol:

Phenol crystals	3.0g (if liquid: 5gm phenol solid weight = 6ml liquid volume)
Distilled water	87ml

Prepared from pure crystals dissolved in distilled water and stored in a tight fitting glass stoppered bottle.

b. **Auramine-Phenol solution:** Warm 100 ml stock of three percent phenol to 40°C. To this add gradually 0.3 gm of Auramine with vigorous shaking for 10 minutes. Filter and store in a dark brown bottle. The stain should not be kept for more than 3 weeks. A standard good quality powder of "Auramine O" should be used (see specifications).

c. **Acid Alcohol**

0.5 ml concentrated hydrochloric acid
0.5 gm Sodium chloride
75 ml absolute alcohol
25 ml distilled water

Dissolve sodium chloride in water, add the concentrated hydrochloric acid, mix with the alcohol and store in a tight fitting glass stoppered bottle. *Always add acid slowly to alcohol, not vice versa*. Store in an amber coloured bottle. Label bottle with name of reagent and dates of preparation and expiry. Store at room temperature for upto three months.

d. **0.1% Potassium permanganate:** Freshly prepared in distilled water and stored in a dark brown bottle. Label bottle with name of reagent and dates of preparation and expiry. Store at room temperature for upto three months. $KMnO_4$ is explosive, therefore, avoid contact with combustible materials.

Specifications for Auramine O and $KMnO_4$

Auramine O:

Auramine hydrochloride;

(1,1-bis(p-dimethylaminophenyl)methylenimine hydrochloride)

Formula: $C_{17}H_{21}N_3HCl.\ H_2O$

Mol Wt. 321.85

Appearance: Yellow to brown powder

Potency (Dye content): approximately 85.0%

Absorbance: 435nm

Auramine O is a yellow fluorescent dye; very soluble in water, soluble in ethanol; used to stain acid-fast bacteria in sputum or in paraffin sections of infected tissue

Potassium Permanganate:

Formula: $KMnO_4$

Mol Wt. 158.04

Potency: $\geq$99%

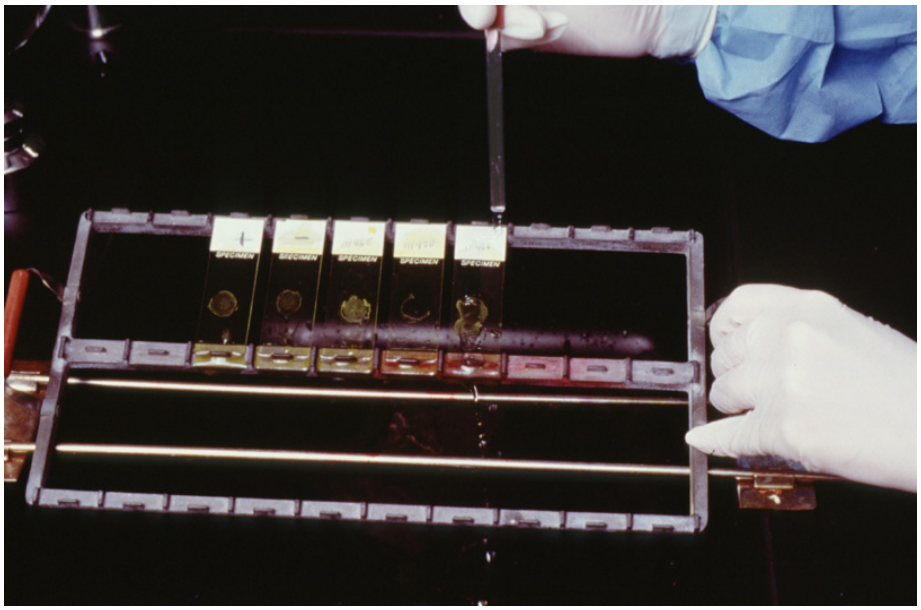
Appearance: Purple solid, dissolves in water to give deep purple solutions.

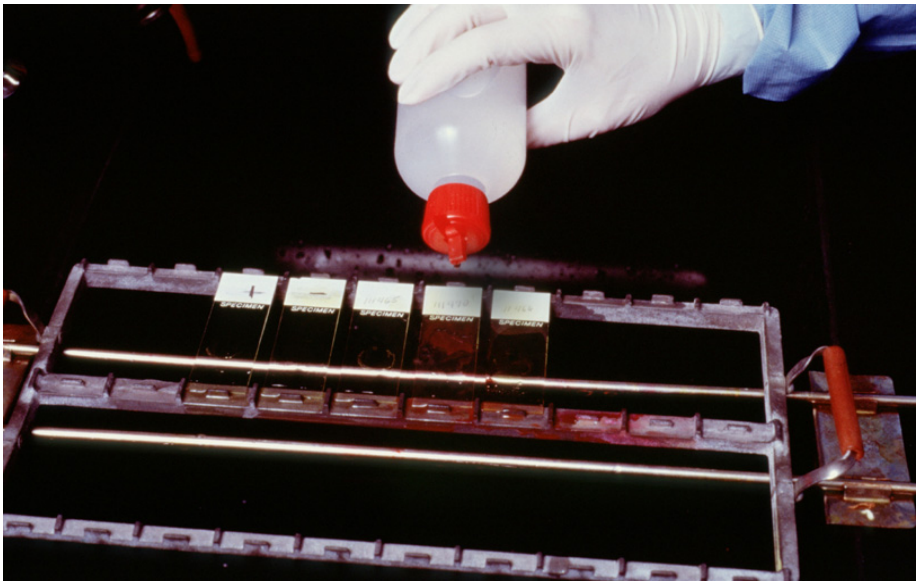
STAINING PROCEDURE

Place the slides on a staining rack, with the smeared side facing up, the slides not touching each other

Flood the slides with freshly filtered auramine-phenol. Let stand for 7-10 minutes

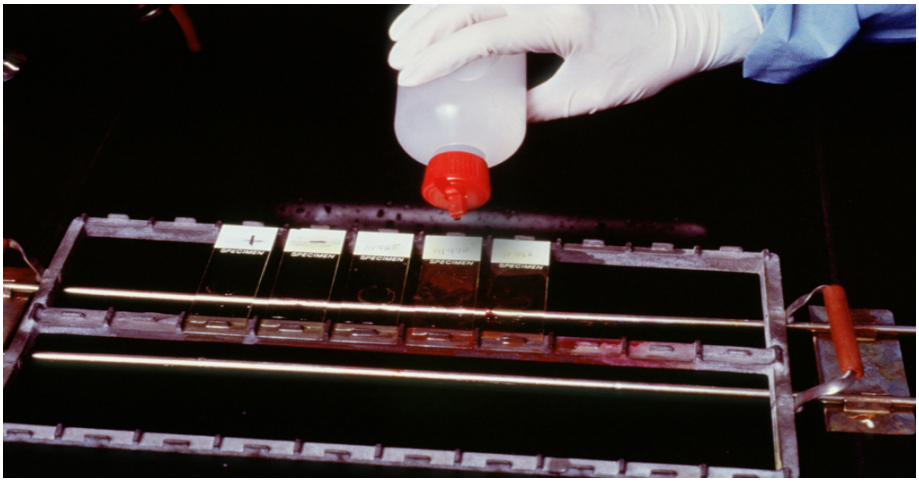
Wash well with running water, taking care to control the flow of water so as to prevent washing away the smear



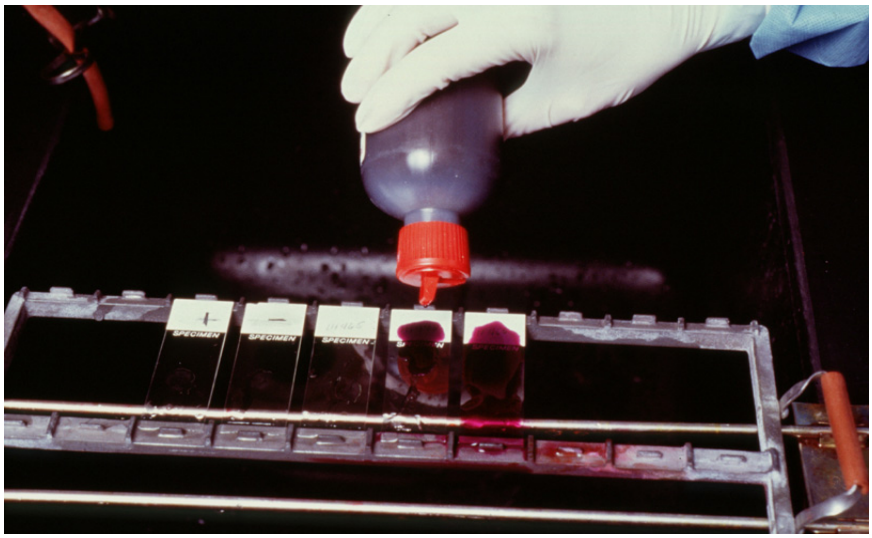


Decolorize by covering completely with acid-alcohol for 2 minutes, twice

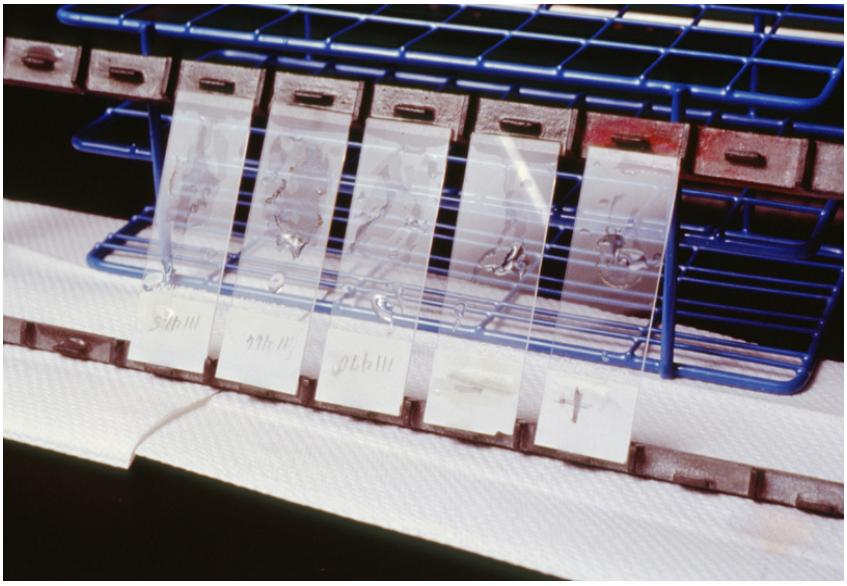
Wash well with running water, as before to wash away the acid alcohol



Counterstain with 0.1% potassium permanganate for 30 seconds



Wash as before with water and slope the slides to air dry



Precautions

- Avoid under-decolorisation with acid-alcohol. Organisms that are truly acid-fast are difficult to over-decolorize since the decolorisation procedure with acid-alcohol is relatively milder than the 25% sulphuric acid used in Z-N staining procedure.
- Avoid making thick smears. This will interfere with proper decolorisation, and counterstain may mask the presence of AFB. Additionally, thick smears have a tendency to flake, resulting in loss of smear material and possible transfer of material to other slides.
- Strong counterstain may mask the presence of AFB.
- Smears that have been examined by FM may be **restained** by Z-N staining to confirm observations. To restain the same smear for Z-N, treat with 5% oxalic acid for 2 min, wash and proceed for Z-N. However, once smears have been stained by Z-N staining, they cannot be used for FM.
- Fluorescent stained smear are to be read within 24 hours of staining because of fading.

- Stained smears have a tendency to fade on exposure to light. The slides are to be stored in the slide box to avoid exposure to light. Alternatively, they may be stored wrapped in brown or black paper and kept away from light.

EXAMINATION PROCEDURE AND REPORTING OF RESULTS

Switch on the mercury vapor lamp. The bulb takes approximately 10 minutes to reach full intensity. Using the low power objective (magnification 100-150x) first examine a known positive slide to ensure that the microscope is correctly set up.

With auramine staining, the bacilli appear as slender bright yellow fluorescent rods, standing out clearly against a dark background. Rule out any artifacts. Grade positive smears into four degrees of positivity using the 20x, 25x objective along with 10x eyepiece (table 1). Smear needs to be observed in “linear pattern”. For a trained and experienced LT, each smear would take approximately a minimum of 2 minutes for 100 fields or three horizontal sweeps. In the fluorescent staining, smears are examined at much lower magnifications (typically 250x) than used for ZN-stained smears (1000x). Each field examined under fluorescence microscopy, therefore, has a larger area than that seen with bright field microscopy. Thus, a report based on a fluorochrome-stained smear examined at 250x may contain much larger numbers of bacilli than a similar report from the same specimen stained with carbolfuchsin and examined at 1000x. For the purpose of uniformity for examination and quantitative reporting of results, a method has been suggested (Reference 1. WHO Manual on Microscopy Part II) whereby the number of acid-fast bacilli observed under fluorochrome staining could be divided by a “magnification correction factor” to yield an approximate number that might be observed if the same smear were examined under 1000x after carbol fuchsin stain. To adjust for altered magnification of fluorescent microscope, when using objectives of x20 or x25 powers, divide the number of organisms seen under FM by the factor of 10. Similarly, if one using a 40 x objective the magnification correction factor is 5, and if one using a 45 x objective it is 4. The magnification correction factors for different FM objectives are given in table 2.

Example: Suppose 50 acid-fast bacilli are observed per field after 50 fields using the 250x magnification. If this number is divided by the magnification correction factor of 10, the comparable number of bacilli that would have been observed under 1000x is 5 per field. The laboratory result should therefore read 2+ Positive.

Table 1: Comparative grading

RNTCP ZN staining grading (using 100x oil immersion objective and 10x eye piece)	**Auramine O fluorescent staining grading (using 20 or 25x objective and 10x eye piece)**	**Reporting /Grading**
>10 AFB/field after examination of 20 fields	>100 AFB/field after examination of 20 fields	Positive, 3+
1-10 AFB/field after examination of 50 fields	11-100 AFB/field after examination of 50 fields	Positive, 2+
10-99 AFB/100 field	1-10 AFB/ field after examination of 100 fields	Positive, 1+
1-9 AFB/100 field	1-3 AFB/100 fields	doubtful positive /repeat
No AFB per 100 fields	No AFB per 100 fields	Negative

Table 2: Magnification correction factor

S.No.	**FM objective magnification (power)**	**Magnification correction factor***
1	20x	10
2	25x	10
3	40x	5
4	45x	4
5	63x	2

* To obtain the comparative grading, divide the observed count of AFB under the FM objective with this factor before grading.

Internal Quality Control Procedures

i. Quality control for staining

- All containers of stains and reagents should show the date received and the date first opened.

- Any material found to be unsatisfactory, for example scratched slides, poor quality of reagents, etc., should be recorded as such and removed from the laboratory immediately.
- Date of preparation and name of the reagent should be labeled on the bottle.
- Whenever any staining reagent is freshly prepared, the quality of the reagent should be tested using panel slides.
- The panel slides should consist of at least one 3+ smear, one 2+ smear, one 1+ smear and 2 negative smears. (These panel slides will be provided by the NRL)
- These results should be recorded and if the results of the panel slides are satisfactory to good, the reagents can be used.

Quality control for smear

- All positive smears should be checked by a senior member(microbiologist) of a laboratory staff.
- A senior lab staff should also check 10% of the negative smears selected by systematic random checking. (i.e only the first slide is selected at random and subsequently every fourth slide is selected)
- Checked smear results should be indicated by putting a tick mark (using red pen) against the lab number of the examined smear in the smear result book.
- Re-stain any doubtful smears by ZN method and examine to confirm morphology. This restaining is done only when the smears are rechecked after 48-72 hours of primary reading of smears.
- If the result is to be amended, correct this in the smear result book and enter in the smear amendment register within 48 hours from the preparation of the smear.

MICROSCOPE

The microscope is a precision instrument and requires careful maintenance from both the optical and mechanical points of view. Laboratory workers should be familiar with the general mechanical and optical principles. Most manufacturers publish manuals

containing useful explanations and information. The following are certain basic requisites in the maintenance of microscopes.

- When not in use, the microscope should be kept in its case or protected from dust by a plastic cover.
- Avoid exposing the microscope to moisture. Humidity may allow fungi to grow on the lenses and may cause rusting of the metal components. To limit exposure to moisture, place a shallow plate containing dry blue silica gel in the microscope case whenever the microscope is stored. When silica gel is unable to absorb more moisture it changes color (from blue to pink). In such situations the silica gel must be replaced or dehydrated in a hot air oven and re-used when its original (blue) color reappears.
- Avoid keeping the microscope in a place where there are chemical reagents, water or discharge of corrosive gases.
- The microscope should be picked up or carried with hands, one grasping the arm firmly and the other under the base for added support. **Never carry a microscope with only one hand.**
- Install the microscope on a sturdy, level surface. Do not place the equipment near instruments generating vibrations (e.g. centrifuges) on the same table.
- If the microscope is to be used every day, do not remove it from the site of installation, but when not in use keep it covered with a polythene or plastic cover.
- Microscope lenses may be scratched by dirt or grit. The lenses should be cleaned only with clean, dry lens tissue. Cloth or other tissues should not be used as they may scratch the lenses. Never use soap, alcohol, or other solvents to clean the lenses.
- The microscope should never be dismantled; if faulty it should be entrusted to a competent person to repair i.e. a Company service person.
- **Mercury Lamp Precautions:**
    - The lamp emits strong UV and visible radiation. Do not look directly into the source or disassemble the lamp housing.
    - Keeping track of mercury lamp usage is vital. Make sure to record the used time and the total hours accumulated usage everyday.

- Mercury lamp lifetime is rated at 100 hr. When the end of lamp's service life is near, flickering is likely to increase. If used beyond that point, risk of explosion and mercury contamination of the room sharply increases. Be very careful while turning the lamp on if the lamp reached its expected lifetime!
- Frequent switching ON/OFF shortens the mercury lamp's life considerably. It is better to leave it on if the next user is going to need it within 1-2 hours.
- After turned on, it takes about 15 min for the lamp to reach full brightness.
- Lamp must be ON for at least 30 min before it can be switched OFF.
- After the lamp has been switched OFF, it must cool down (at least 30 min) before it may be switched ON again.

- When switching between objectives, hold on the collar of the nosepiece; NEVER turn the nosepiece turret by grabbing the objectives. The objectives are precision-mounted and this force could disturb their alignment.
- A few seconds spent properly adjusting the condenser will greatly improve the resolving power of the microscope.

References

1. Laboratory services in tuberculosis control: Part II: Microscopy. WHO/TB/98.258 (1998)
2. SOPs adopted at NTI and TRC for fluorescence microscopy.