Photography

- **Aim:** complete & faithful reproduction of image detail.
- For EM – During photography & development process we want to maximize negative density, enhance contrast, & reduce noise.
- Photographic emulsions respond to electrons & light in a different manner
  - -electrons = single-hit process
  - -photons = multiple-hit process.

Kodak EM Film 4489

- Polyester base (ESTAR™).
- Microfine Grain.
- Blue Sensitive Emulsion.
- Most widely used film for EM

Kodak Electron Image Film SO-163

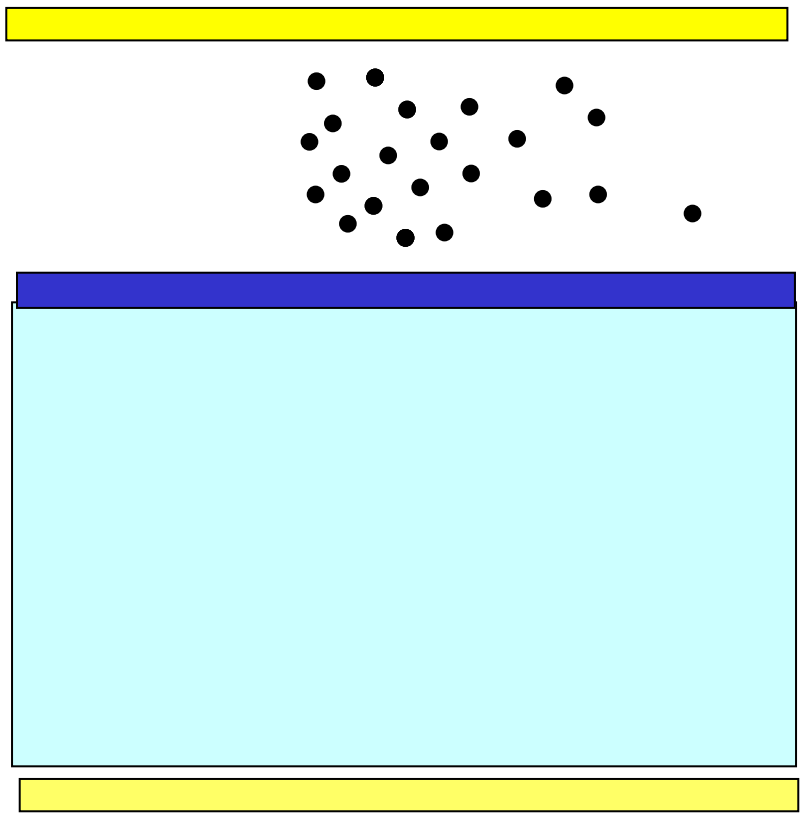
- Fine grain, blue sensitive emulsion.
- Polyester base (ESTAR™).
- Twice as fast as EM film 4489.
- Designed so that you can change the speed & granularity of the film by changing the exposure & processing conditions

Agfa Scientia 23D56 film

- Similar to SO-163 in exposure & development range

The photographic process

- Photographic EM emulsions ~ 10 & 30 μm thick.
- **Emulsion** = suspension of Ag halide crystals (i.e. Ag bromide) in gelatin**.**
- Mean crystal size varies from ~ 0.05 - 2 μm. Emulsion has clear gelatin overcoat -protects Ag grains against abrasion (abrasion can render them developable without exposure).
- Film has a gelatin coating on layer opposite the emulsion - prevents curling during processing & drying



Hard gelatin-protective coat

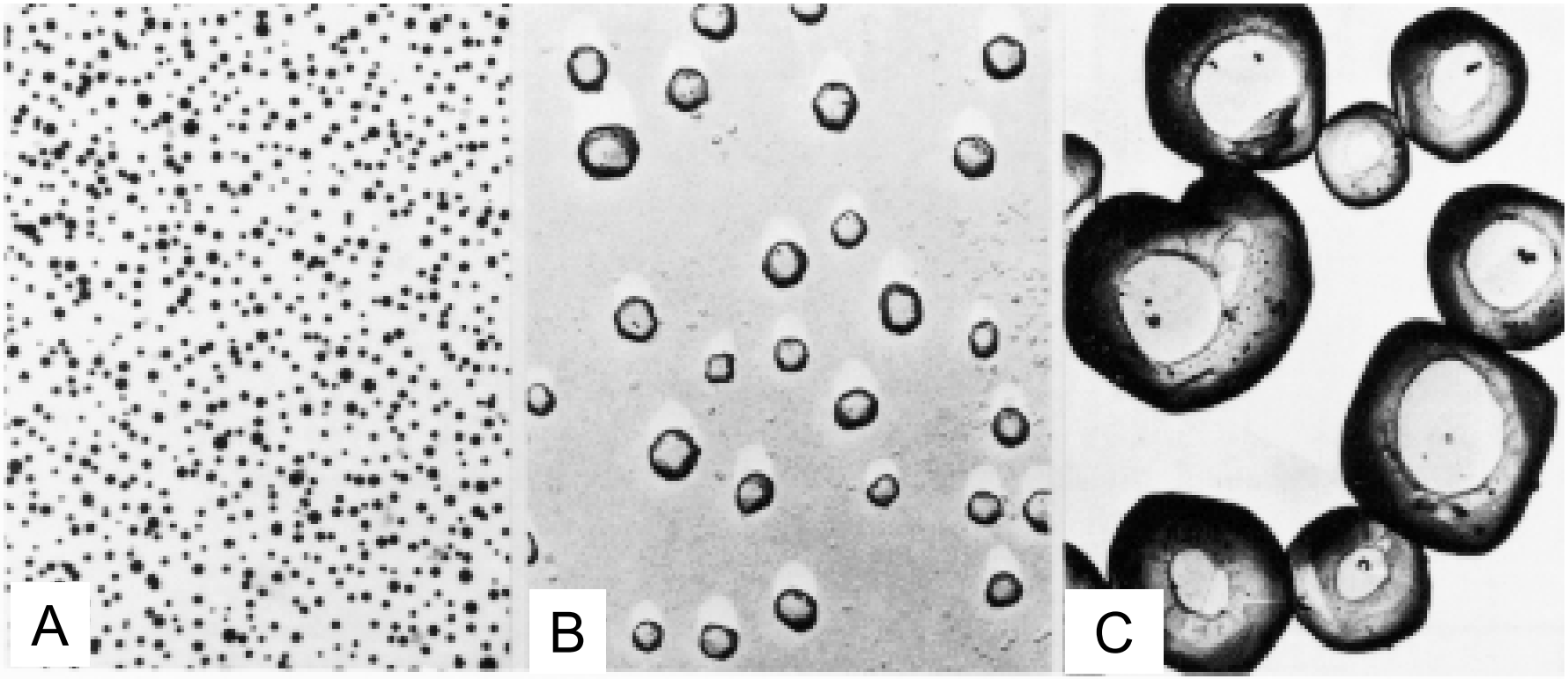
Ag halide crystals

Substrate-adhesive

Emulsion support

Anti-curl layer

Structure of the photographic material.



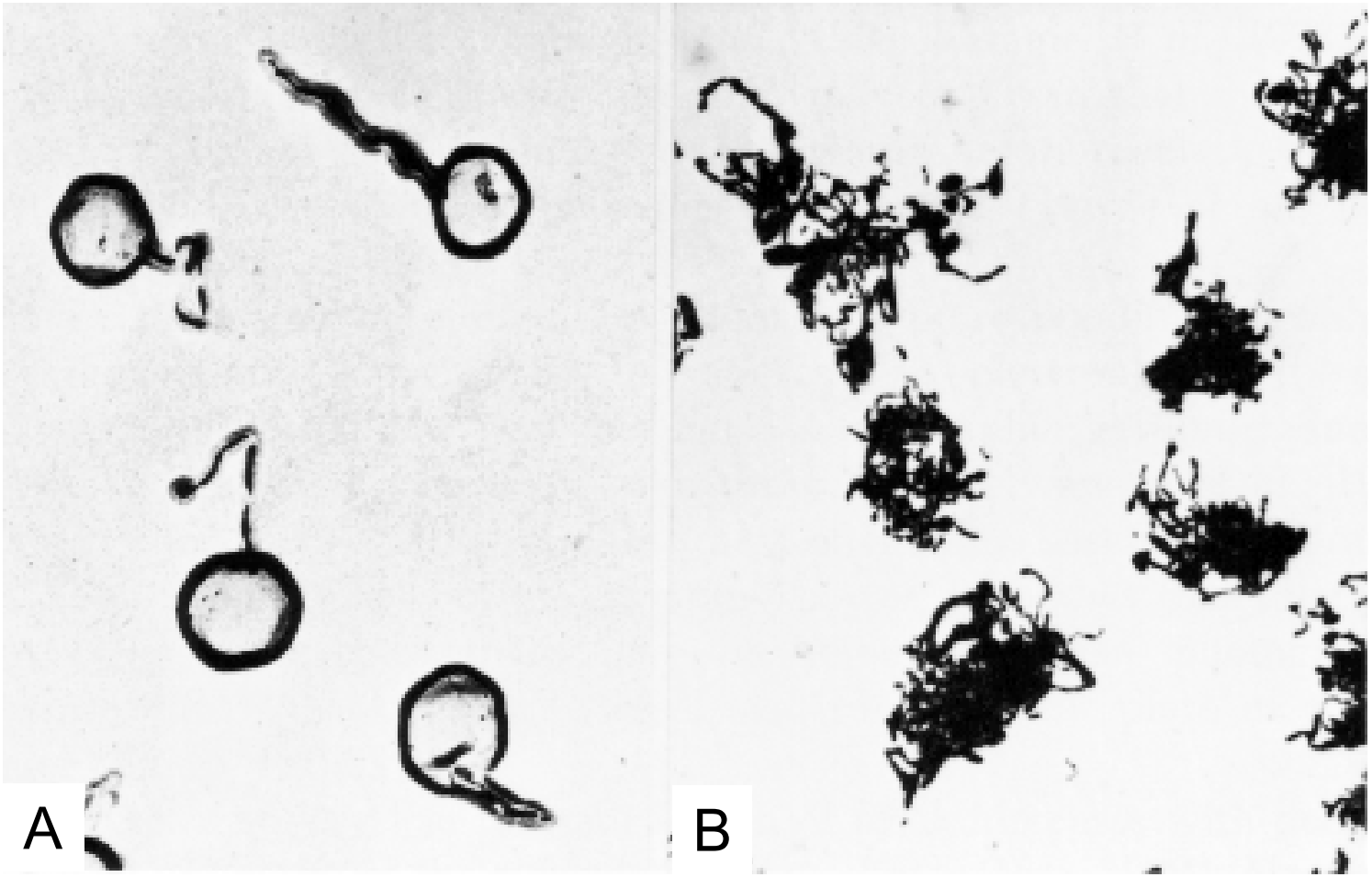
Electron micrographs of emulsions

(A) High resolution emulsion
(B) Emulsion of medium grain size - type commonly used in EM.
(C) X-ray emulsion of high sensitivity. The micrographs shown at the middle & right are of Au/Pd shadowed carbon replicas.

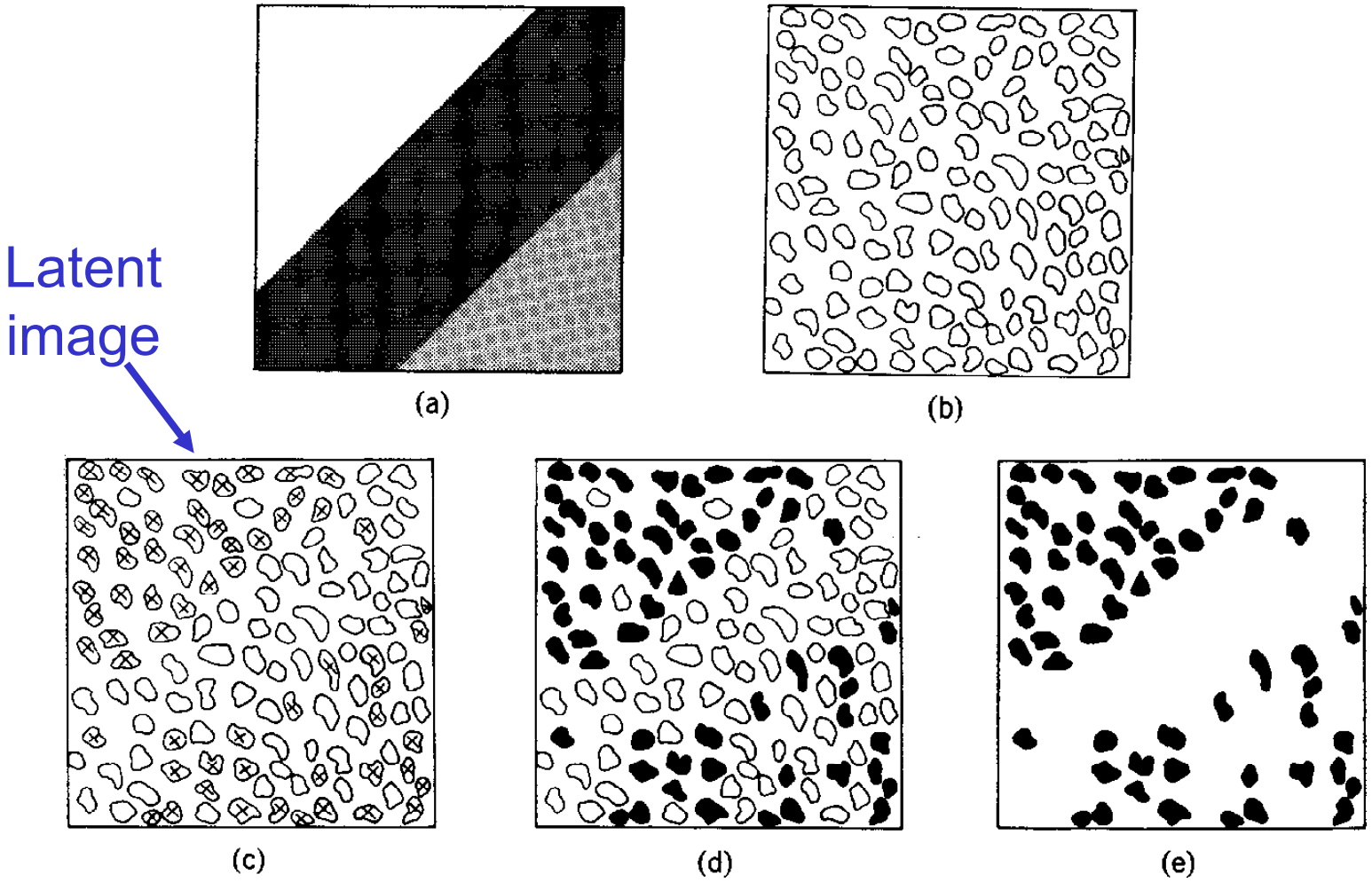
From Farnell and Flint, p.20

- If emulsions exposed to photons with an energy of > 2.5eV (blue or shorter λ) or to moving charged particles, Ag atom specks form in the crystal.
- Photons - required energy is ~ 30eV (10 photons)
  -with charged particles such as e^- ~ 500eV needed.
- E^- beams in TEM (100kV) sufficiently ionizing - a single e^- through a Ag halide crystal transfers more than enough energy.

- Ag specks formed in irradiated crystals = latent image.
- Emulsion developed by developer (chemical reducing agent (i.e. hydroquinone or p-methylaminophenol sulfate)
- Crystals with latent image more rapidly converted to metallic Ag than the others.
- As little as 3 Ag atoms in latent image sufficient to cause development of the Ag halide crystal into larger, filamentous mass of metallic silver, called **silver grains**.



Electron micrographs: (A) exposed grains at early stage of reduction by developer (B) grains of the same emulsion fully reduced to tangled masses of silver filaments. Magnification ~28,000X. From Farnell and Flint, p.21



Stages in photographic processing: (a) object; (b) unexposed emulsion; (c) exposed but unprocessed emulsion; (d) developed but unfixed emulsion; (e) final image after fixation. From Slayter, p.462

- Emulsions for normal photography have dyes added - extend sensitivity to include **red light** (panchromatic emulsions). Developed in total darkness.
- Non-panchromatic (orthochromatic) emulsions used for TEM– can use red / yellow safelight After development film is **fixed** (sodium thiosulfate)- dissolve unexposed Ag halide crystals & harden gelatin matrix.
- Emulsion washed to remove fixative & avoid discoloration & eventual disappearance of the image.

Quality of EM negative

- A combination of **emulsion type** coupled with the **exposure time**, the **developer type** & **development time,** as well as the **accelerating voltage** work together to influence the quality of the final EM negative.
  - -Maximize density
  - -Enhance contrast
  - -Reduce noise

Optical density (OD)- processed emulsion

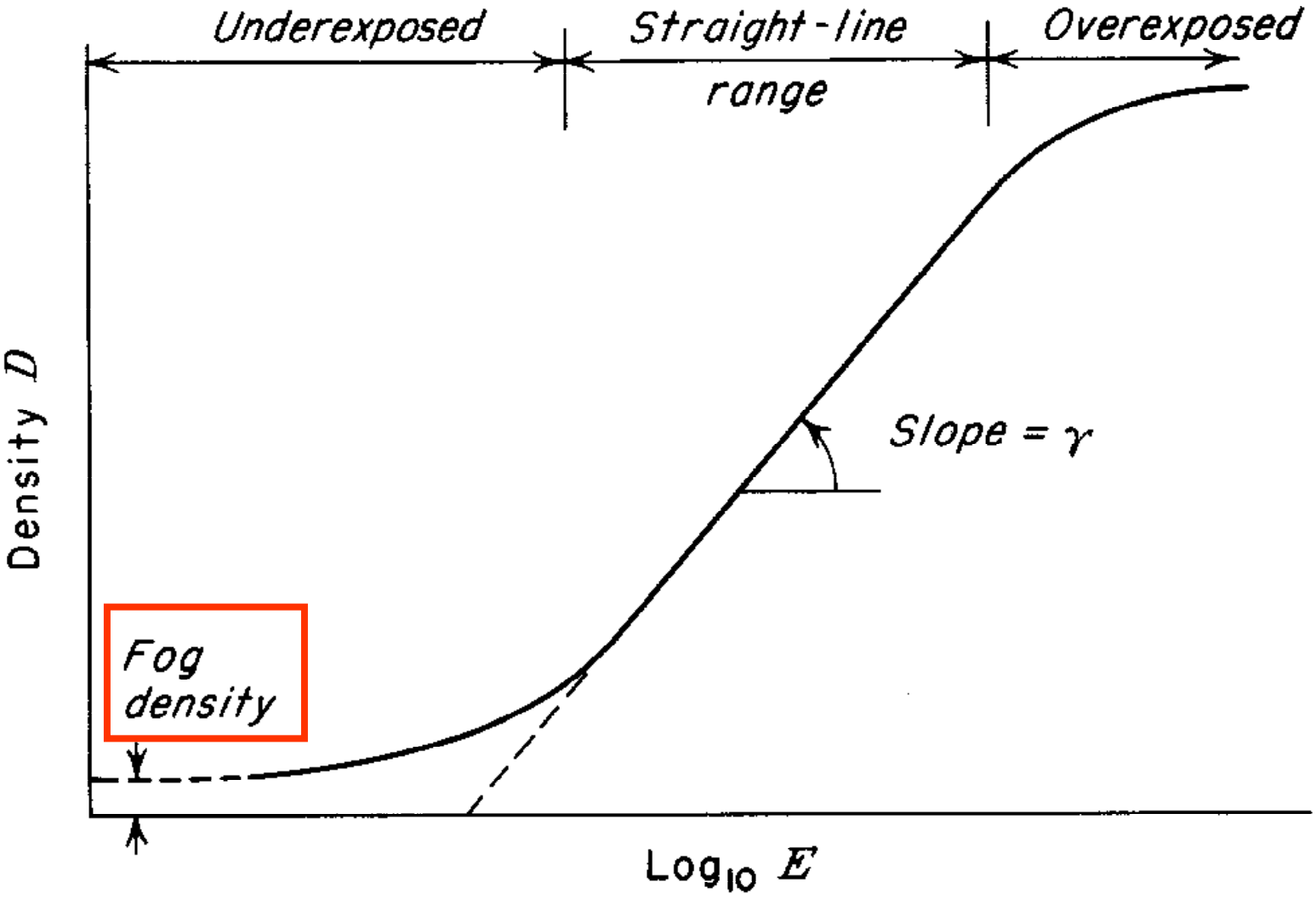
- Emulsion response expressed by **optical density** defined as $D = \log_{10}(I_o/T)$
  I_o = intensity of incident radiation & T = intensity of transmitted radiation.
- *OD = quantitative measure* -blackening of the photographic emulsion.
- If negative transmits 10% of the incident light $D = 1.0$. For $T = 0.01$ (1% transmitted), $D = 2.0$.
- Density of an emulsion exposed to e^- directly proportional to the exposure up to a D of ~1.0

Density related to exposure

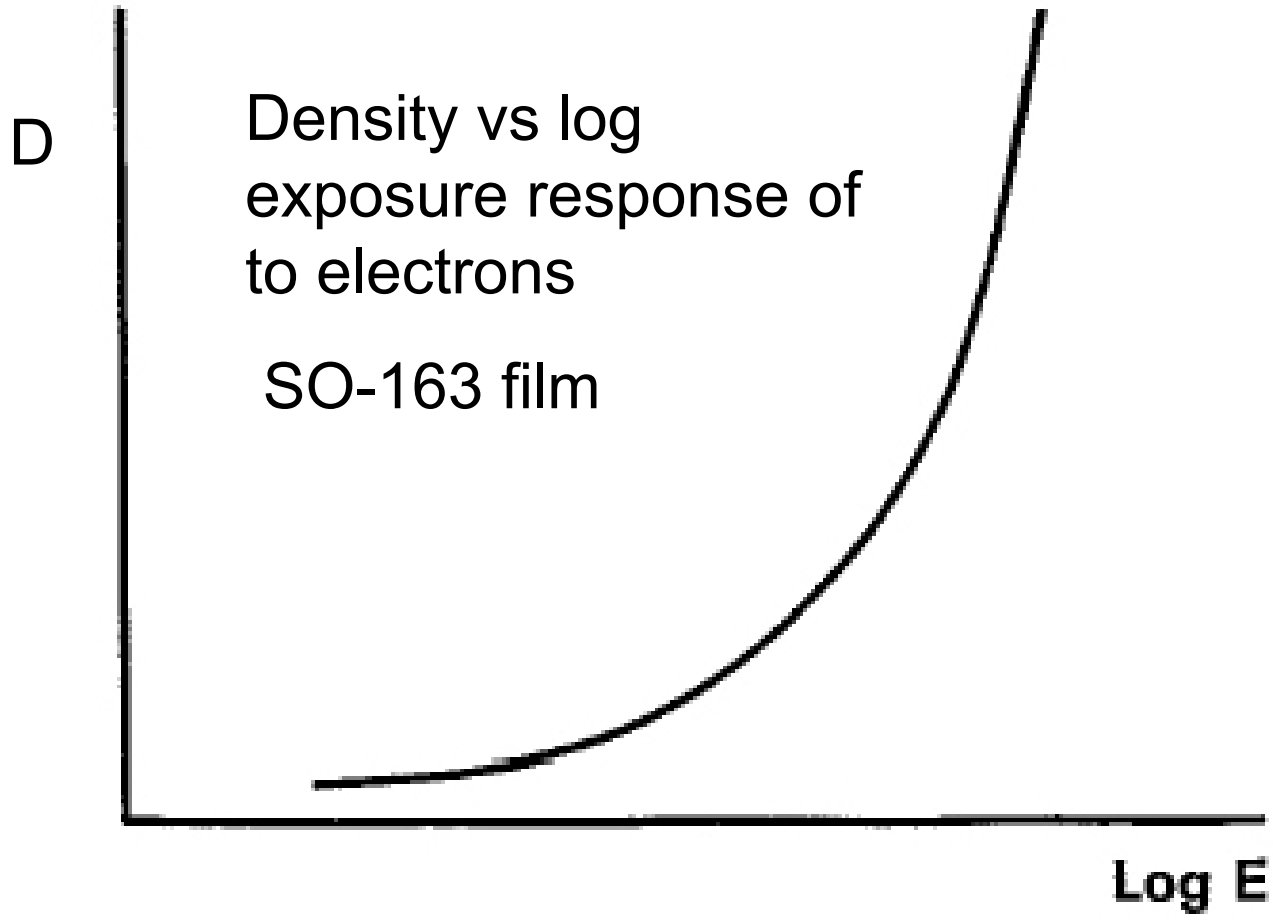
- Exposure is the quantity of electricity per unit area in Coulombs/cm2.
- Exposure to e^- is a single-hit process ∴ virtually every halide crystal hit by an e^- is rendered developable.
- Subsequent hits of the same crystal by other e^- irrelevant. A single e^- normally hits more than one crystal in its passage through the emulsion (may hit up to 10 different silver halide crystals)

Density/Exposure curves

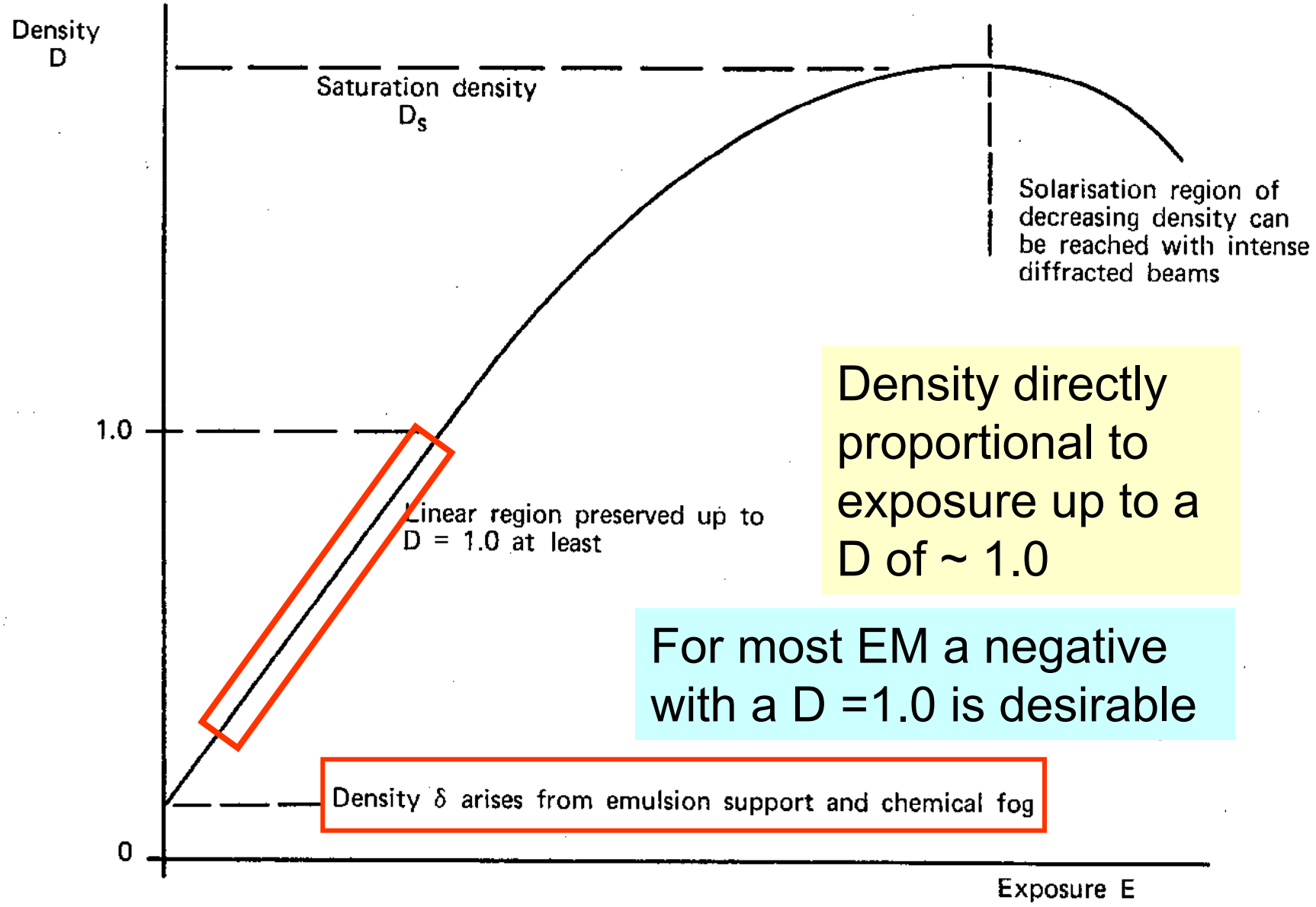
- Relationship between photographic emulsion optical density & exposure to light demonstrated in the **H and D curve** (after Hurter and Driffield, 1890).
- D is plotted against $\log_{10}E$. For photons curve = sigmoidal with 3 regions.
- The **slope**, γ (**gamma**), in the straight-line portion (working region of the emulsion) is a measure of the contrast of the emulsion.



Idealized HD characteristic for photographic film exposed to visible light (sigmoid curve). The exposure E = intensity x exposure time. From Hall, p.172



For emulsions exposed to electrons the response is exponential. Density increases with exposure.



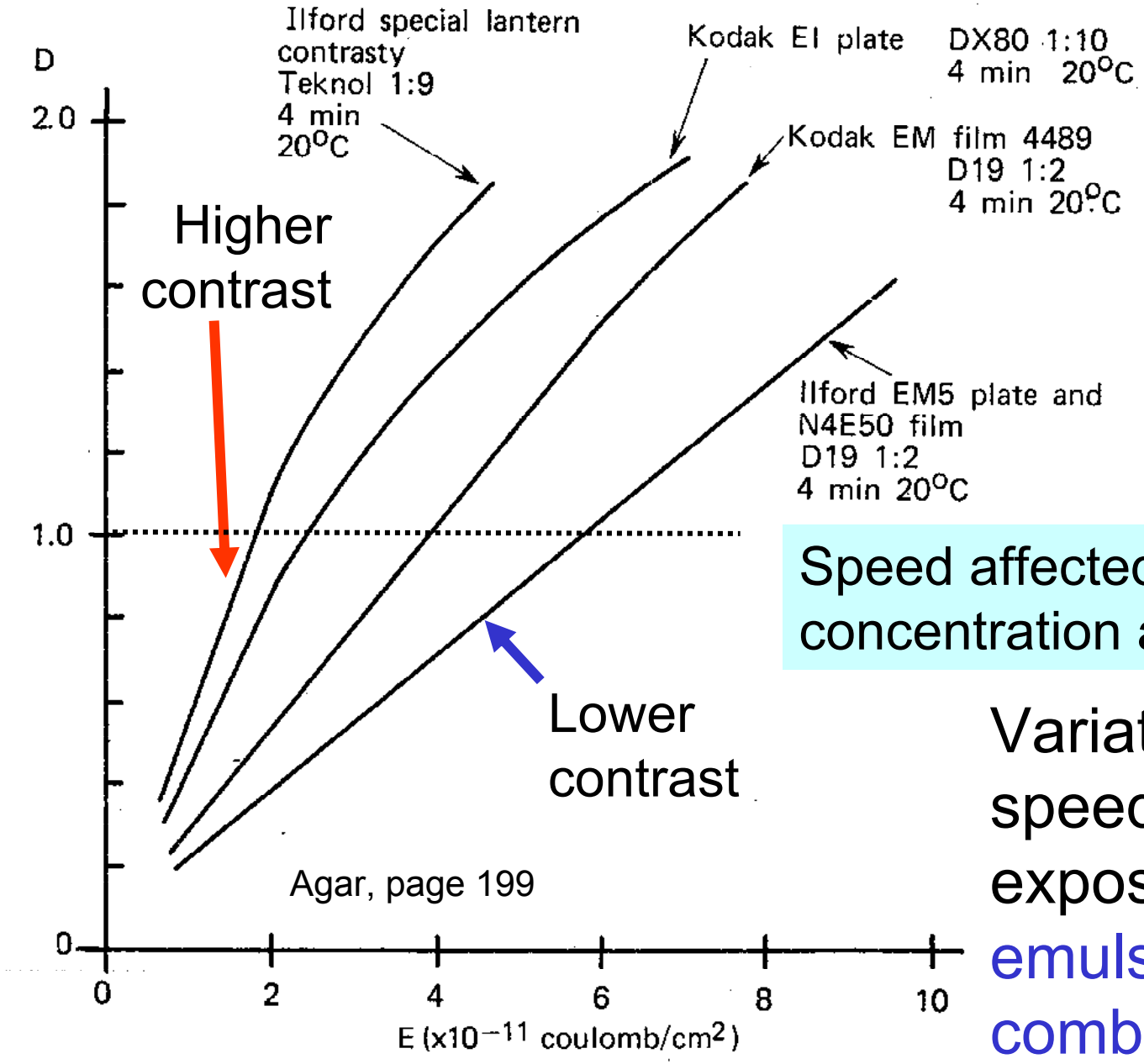
Useful to display density/exposure relationship for electron image recording with linear plot. (From Agar, p.197)

Contrast

- **Contrast** =difference in density for a given ratio of intensities in the image. Equal to the slope of the *D* vs log*E* curve (gamma (γ) = $\Delta D/\Delta \log E$).
- For e^-, contrast linearly related to density in the linear region of the *D* vs *E* curve
- Contrast does not go on rising indefinitely with *D* because at high densities the linear relationship fails.
- Generally - for high resolution EM a more contrasty, denser negative is required.

Speed of electron emulsion

- For e^-, there is linear relation between density & exposure over the useful working range of the emulsion.
- **Speed** = exposure required to produce a given density.
- **Speed** = reciprocal of the # of e^- required per unit area to produce a density above the fog level equal to 1.0.
- For low *E*, in the linear region of the *D* vs. *E* curve, speed is equal to the slope of the straight line.



Agar, page 199

Speed affected by **developer** concentration and formulation

Variation of speed/density with exposure for 4 emulsion + developer combinations.

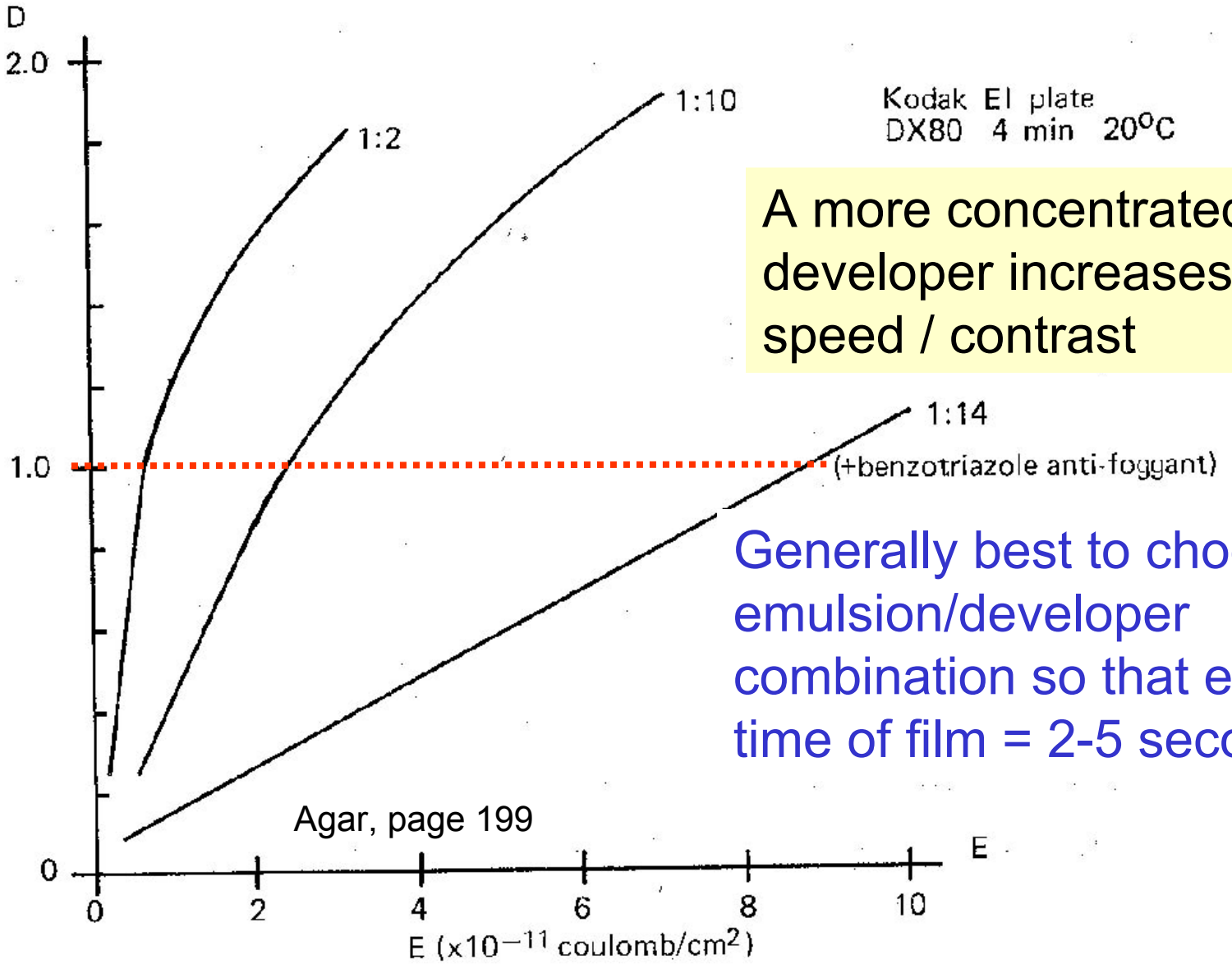
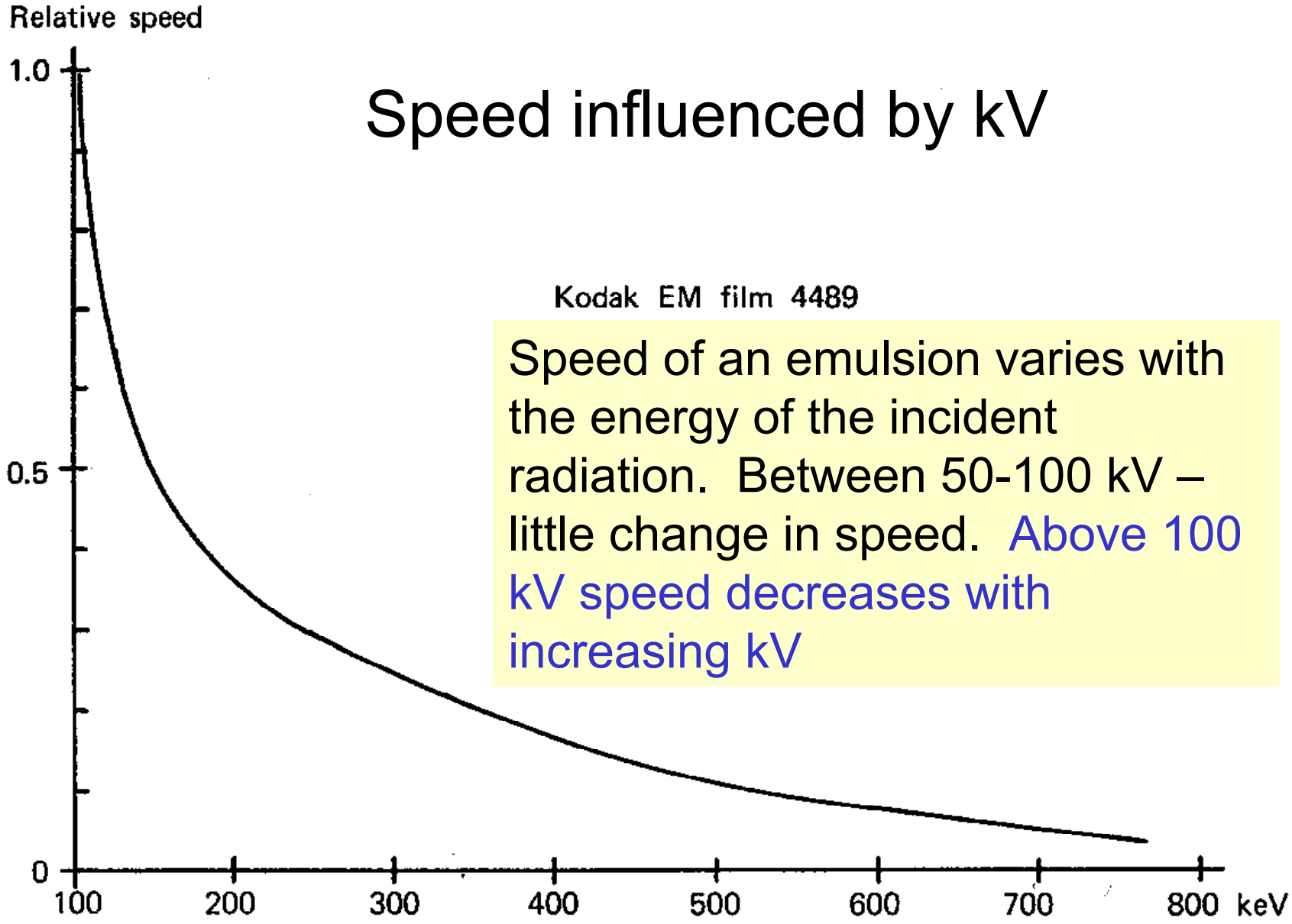


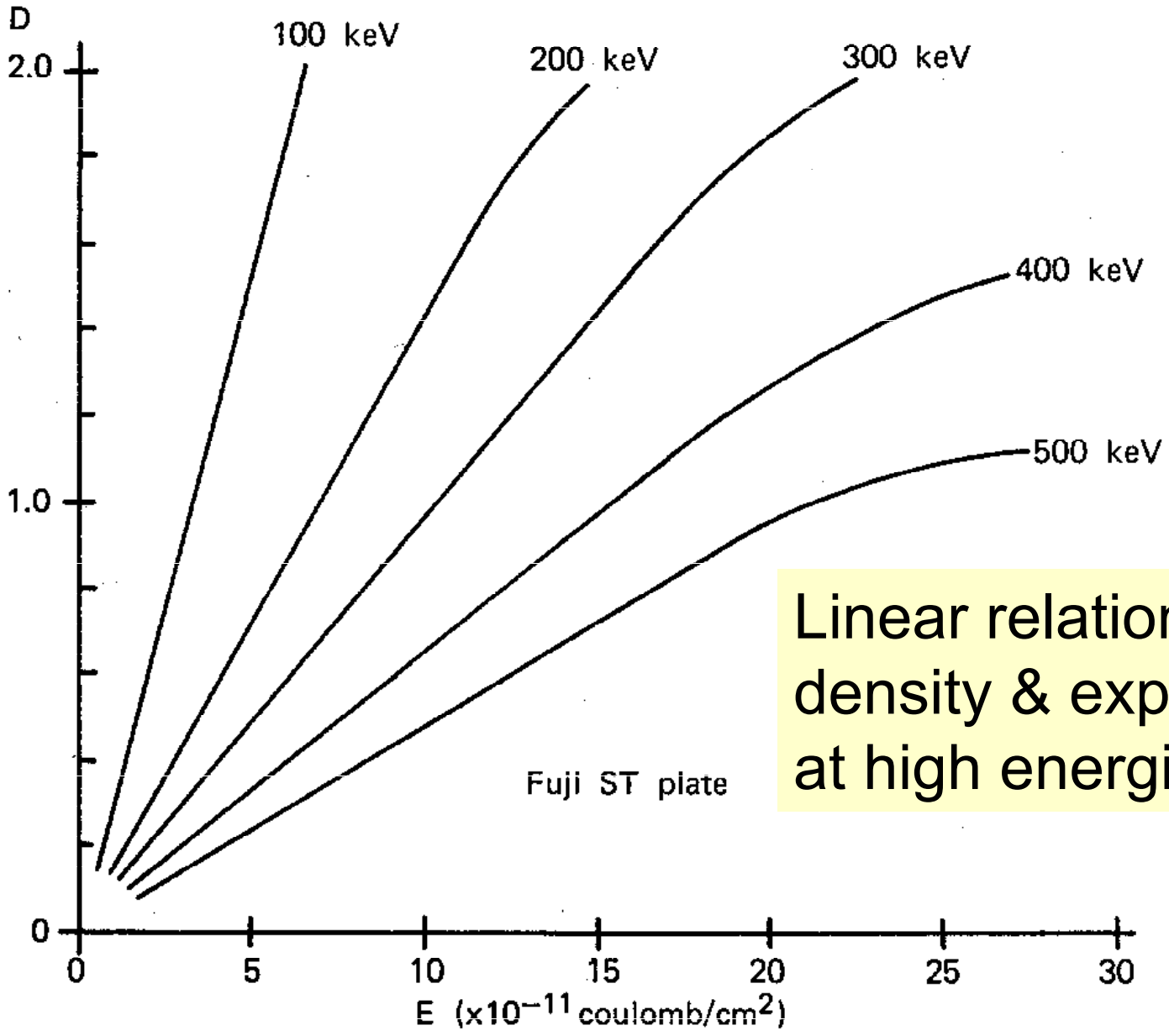
Fig. 7.5. Variation of speed with developer concentration.

Speed influenced by kV



Speed of an emulsion varies with the energy of the incident radiation. Between 50-100 kV – little change in speed. Above 100 kV speed decreases with increasing kV

Variation of relative speed with e^- energy in the range 100-800keV.



Linear relationship of density & exposure at high energies.

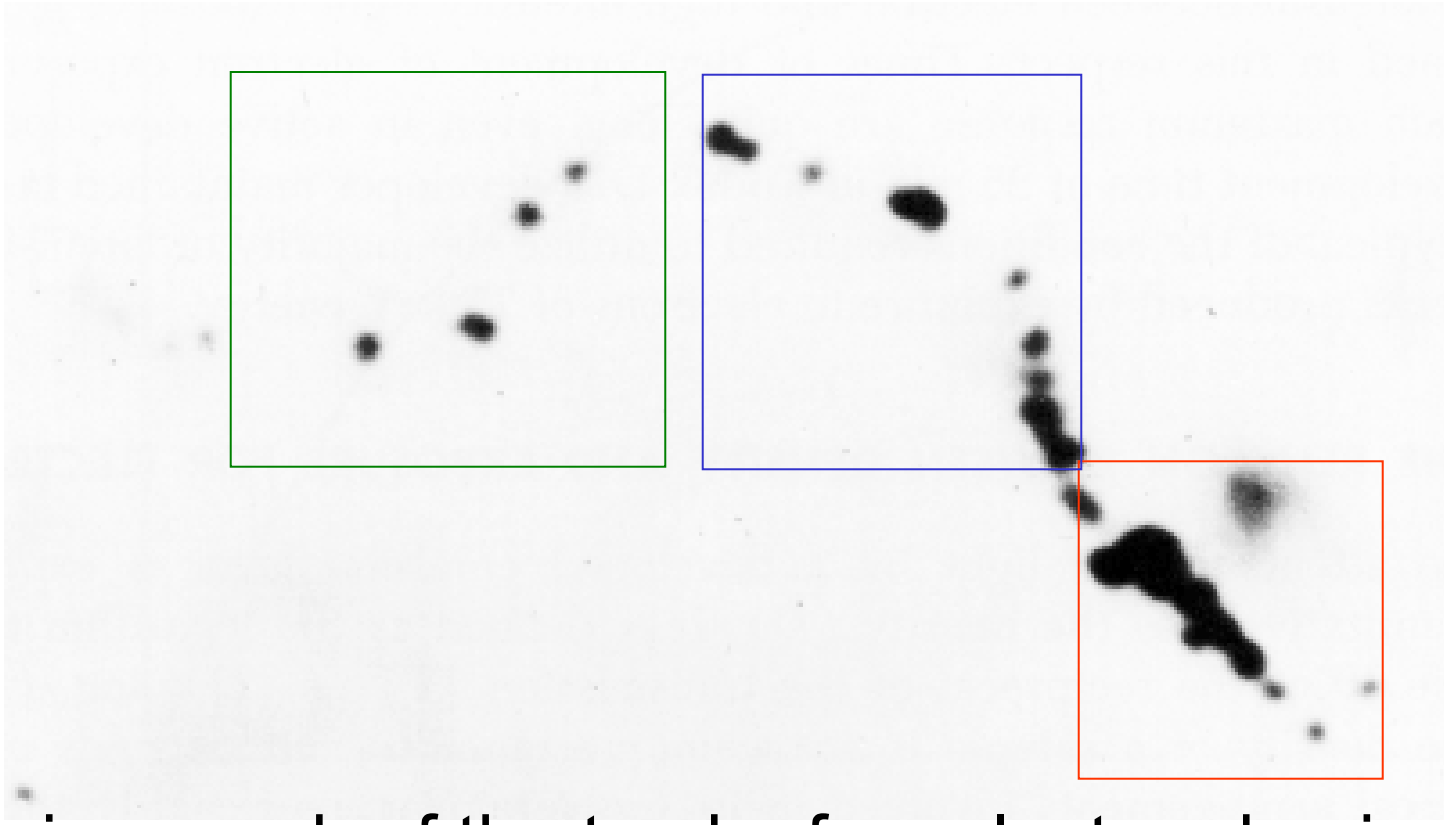
Reducing Noise - Graininess

- Graininess limits the amount of information recorded on the negative.
- **Graininess** - not a photographic emulsion defect. (excessive underfocus can appear "grainy")
- Graininess = statistical phenomenon.
- Granularity due to statistical fluctuation in the number of grains hit by each e^- is negligible – therefore granularity due mostly to electron noise.
- **Granularity (Graininess) due to random distribution of e^- "particles" in the beam** (electron noise - poor S/N ratio).

Number of grains / electron

- Each e^- may hit more than 1 Ag halide crystal.
- A medium-speed emulsion used for EM has ~ 10% halide by volume & the crystal diameter is ~ 0.3μm.
- ∴ theory predicts 1 grain per 2 μm, or, for a 20 μm thick emulsion, about 10 grains per electron track.
- Each e^- may pass through as many as 100 Ag halide grains, losing some energy in each. Energy loss per grain ⇑ along the path & becomes high at the end.

- Quantity of energy transmitted to each grain at the start of its track will be much less than towards the end.
- $\therefore$ the # of developed grains per unit length of track will be small at the start, but increase towards the end of the track.
- In almost any emulsion, a few grains near the path end are made developable. Whether or not those at the path beginning develop depends on development conditions.



Photomicrograph of the track of an electron having an initial energy of 75keV recorded in a nuclear track type emulsion layer. The dense aggregation of grain occurs towards the end of the track. Magnification ~4,500X.
(Farnell and Flint, p.23)

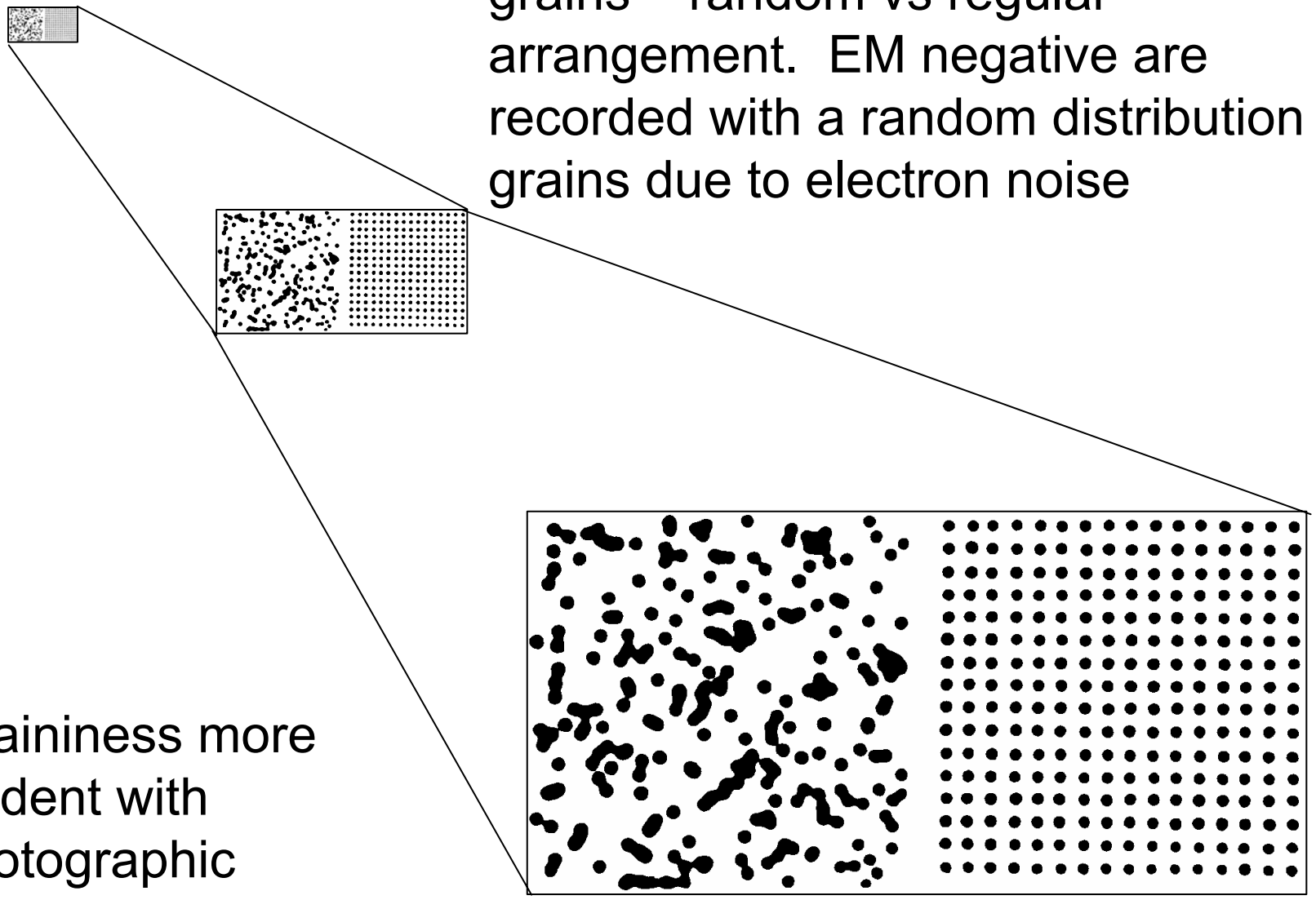
- Graininess = product of 2 random processes.
- Random arrival of quanta or e-.

  - Over a large area radiation is uniform,

  - Over small regions radiation distribution is not uniform
- Random fluctuation in irradiation = fluctuation in negative density & the impression of graininess.
- Random fluctuations = **electron noise**.

Agar page 201

Images below have the same # of grains – random vs regular arrangement. EM negative are recorded with a random distribution of grains due to electron noise



Graininess more evident with photographic enlargement

Solutions to graininess

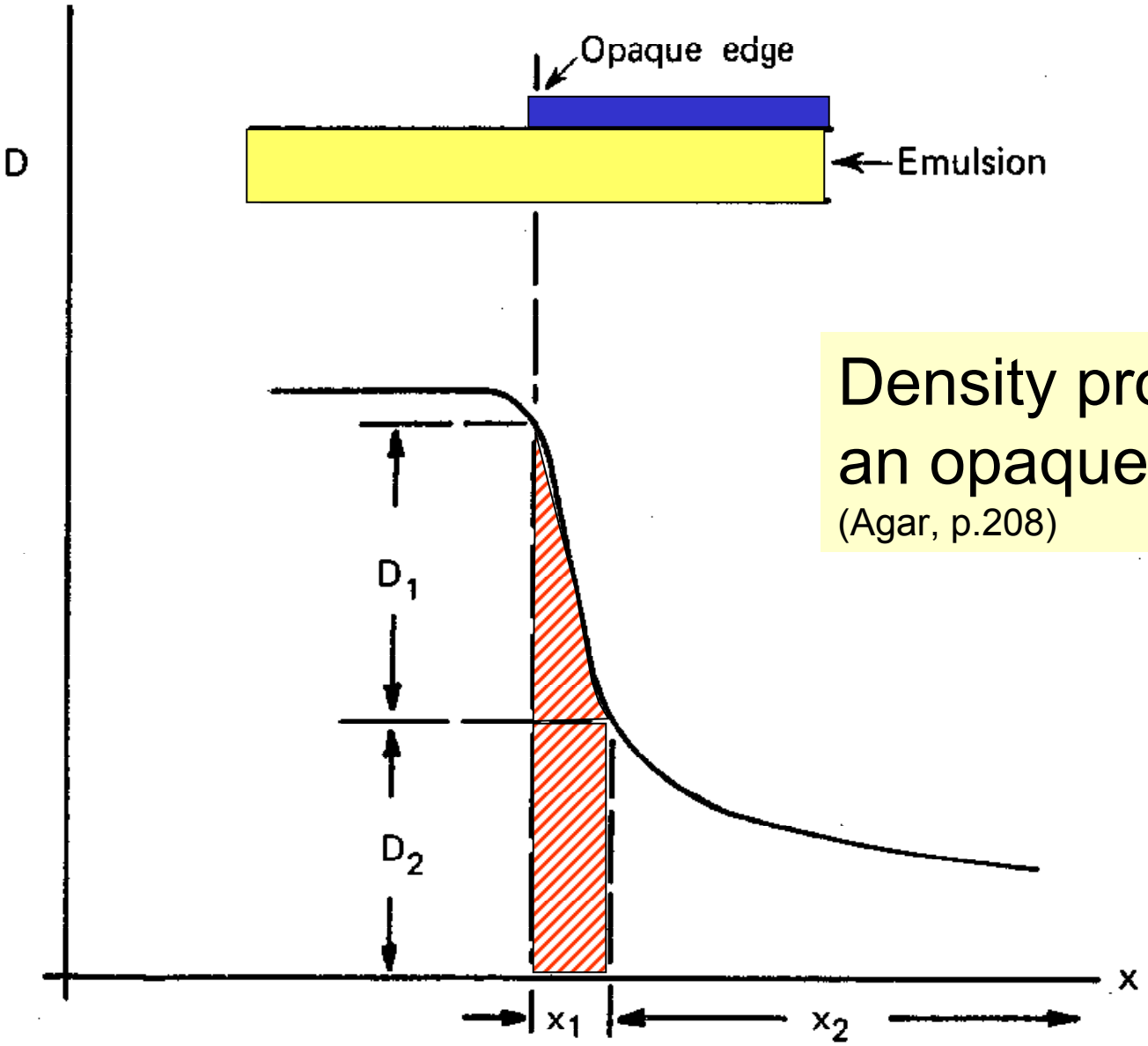
- **To increase *S*/*N*, exposure must be ↑.** Use more e^- to record image (preferred)
- Use image processing to add images together, - effectively increases the # of e^- per image.
- Development techniques = small effect on e- noise but can ⇓ grainy appearance.
  - Develop emulsion longer (or use a more concentrated developer).
  - Use slower emulsion developed longer to give the same *D*.
  - Use slower developer for a longer period of time.

Resolution - Image spread

- Emulsions have limited resolution. E^- track through emulsion is not a straight line perpendicular to the surface
- E^- scattered from incident direction by interaction with halide grains.
- E^- path includes sideways scatter in the emulsion $\therefore$ image points are spread out & contrast is reduced = **electron diffusion**.

Resolution - Image spread

- The magnitude of the image spread depends on the kV.
- Spread small for low voltage e^- due to limited track length. Larger for medium energy e^- more grains hit in the non-linear path
- At higher voltage, spread becomes small again - less scattering of the e-, & fewer grains are hit.
- Image spread will ⇑ at all voltages as emulsion thickness increases.

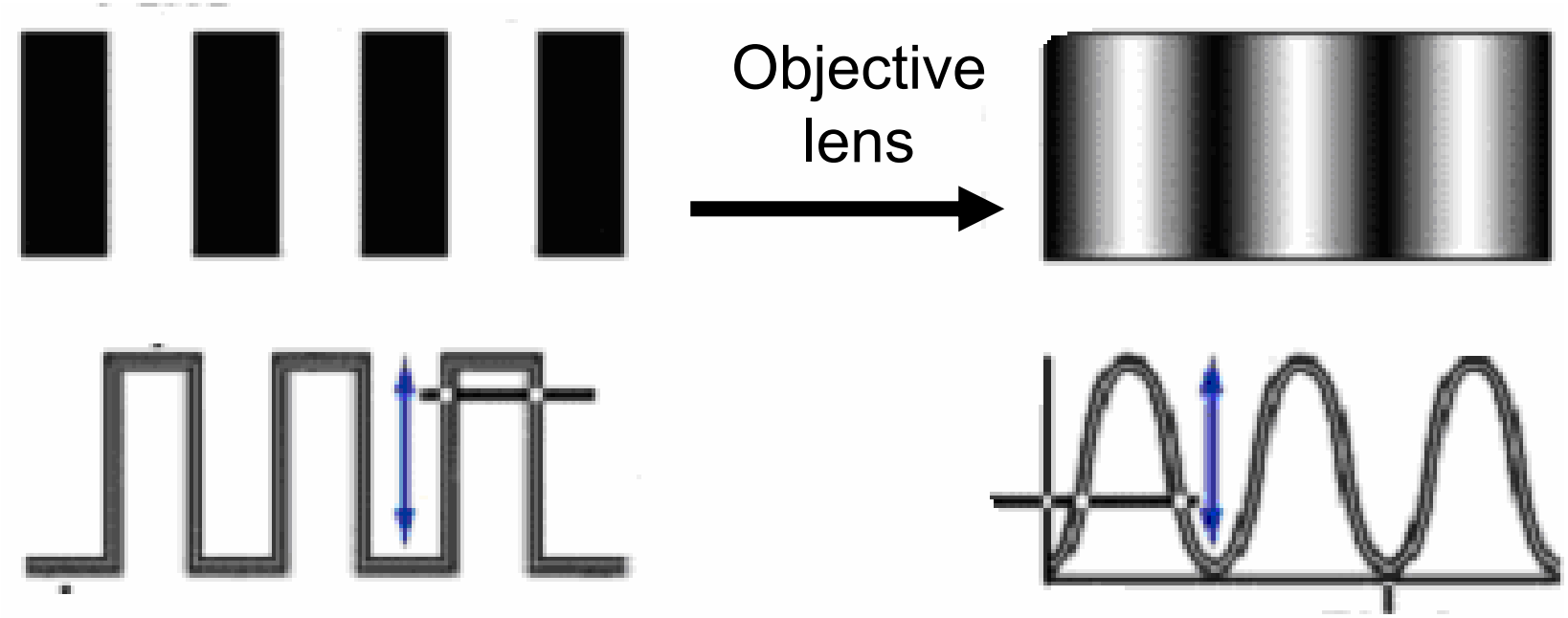


Density profile at an opaque edge.
(Agar, p.208)

Recording of image to film

- Not all the information in the image is recorded on the film. Therefore, the resolution on the film will be less that what is contained in the image plane of the lens.
- The recording of information on film is based upon a **contrast transfer function (CTF)**
- Important factors affecting the CTF:
  -***defocus*** - deviation in objective lens focus from the Gaussian image plane
  -***spherical aberration coefficient*** of the objective lens at a specific kV.

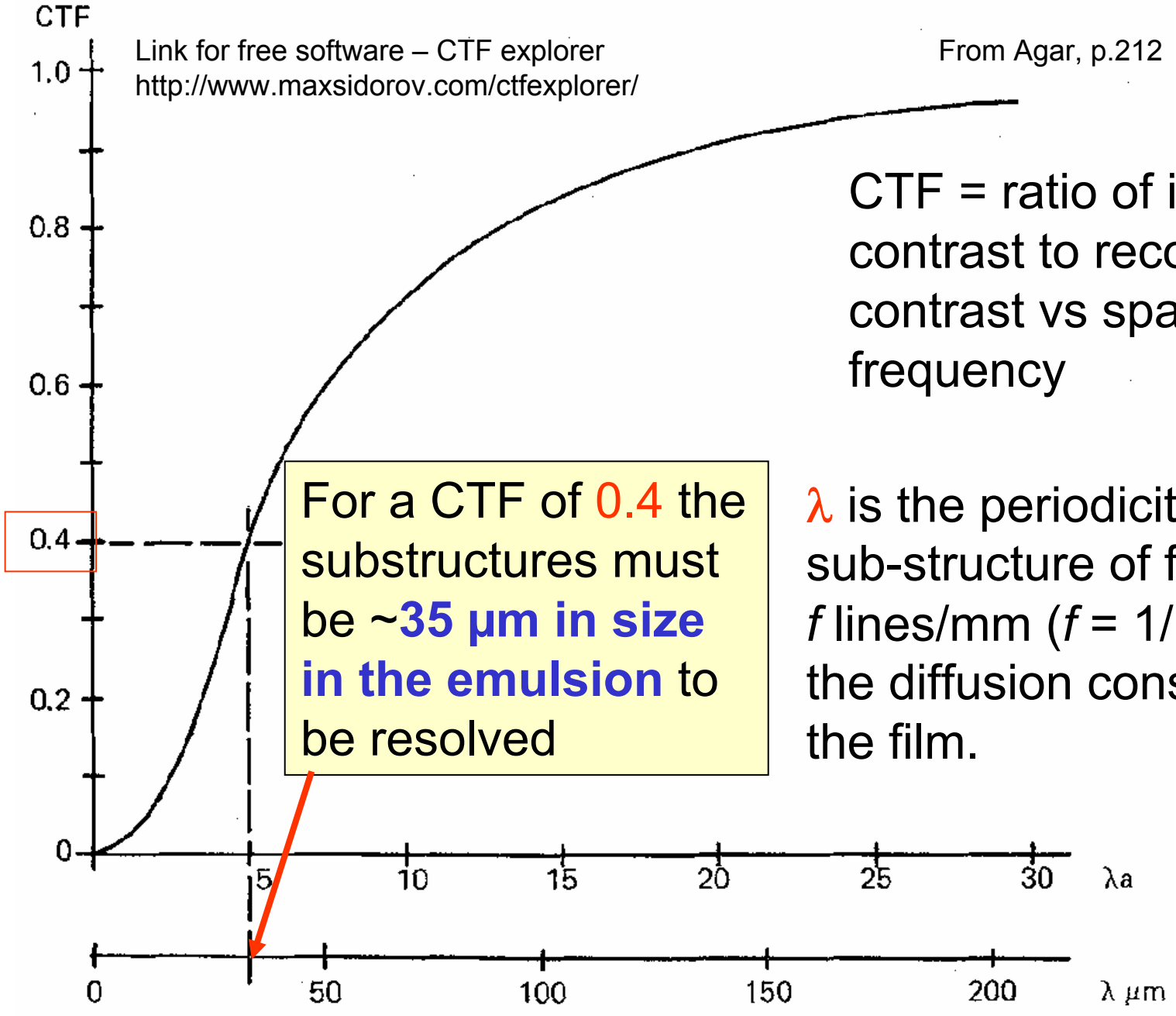
Light microscope lenses also have a CTF analogous to the CTF in electron microscopes. The CTF in a light microscope measures the microscope's ability to transfer contrast from the specimen to the intermediate image plane at a specific resolution



- Most specimens consist of variations in intensity rather than sharp-edges. Resolution limit best described in terms of a contrast transfer function (based on concept that a structure can be resolved into a spectrum of spatial frequencies)
- **Contrast transfer function (CTF)** of an emulsion defines the amount of contrast that can be recovered at given resolution limits.
- Perfect emulsion has a CTF = 1.0 at all resolutions.
- In practice, CTF of a typical electron emulsion below 0.5 (contrast = 1/2 of what is in the image.)

Link for free software – CTF explorer
http://www.maxsidorov.com/ctfexplorer/

From Agar, p.212



CTF = ratio of incident contrast to recorded contrast vs spatial frequency

For a CTF of 0.4 the substructures must be ~**35 μm in size in the emulsion** to be resolved

λ is the periodicity of a sub-structure of frequency *f* lines/mm (*f* = 1/ λ). ***a*** is the diffusion constant of the film.

4489 film-diffusion constant

Fig. 7.12. The contrast (or modulation) transfer function.

Use of the CTF

- If we know the spherical aberration coefficient & the amount of defocus & other scope imaging parameters then a series of image processing steps can be used to extract greater detail (resolution) from pure phase objects imaged by the electron microscope.

Photographic enlargement of EM negatives

- EM negatives are fine grain, high resolution negatives - very best enlargers necessary to produce enlargements.
- The enlarger light source should be the "**point source**" type.

  - small clear bulb -produces a small intense source of light.

  -Point source enlarger produces a sharper & more contrasty image.

Photographic enlargement of EM negatives

- EM negative placed in enlarger so that the emulsion surface faces the emulsion on the photographic paper (**emulsion to emulsion**).
- Photographic paper should be of the "cold tone" type. Contrasty papers that produce true blacks & whites.
- The papers have different contrast levels designated by "F" grades. F-grades range from 1-6 depending upon the manufacturer. Kodak papers from F1 to F5, with F1 being the least contrasty and F5 being the most contrasty.

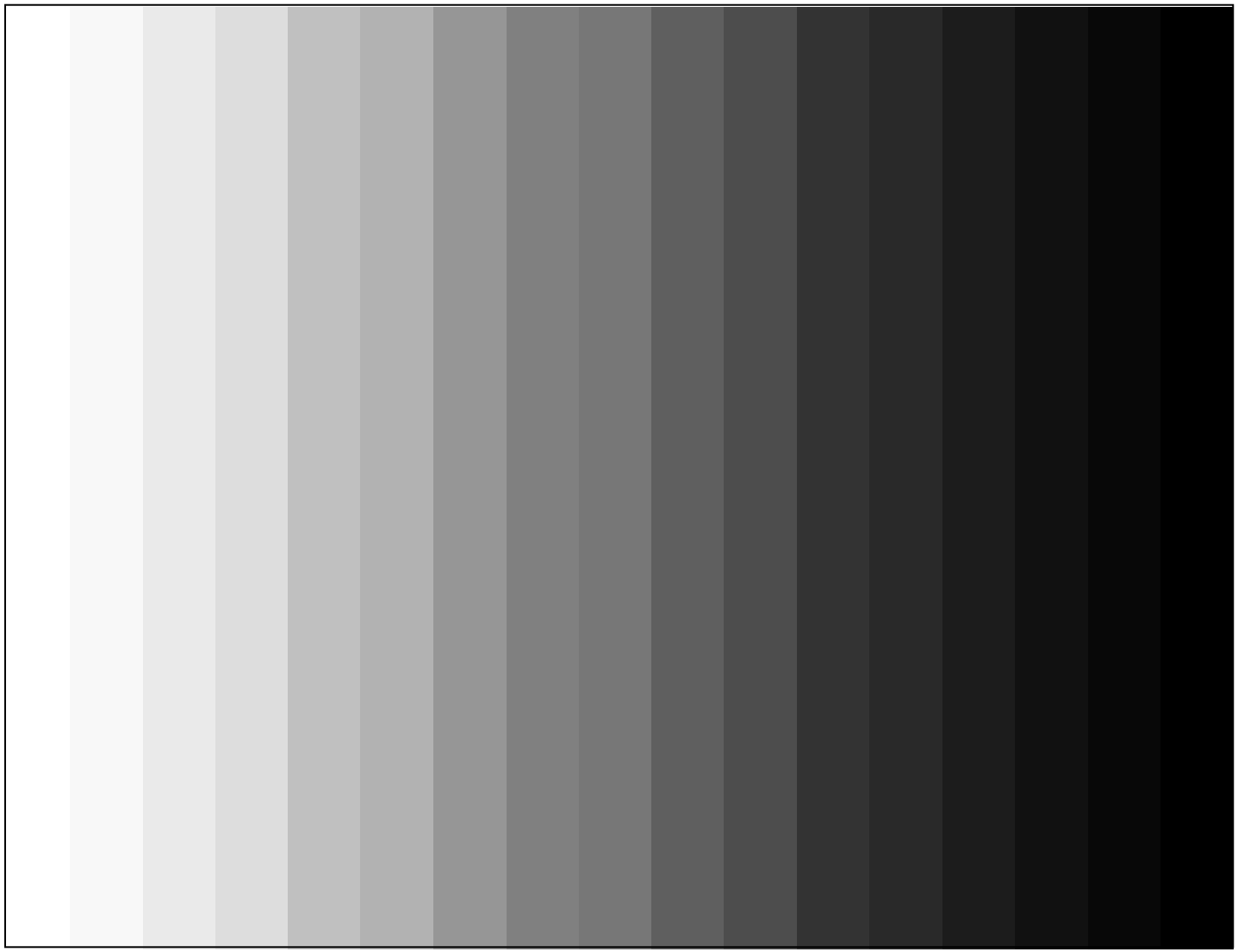
Photographic enlargement of EM negatives

- **Polycontrast papers** use different colored filters to achieve the different contrast levels of single contrast grade papers. An F1 would correspond to a yellow filter, and F5 to a magenta filter.
- Grades in between F1 and F5 use progressively less yellow and more magenta to increase the contrast in the paper.
- A low contrast negative will require a high contrast paper and a high contrast negative will require a lower contrast paper.

Photographic enlargement of EM negatives

- The goal in developing is to produce images that have a full range of gray tones, with the darkest being almost black & the lightest being almost white.
- In biological tissues, a white space would indicate an empty space, therefore there should be no pure white in your images.

Micrographs should be printed to display as full a range as possible of gray levels. Contrast extremes (high/low) obscure of cause loss of detail.

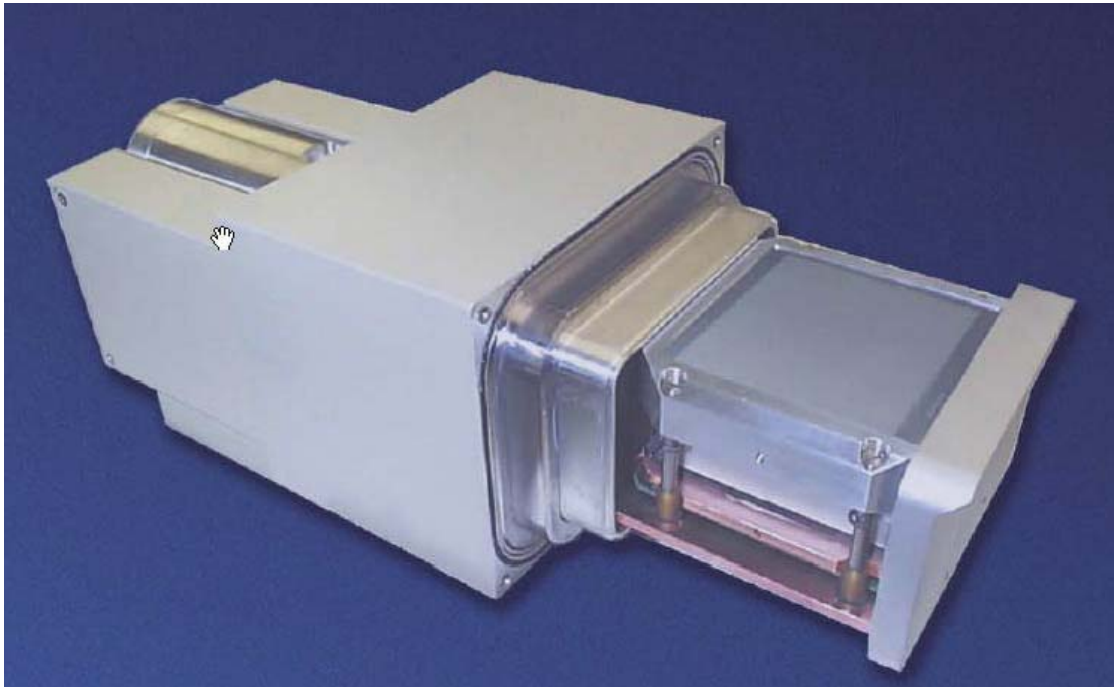


Photographic enlargement of EM negatives

- May be enlarged up to 10X. Photo graininess ↑ with the enlargement factor.
- Best results achieved with 2-3X enlargement.
  - A 3¼ x 4" negative will produce an 8 X 10 print when enlarged 2.5 X.
- The aperture setting on the enlarger lens should be wide open. This produces the shallowest depth of field & the sharpest focus.
- Use the rheostat & not the lens aperture to adjust the exposure lighting.

Digital microscopy

- Digital cameras such as the Gatan Ultrascan™ (4080 x 4080 pixels) -16 million pixel - capable of producing high quality digital images in the electron microscope.



Digital microscopy

- One advantage of CCD cameras is that they can perform binning operations. The output from an array of pixels (e.g. 6 x 6 pixels) may be combined into one pixel. This reduces resolution, but increases sensitivity. Allows rapid collection of information from beam sensitive specimens using minimal illumination. If the objects of interest are present, then a final high resolution image that does not utilize binning may be captured.

Digital microscopy

- Film has the equivalent of 20-30 megapixels of resolution.
- The best digital cameras have ~16 megapixels.
- The advantage of CCD is greater sensitivity & a more linear response & greater dynamic range. There is no image distortion with CCD.

Dynamic Range

- 8 bit = 256 gray levels; 10 bit = 1024 gray levels, 12 bit = 4096 gray levels; 14 bit =16384 gray levels.
- What is the benefit of 14-bit dynamic range vs. 12-bit vs 10-bit?
  - -You can record strong & weak intensities in a single image (e.g., diffraction pattern).
  - -Useful for image processing.
- If your sample does not have the dynamic range, an 8 bit image is usually sufficient.

Advantages of digital imaging

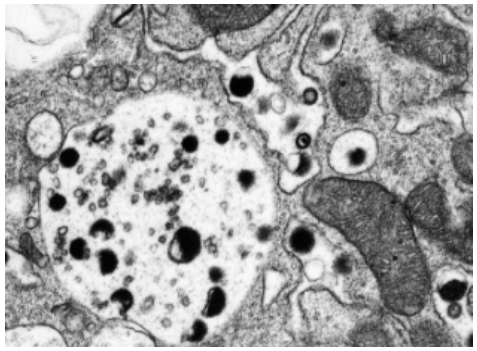
- There are also some inherent methods in which the human eye perceives images that can be used to great advantage in digital imaging, & although point to point resolution may be lower in most digital images used today, the ability to take advantage of the contrast range of a digital image provides greater visual enhancement over traditional photographic techniques. The human visual system is very sensitive to changes in contrast.

Digital microscopy

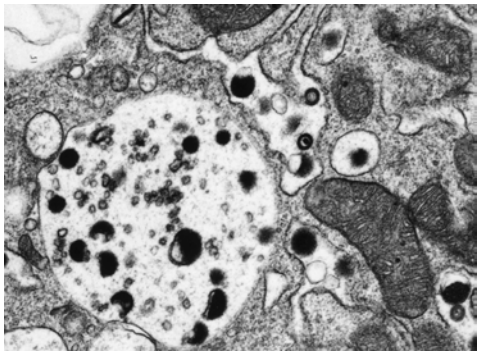
- Associated with digital imaging is the manipulation of the digital image via image processing.
- Important to note: microscopists new to digital imaging have been performing optical image processing all along in the wet darkroom, by the use of filters, different paper grades, & dodging & burning-in methods of development.

How much resolution is enough? Converting film to digital image

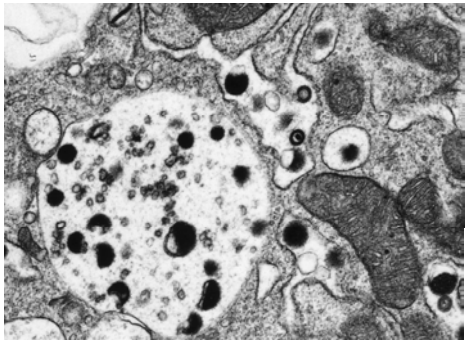
- Nyquist's criterion: to digitally preserve the resolution of detail (spatial frequency) in an image - necessary to sample the image at a rate twice as high as the highest spatial frequency of the detail we wish to preserve.
- In practice, sampling rate (dpi) will also depend upon how the image is going to be displayed. Computer screen vs. printed copy, & enlargement factor.



100 dpi

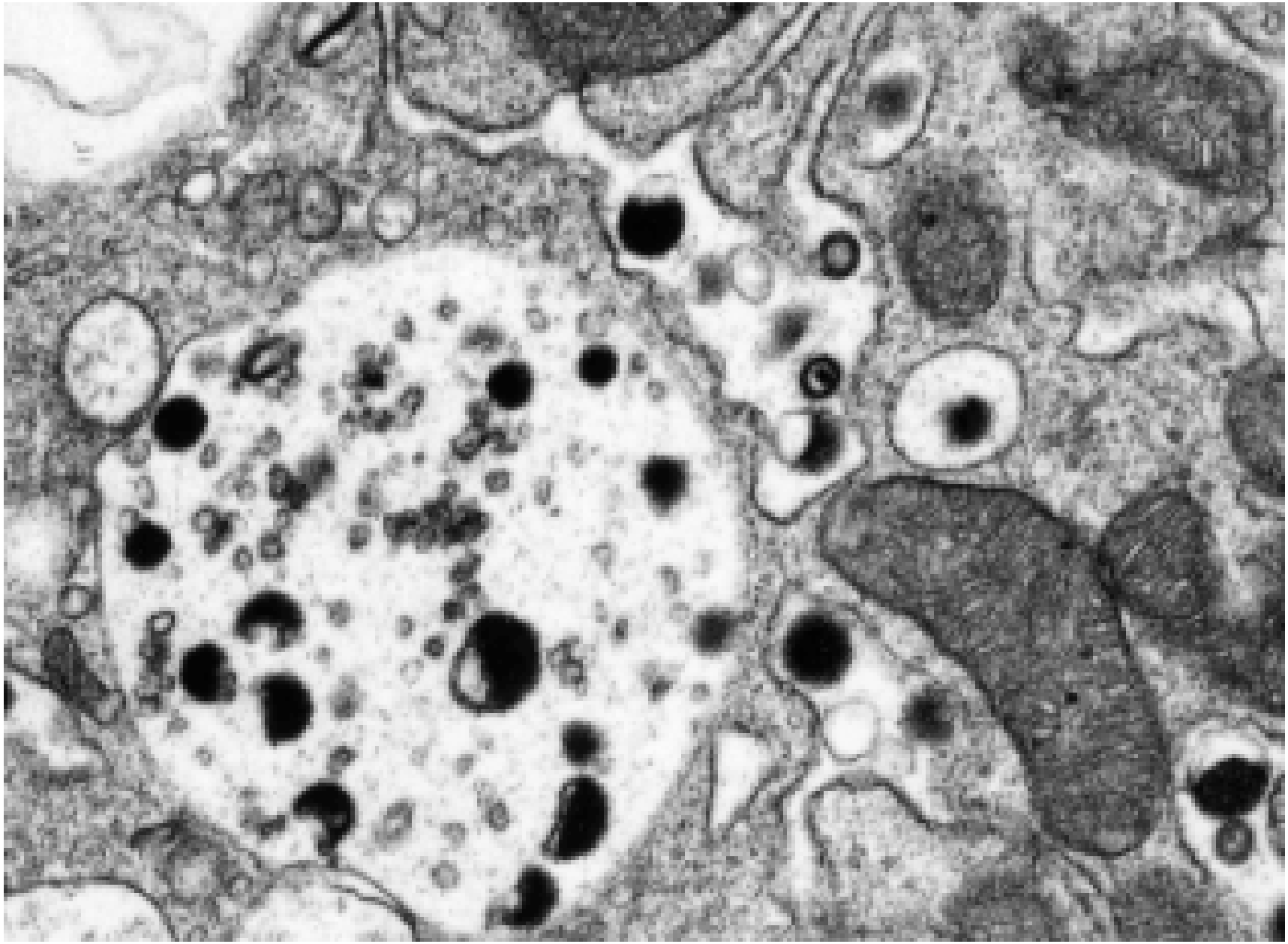


300 dpi

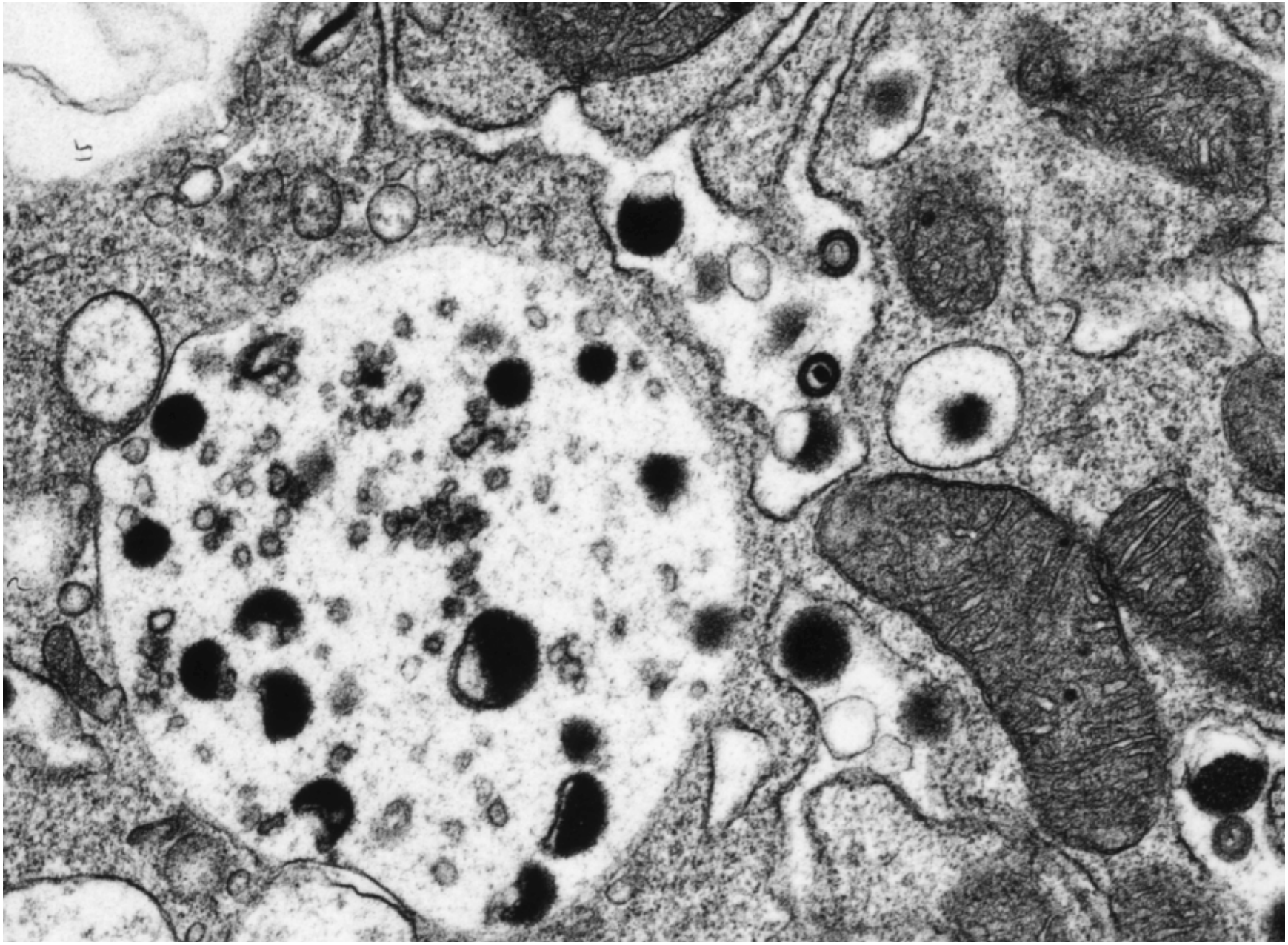


600 dpi

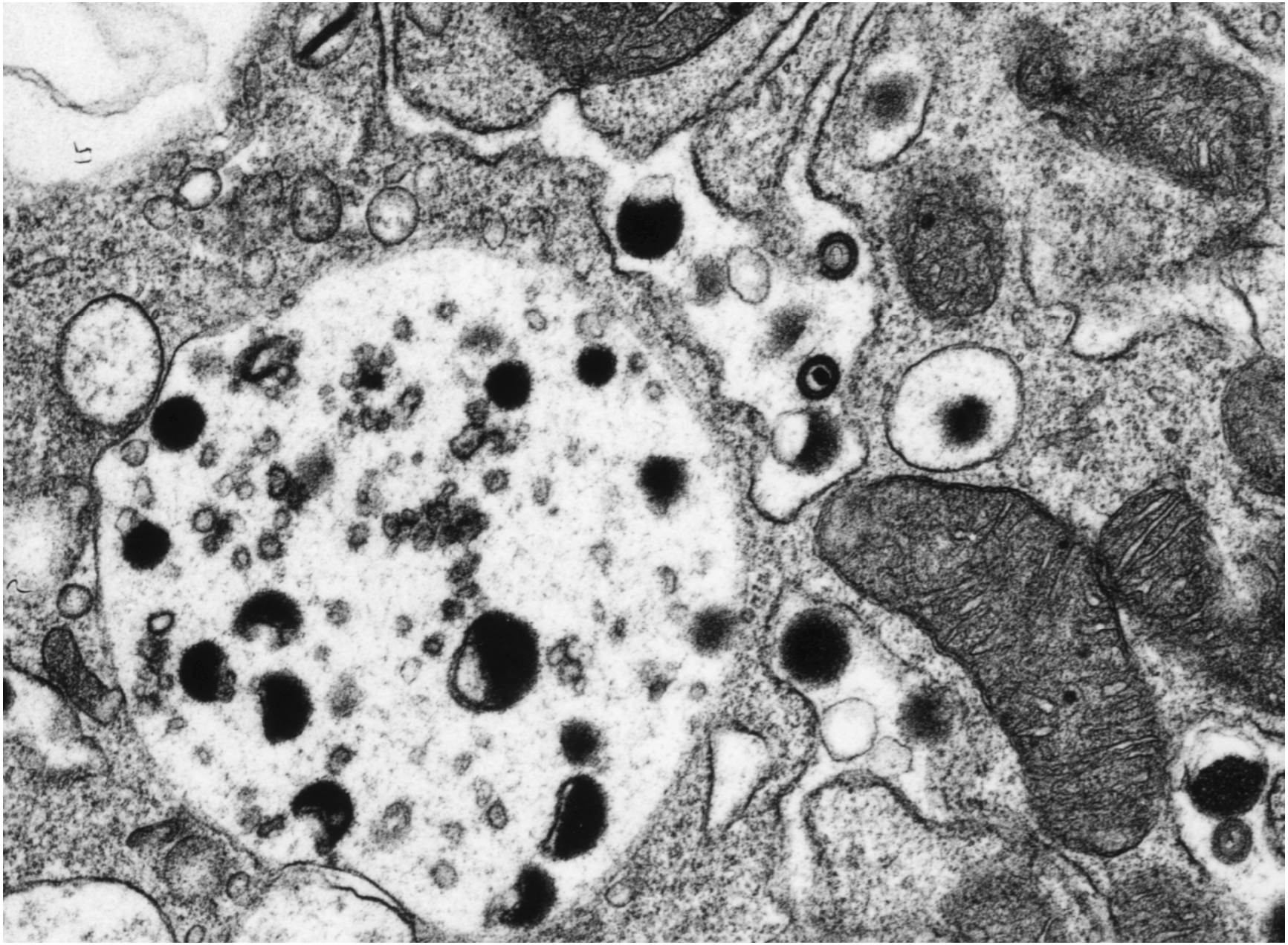
Negative digitized at 1200 dpi ~ 150MB



100 dpi



300 dpi



600 dpi

Converting negative to digital image

- Flat bed scanners with transparency adapter are cost effective & adequate for routine TEM (tissue sections). Sampling rates higher than 600 dpi are often interpolated. Resolution of scanner often degraded by lens distortion at the edges of a scan.
- High resolution microscopy requires use a drum scanner. Not CCD technology.

Negative is “oiled”. This produces an image free of defects.

EM55 MultiDodge Enlarger

Flying spot CRT system of exposure - improves photo detail while permitting the use of a variety of higher grades of paper to further increase speed & detail contrast while maintaining an acceptable level of gross contrast. The constantly variable light source, "produces detail not seen in any other enlargement system" - & corrects for uneven exposures.