Bridging the Resolution Gap: Imaging the Same Transcription Factories in Cryosections by Light and Electron Microscopy

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SUMMARY The resolution of conventional light microscopy is limited to ~200 nm in the x- and y-axes and >500 nm in the z-axis. A simple way of improving z-axis resolution is to analyze thin sections of 100–200 nm. The utility of such an approach is illustrated by reference to transcription sites imaged in cryosections of human nuclei. Cells are permeabilized, allowed to extend nascent transcripts in Br-UTP, fixed, cryosectioned, and Br-RNA-immuno- labeled with fluorochromes and gold particles. As expected, physical sectioning improves resolution and brings other advantages. First, sections allow improved antibody access and better immunolabeling. Second, more sites (with a more representative range of intensities) can now be resolved against lower backgrounds, facilitating quantitative analysis. Third, problems associated with chromatic aberration when two differently colored images of the same objects are collected can be sidestepped by refocusing between image collection. Fourth, exactly the same sites can be imaged by light and electron microscopy, allowing direct comparison between the two techniques. Immunogold labeling and electron microscopy provided the most accurate counts of site number. The results confirm that nascent transcripts in the nucleoplasm are confined to several thousand sites, or “factories,” with diameters of ~40 nm.

KEY WORDS
BrUTP
CLSM
cryosection
EM
fluorescent-gold
immunoprobe
immunofluorescence
Tokuyasu
transcription factories

The resolution of the conventional light microscope is at best ~200 nm in the x- and y-axes, and 500 nm or more in the z-axis (Pawley 1995). The poor resolution in the z-axis can be improved by collecting a series of images and then removing out-of-focus flare with sophisticated algorithms (e.g., Scalettar et al. 1996). Such algorithms are evolving rapidly, and existing ones enable accurate counts of small numbers of bright objects (where thresholds between background and an object, and between objects, are easily drawn). However, they are applied with less confidence to thousands of small faint objects of differing intensities if many appear to overlap in the primary optical sections. We now describe a powerful approach to improve z-axis resolution from >500 to ~100 nm. We analyze cryosections with a (z-axis) thickness that can range from 75 to 250 nm and demonstrate the utility of the approach by examining sites of transcription in the nuclei of human cells. Note that the term “resolution” can be defined in both theoretical and practical senses. It is used here in the practical sense to describe whether or not individual transcription sites can be identified.

Sites of transcription in mammalian nuclei have traditionally been imaged after growing cells for short periods in [3H]-U and then localizing the resulting [3H]-RNA by autoradiography. However, it was difficult to resolve individual extranucleolar sites because pulses giving sufficient incorporation for detection often allowed time for completed transcripts to move away from synthetic sites, and the resolution of autoradiography is poor (silver grains can lie >100 nm away from the tritium source)(Rogers 1979). Recently, transcription sites have been imaged with higher resolution after allowing intact or permeabilized cells to extend nascent transcripts by a few nucleotides in the presence of Br-U, Br-UTP or biotin-CTP, and then labeling the resulting Br- or biotin-RNA with antibodies tagged with fluorochromes or gold particles. Surprisingly, nascent transcripts are not spread diffusely.
throughout the nucleoplasm but rather are con- 
centrated in 2000–10,000 sites with diameters of 40–80 
nm (Jackson et al. 1993,1998; Wansink et al. 1993; 
Iborra et al. 1996a; Fay et al. 1997; Iborra and Cook 
1998; and Pombo et al. unpublished data). Because a 
site typically contains 10 or more transcription units 
and associated transcripts, such sites have been chris-
tened transcription “factories” (e.g., Iborra et al. 1996b).

Figure 1 illustrates the problems encountered in im-
aging and then counting objects like these transcription 
sites. In a conventional light microscope, an object 
of ~50 nm diameter appears wider (i.e., >200 nm) and more elongated (i.e., >500 nm) than it truly 
is (Figure 1A). This makes it difficult to distinguish 
faint objects in the optical section from out-of-focus 
flare from brighter objects lying above or below, and 
to resolve an object lying immediately above another 
in the same optical section. In the confocal micro-
scope, objects appear both narrower and less elon-
gated, and optical sectioning reduces but does not 
eliminate flare (Figure 1A, confocal). The precise ap-
pearance depends on the point-spread functions asso-
ciated with the different microscopes (Shaw 1995; 
Wilson 1995). However, in a thin physical section, all 
signal (strong or faint) must come exclusively from 
objects within the section (Figure 1B, conventional).
Moreover, because all objects lie well within the opti-
cal section, the intensities seen more directly reflect the 
intensities of those objects. In theory, therefore, a 
combination of physical and optical sectioning should 
Improve axial resolution (Figure 1B, confocal) (Shaw 
1995; Wilson 1995), and we show that this is so in 
practice.

Sections of the appropriate thickness (i.e., 100–200 
nm) can be prepared from whole cells by infiltration 
with a medium that hardens sufficiently (e.g., by poly-
merization or freezing) to permit sectioning. We used 
an approach borrowed from electron microscopy— 
freezing, cryosectioning, coupled to “dry knife, wet re-
trieve” (Tokuyasu 1980)—because ultrastructure is 
preserved and epitopes can be immunolabeled with 
high sensitivity (Griffiths et al. 1984; Tooze et al. 
1991). This procedure does not require dehydration 
or detergent treatment until sections are fixed to the 
glass coverslip or grid. Our basic procedure to label 
transcription sites is as follows. Cells are permeabi-
лизated with saponin in a “physiological” buffer, al-
lowed to extend nascent transcripts in the presence of 
Br-UTP, fixed in paraformaldehyde, infiltrated with 
sucrose, frozen in liquid nitrogen, cryosectioned, and 
the sections placed on coverslips (or grids) before Br-
RNA is immunolabeled with fluorochromes (or gold 
particles). Cryosectioning brings improved resolution 
as well as other advantages. First, the thin sections al-
low improved antibody access and therefore better im-
umolabeling. Second, fewer sites (with a more repre-
sentative range of intensities) are seen against lower 
backgrounds, facilitating quantitative analysis. Third, 
problems associated with chromatic aberration when 
two differently colored images of the same objects are 
collected can be solved simply by refocusing between 
image collection. Fourth, it proves possible to image 
the same sites by light and electron microscopy, allow-
ing direct comparison between the two techniques. 
Immungold labeling and electron microscopy can 
provide both the highest sensitivity and resolution, 
and therefore the most accurate counts of site number. 
The results also confirm that essentially all nascent 
transcripts within the nucleoplasm are confined to 
several thousand “factories” that have diameters of 
~40 nm.

Materials and Methods

Transcription Reactions

HeLa cells were generally grown as suspension cultures 
(Jackson et al. 1988). Monolayer cultures grown in MEM 
plus 10% fetal calf serum were used for Figures 2A and 2B 
to provide the best conditions for imaging whole cells. Cells 
in suspension were washed successively in PBS, ice-cold 
physiological buffer (PB; Iborra et al. 1996a) containing ri-
bonuclease inhibitor (5 U/ml; Amersham-Pharmacia, Upp-
sala, Sweden), pelleted (200 × g, 5 min), lysed with saponin 
in PB (100 µg/ml, 5 min, 4C; Sigma, Poole, UK), and washed 
three times in PB. Lysed cells were preincubated (2 min, 
33C), and a 10× concentrated solution of ribonucleotide 
triphosphates (33C) added to give final concentrations of
1.1 mM ATP, 0.1 mM Br-UTP, CTP, GTP, 1.4 mM MgCl₂, and 25 U/ml ribonuclease inhibitor. After 15 min at 33°C, reactions were stopped with 2 volumes ice-cold PB and cells were fixed and cryosectioned as described below. Cells on coverslips were washed and lysed as above, but overlaid with a 1 × concentrated solution of ribonucleotide triphosphates. Then reactions were stopped by addition of ice-cold PB. Cells were fixed in 4% paraformaldehyde in 250 mM HEPES (pH 7.4, 10 min, 4°C), pelleted, refixed in 8% paraformaldehyde in 250 mM HEPES (pH 7.4, 2 hr, 4°C).

**Cryosections**

Cryosections were prepared using a modification of existing methods (Tokuyasu 1980; Griffiths et al. 1984; Tooze et al. 1991). After stopping the reactions, cells were pelleted as above, fixed with 4% paraformaldehyde in 250 mM HEPES (pH 7.4, 10 min, 4°C), pelleted, refixed in 8% paraformaldehyde in 250 mM HEPES (pH 7.4, 20 min, 4°C), refixed (8000 × g, 5 min), and fixation allowed to continue in the pellet for 20 min before the pellet was dislodged and left for 80 min. Next, the pellet was washed in PBS, transferred through 3 drops of 2.1 M sucrose in PBS over 2 hr and then onto a copper block, and excess sucrose removed with a filter paper. After shaping into a cone with forceps, pellets were frozen by immersion in liquid nitrogen and stored in liquid nitrogen until use. Cryosections (100–200 nm, dehydrated from interference color) were cut (Reichert Ultracut S) and cryosections were frozen by immersion in liquid nitrogen and stored in liquid nitrogen until use. Cryosections (100–200 nm, dehydrated from interference color) were cut (Reichert Ultracut S) and cryosections were frozen by immersion in liquid nitrogen and stored in liquid nitrogen until use. Cryosections (100–200 nm, dehydrated from interference color) were cut (Reichert Ultracut S) and cryosections were frozen by immersion in liquid nitrogen and stored in liquid nitrogen until use. Cryosections (100–200 nm, dehydrated from interference color) were cut (Reichert Ultracut S) and cryosections were frozen by immersion in liquid nitrogen and stored in liquid nitrogen until use.

**Immunolabeling and Imaging**

Before addition of antibodies, free aldehyde groups were quenched (20 min) with 25 mM glycine in PBS. Then whole cells on coverslips were treated with 0.5% Triton X-100 in PBS for 20 min and cryosections with 0.1% Triton X-100 in PBS for 2 min. After washing in PBS and incubation (30 min) in PBS + 1% BSA + 0.2% fish gelatin (PBS-), Br-RNA was indirectly immunolabeled (1–2 hr) using a mouse monoclonal anti-Br-dU (Boehringer Mannheim; Lewes, East Sussex, UK) as a primary antibody diluted in PBS+ (pH 8.0) to 1 μg/ml (whole cells) or 5 μg/ml (cryosections), and washed in PBS+ (1 hr). (Antibodies were titrated and the maximal concentration that did not give significant background was used.)

For Figure 2, samples were incubated (1–2 hr) with a donkey anti-mouse IgG conjugated with FITC (fluorescein isothiocyanate, 10 μg/ml; multiple-labeling grade from Jackson Immunoresearch Laboratories, West Grove, PA), washed (1 hr) in PBS+ and then in PBS supplemented with 0.1% Tween 20, incubated in TOTO-3 (20 μM in PBS/Tween for 20 min; Molecular Probes, Eugene, OR), washed successively in PBS/Tween and PBS, and mounted in Vectashield (Vector Laboratories; Peterborough, UK) on an England finder (BDH; Poole, Dorset, UK). Images shown in Figures 2A and 2C were collected using a CCD camera (Hamamatsu Photonics C4742; Hamamatsu City, Japan; 1000 × 1018 pixels, 10-bit images, pixel size 12 μm), run under Image v1.41 (NIH, Bethesda, MD), and a × 63 Plan-Neoflaur objective (NA 1.4) on a Zeiss Axioskop microscope (Carl Zeiss-Oberkochen; Welwyn Garden City, Herts, UK). Images shown in Figures 2B and 2D were collected using a confocal microscope (hybrid BioRad M R C-1000/1024; Hemel Hempstead, Herts, UK) running under Comos software (v. 7.0a), and fitted with an argon/krypton laser, a x 60 PlanApo objective (NA 1.4), and a Nikon Diaphot 200 inverted microscope. Images were collected with a closed iris (to obtain best resolution), Kalman filtering (n = 6–10), and the minimal laser power that filled the whole gray scale in the low scan/low signal mode.

For Figure 3, immunolabeling was as above, except that two secondary antibodies were used together—the IgG–FITC described above and a donkey anti-mouse IgG conjugated with Cy3 (iodocarbocyanine, 1 μg/ml; multiple-labeling grade from Jackson ImmunoResearch)—and images were collected sequentially on the confocal microscope (as above). For Figure 4, Br-RNA was indirectly immunolabeled using the mouse anti-Br-dU antibody, followed by rabbit anti-mouse IgG (1 hr, 1 μg/ml; Cappel Laboratories, supplied through Organon Teknika, Turnhout, Belgium) and then FITC–protein A adsorbed to 6-nm gold particles (3 hr, 1:100 dilution); the conjugate was made as described by Griffiths (1993) but using FITC–protein A (Amersham-Pharmacia; Little Chalfont, UK). (The sensitivity, stability, and storage properties of the conjugate were similar to those of protein A–gold.) After washing in PBS+, cryosections were stained with TOTO-3 and imaged on the confocal microscope (as above). Then coverslips were floated off the slide, washed in PBS, treated (10 min) with 0.5% glutaraldehyde in PBS, stained (1 hr) in 1% OsO₄ and 1.5% potassium ferrocyanide, washed in water, incubated (4C, 10 hr) in 0.5% magnesium uranyl acetate, washed in water, stained (30 sec) with Reynolds’ lead citrate, and washed in water. The cryosection on a coverslip in a Petri dish was dehydrated through ethanol, flat-embedded in Epon (Tooze and Hollinshead 1992), and the first 150-nm Epon section (which contained the original cryosection) imaged in a Zeiss EM 912 Omega transmission electron microscope equipped with a cooled slow-scan CCD camera (1024 × 1024 pixels; Proscan, Proscan Elektronische Systeme, Penzing, Germany) running under analySIS 2.11 software supplied with the microscope. The FITC–gold conjugate described above penetrates as efficiently into cryosections as a donkey anti-rabbit IgG conjugated with LRSC (lissamine rhodamine sulfonyl chloride, 1 μg/ml; multiple-labeling grade from Jackson Immunoresearch). After labeling of Br-RNA with the primary mouse monoclonal antibody, simultaneous incubation with both reagents led to 82% foci being doubly labeled with both fluorochromes, 13% only with LRSC, and 5% only with FITC (not shown). Rough estimates showed that conjugates typically contained FITC, protein A, and gold particles in the ratio 60:10:1. These ratios were calculated using (a) the FITC:protein A ratio (6:1, manufacturer’s information), (b) particle numbers (from particle counts of known volumes dried on grids), and (c) the protein A:particle ratio (i.e., 10:1) (from...
the minimal protein concentration required for stabilization; Griffiths 1993). We found that the FITC-protein A-gold conjugate gave better resolution and sensitivity than commercial reagents (i.e., FluoroNanogold-Fab from Nanoprobes).

For Figure 5, Br-RNA on grids was immunolabeled and treated with glutaraldehyde as for Figure 4. Then grids were washed eight times in water, incubated (4°C, 10 min) with 0.3% uranyl acetate in 2% methylcellulose, captured on a wire loop, excess methylcellulose blotted onto a filter paper, washed eight times in water, incubated (4°C, 10 min) with Dbins (e.g., 0–5 nm, 5–10 nm, 10–15 nm in Figure 5) of size because they are too small to be detected. The radii of the radii is deduced by sequential subtraction. Starting with the smallest category; then the next largest bin is treated in the same way, and so on.

Numbers of sites in 3-D space in a nucleus were calculated as follows (Weibel 1979): (a) the density (n_r) of clusters in 2-D images of 3-D sections was measured, (b) the volume density (n_v) was calculated using Abercrombie’s formula

\[ n_v = \frac{n_s}{D + T}, \]

where T is the section thickness, and (c) the number of sites per nucleus calculated with knowledge of nucleoplasmic volume. The volume of nucleoplasm (i.e., 554 ± 163 μm^3; range 420–921; n = 11) in permeabilized HeLa cells was determined using Adobe Photoshop from serial optical sections collected at nominal 0.4-μm intervals through whole cells stained with TOTO-3. Each voxel had x and y dimensions of 45 nm (calibrated according to BioRad’s instructions), and a z dimension of 0.39 μm and not 0.4 μm (determined by reference to 10-μm latex Nile Red FluoroSpheres from Molecular Probes, as described by Visser et al. 1991). A lower value for nucleoplasmic volume (i.e., 380 μm^3) was obtained by stereological analysis of cryosections, assuming that nuclei and nucleoli are round (Iborra et al. 1996a). Note that the calculated values for the number of sites in 3-D space is sensitive both to values of site radius and to nucleoplasmic volume.

**Results**

Improving Z-axis Resolution of the Light Microscope

The improved resolution given by sections is illustrated in Figure 2. HeLa cells were permeabilized in a physiological buffer, allowed to extend nascent transcripts by ~1100 nucleotides in the presence of Br-UTP, fixed, and Br-RNA indirectly immunolabeled with FITC. All four cellular RNA polymerases incorporate the analogue (unpublished data), so Br-RNA is found in nucleoli (made by polymerase I), the nucleoplasm (made by polymerases II and III), and the cytoplasm (made by the mitochondrial polymerase). When a whole cell is viewed with a conventional microscope, fluorescence (marking Br-RNA) appears distributed throughout the cell in a complex pattern. Bright foci in the nucleolus and nucleoplasm are seen against a diffuse nucleoplasmic signal, and these are surrounded by a few mitochondrial foci (Figure 2A). Here (and in all other fluorescence images shown), the primary image has been stretched to fill the gray scale but not treated in any other way. As a result, foci appear more blurred than those presented after subtracting “background” (e.g., Jackson et al. 1993). A confocal view of the same cell gives a simpler pattern. Because the optical section is thinner, some individual nucleoplasmic foci can now be resolved, although many still appear to fuse one into another (Figure 2B). Views of a cryosection (~150 nm thick) yield still simpler images; even in the conventional microscope, foci are well resolved (Figure 2C), and in the confocal microscope they appear even sharper (Figure 2D). In Figure 2D, no signal other than noise could be detected between foci (not shown; see also Iborra et al. 1996a). (Noise is
defined as the average intensity seen over noncellular regions.) As a result, nucleoplasmic foci can easily be counted, and the poorer resolution given by the conventional microscope is reflected by an underestimate of the number of foci by 27% (average of counts made on 12 images of six cells like those in Figures 2C and 2D; not shown). These results confirm the theoretical expectations that foci should appear sharper and better resolved when thin cryosections are imaged using a confocal microscope.

Minimizing Chromatic Aberrations when Doubly-labeled sites Are Imaged

Multiple fluorochromes are often used to localize different objects within cells. The approach requires that there is little misregistration or difference in magnification between the two resulting images. This is difficult to achieve in practice because fluorochromes emit light of different wavelengths, which is diffracted to different degrees (Keller 1995). As a result of such chromatic aberration, objects in the same plane are never exactly in focus in both images. This problem becomes even more acute when objects lying further within cells (or confocal sections) are imaged; microscope objectives are designed to image with maximal quality objects immediately below glass coverslips ~0.17 mm thick (Keller 1995). Such problems can be minimized by refocusing, as follows.

Br-RNA in cryosections was labeled with both red and green fluorochromes. After focusing on sites through a red filter, an image was collected (Figure 3A). If the same sites are then imaged using the green filter, they appear more blurred (Figure 3B). They become sharper after refocusing (Figure 3C), and changing back to the red filter then defocuses them again (not shown). We compared foci in Figures 3A–3C to gauge how great the differences in misregistration, magnification, and brightness were in these thin sections. Such comparison is complicated by the fact that antibodies bind to different sites in differing ratios; some in-focus foci appear slightly brighter when viewed through the green filter (e.g., focus 1), others through the red filter (e.g., focus 2). (This also indicates that sites are detected with similar sensitivities using the two fluorochromes.) Switching filters leads to some increase in the size of sites, with adjacent sites apparently fusing (e.g., adjacent foci 3); some faint sites may even be lost (e.g., focus 4). A combination of both effects leads to a ~20% reduction in the number of sites counted after switching filters without refocusing. Roughly equal numbers are seen with refocusing.

It remained formally possible that starting and stopping scans, and/or the z-axis movements required for refocusing, might alter x-y positions, so we tested whether this was so. First, we established how x-y position varied after collecting a series of images like those in Figure 3A without any refocusing. After contrast-stretching to fill the gray scale and subtraction of background noise (the average intensity outside cells), the center of gravity of the remaining foci (defined as ≥2 touching pixels that contained any intensity) was determined (not shown). The centers of gravity of only 8% foci moved by >1 pixel from scan to scan, and this movement was not in any one direction. After refocusing between scans (as in Figures 3A and 3C), no further movement was seen (i.e., the center of gravity of only 6% foci moved by >1 pixel). These results show that refocusing has no detectable effect on x-y registration in our system and that this approach minimizes the chromatic aberrations associated with imaging multiply-labeled sites.

Imaging the Same Sites by Light and Electron Microscopy

Because the cryosectioning technique was borrowed from electron microscopy, we investigated whether it was possible to image exactly the same sites by both light and electron microscopy. For this purpose, we prepared a probe labeled with both a fluorochrome and gold particle (see Materials and Methods). This
reagent allows direct correlation without the need for amplification. Because large gold probes apparently do not penetrate efficiently into cryosections (Stierhof et al. 1986; Slot et al. 1989; Stierhof and Schwarz 1989), we improved an existing procedure in two ways: (a) by adding a mild detergent treatment after cryosections are stably attached to the glass coverslip, and (b) by incubating sections with probes for longer. As a result, conjugates containing colloidal gold of \(\leq 10\) nm penetrate as efficiently as immunoglobulin–fluorochrome conjugates (see Materials and Methods). Sections containing Br-RNA were prepared as before, incubated with FITC–protein A adsorbed onto 6-nm gold particles, and fluorescence images collected on a confocal microscope (Figures 4A–4C). Next, the embedding resin used for electron microscopy was poured onto the section, allowed to set, and the resulting block re-sectioned so that the first section (\(~150\) nm) contained the original cryosection. Finally, the same area was identified and an image was collected using the electron microscope (Figures 4D and 4E). Transcription sites were seen either as fluorescent foci or as clusters of gold particles. These clusters appear in stereo views to be spread throughout the thickness of the cryosection (not shown). Foci and clusters colocalize almost perfectly (e.g., Figure 4F). Moreover, lone gold particles did not always give foci visible by light microscopy, and two gold particles gave visible foci if they lay within 100 nm of each other (not shown). (Note that even if gold particles quench fluorescence, there remains sufficient to enable all clusters of gold particles to be detected by light microscopy.)

We next compared the strengths and weaknesses of the different approaches used to analyze light and electron micrographs. Consider, for example, how the number of transcription sites in a nucleus would be determined. In analyzing fluorescence images like that in Figure 4C, we are confronted with the problem of how to define a focus. We must make (often arbitrary) decisions on how many contiguous pixels are needed to constitute a focus, where to draw the threshold between signal and noise, and whether two peaks of intensity are part of one extended focus. In practice, most observers agree that the image contains the five foci shown by the ovals in Figure 4F, largely because their eyes and brains are so well adapted to analyze complex images (Russ 1990; see also Iborra et al. 1996a). (Part of one focus lying on the right edge of the field is excluded in this discussion.) Perhaps surprisingly, it turns out that transcription sites can be counted more rigorously in the corresponding electron micrograph (Figure 4E). We have previously shown that clusters (defined as two or more particles lying within 40 nm of another) mark transcription sites and that lone particles represent background (Iborra et al. 1996a). These criteria are justified because (a) \(~40

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Figure 3  Reducing chromatic aberration by refocusing. Permeabilized cells were allowed to extend nascent transcripts in Br-UTP for 15 min, fixed, cryosections prepared, Br-RNA indirectly immunolabeled with both Cy3 and fluorescein, and a series of “raw” images of the same foci collected using the confocal microscope. Selected foci are numbered. (A) One optical section taken through a red filter. (B) View of same section taken after changing to the green filter (but without refocusing). (C) View of same foci taken after refocusing (but without changing the filter). Bar = 500 nm.
nm is the maximal separation possible between two particles marking adjacent bromine residues in a transcript (i.e., roughly the length of two fully extended immunolabeling probes, each containing two immunoglobulins and a protein A molecule), and (b) the same density of lone particles (i.e., <1 particle/μm², representing <6% total number of particles) is found in controls incubated without Br-UTP and outside of cells (not shown). Using this simple but rigorous definition of what constitutes a cluster, Figure 4E is found to contain six clusters, which can be seen more clearly in Figure 4F. Not surprisingly, the electron microscope resolves some foci into more than one cluster (e.g., two clusters are contained within the large focus at the top center in Figure 4C). Further analysis of 48 foci showed that 39 contained one cluster of particles, eight contained two, and one contained three (not shown). As a result, the number of sites in such samples is only underestimated by ~17% in the light microscope (not shown). The density of sites seen by electron microscopy (i.e., 1.6 cluster/μm²) corresponds to 6500 extranucleolar sites/nucleus, taking into account the average size of clusters (see below) and nucleoplasmic volume (see Materials and Methods).

These results demonstrate that the same objects can be imaged by both light and electron microscopy without amplification. They also show that electron microscopy can yield the most accurate counts.

Diameter of Transcription Sites Determined by Electron Microscopy

The diameter of transcription sites, seen as clusters of gold particles in cryosections, was determined using the sequential-subtraction method (Weibel 1979, 1980). This allows the radius, R, of spheres to be deduced from the radii (r) of circular profiles seen in 2-D images of 3-D sections cut through the sample. Spheres lying completely within the section appear as circles with radius R; any cut nonequatorially may give polar “caps” that either appear smaller or are missed because they are too small to be detected. Clusters of particles marking nucleoplasmic transcription sites had a mean (corrected) radius of 18 nm (Figure 5). Because the length of the immunolabeling probe used during this analysis represents a significant fraction of the diameter of the underlying structure, these are likely to be overestimates. [See Iborra and Cook (1998) for an investigation of possible errors.]

The sequential-subtraction method also provides information on sensitivity of detection; undetected sites and caps would give negative frequencies in Figure 5. Because none are seen here, the method must be very sensitive. Moreover, detected sites range in volume from 1/373 to 9 times the average, so Br-RNA can be detected over a ~3000-fold range. However,
sites soon become saturated with gold particles (i.e., 35 at most with the immunolabeling procedure described here; not shown), so the number of gold particles per site is a poor measure of Br-RNA content.

Discussion

The Use of Cryosections Improves Resolution of Light Microscopy

The resolution of the conventional light microscope is limited to ~200 nm in the x- and y-axes, and at least 500 nm in the z-axis (Pawley 1995). We now describe a simple way of sidestepping problems associated with the poor z-axis resolution. Thin cryosections of 100–200 nm were prepared, using a technique borrowed from electron microscopy, and then viewed in light microscopes equipped with conventional or confocal optics. We demonstrate the utility of the approach by examining the thousands of transcription sites found in the nuclei of human cells. These sites can be imaged after allowing permeabilized cells to extend nascent transcripts by ~1100 nucleotides in the presence of Br-UTP, before the resulting nascent Br-RNA is marked with antibodies tagged with fluorochromes (Jackson et al. 1993; Wansink et al. 1993; Iborra et al. 1996a). When whole cells are viewed in the conventional light microscope, the nucleoplasmic sites are so small and numerous that they appear as a diffuse blur (Figure 2A). The optical sectioning provided by the confocal microscope enables some discrete sites to be resolved, although it remains difficult to count them accurately because so many still merge into each other (Figure 2B). Cryosectioning provides a striking simplification of the pattern in both conventional and confocal microscopes, with the latter giving the sharpest images (Figure 2C and 2D). This is in accord with theory (Shaw 1995; Wilson 1995), and results mainly because sites originally lying above and below the section cannot contribute to the image, so that fewer foci with a more representative range of intensities are seen against a lower background.

The use of thin cryosections confers several other advantages. For example, two different fluorochromes are often used to mark two different antigens (e.g., a nascent transcript and a polymerase or transcription factor) to see if they co-localize (e.g., Grande et al. 1997; Zeng et al. 1997). This approach requires that there is little misregistration or difference in magnification between the two resulting images. However, the two fluorochromes cannot be exactly in focus in both images because they necessarily emit light of different wavelengths that is diffracted to different degrees (Keller 1995). This chromatic aberration becomes more acute when objects lying at different depths in cells or confocal sections are imaged. Although such distortions can be partially corrected using the appropriate algorithms (e.g., Scalettar et al. 1996), the problem can be minimized using cryosections: an in-focus image of one fluorochrome is collected, filters are switched, the microscope refocused, and an image of the second fluorochrome collected (Figure 3). Then, both images are in focus, easing comparison. Therefore, this approach reduces the chromatic aberration inevitably associated with the simultaneous collection of multicolor images.

Cryosections also facilitate imaging of the same objects by light and electron microscopy (Figure 4). This was demonstrated by indirectly immunolabeling sites containing Br-RNA with FITC–protein A adsorbed to 6-nm gold particles. After collecting a fluorescent image on the confocal microscope, the same section was transferred to the electron microscope so that the gold particles could be seen. This enables comparison of the sensitivities of the two methods and assessment of the strengths and weaknesses of the different approaches used for analysis. The two methods had similar sensitivities, but electron microscopy gave the highest resolution. For example, the number of sites was underestimated by ~17% by light microscopy, largely because several small sites that some contained could not be resolved. Of course, electron microscopy is required to determine precisely the diameters of small objects.

The use of cryosections to analyze spherical structures, not much smaller than the thickness of the section, has one major disadvantage: some spheres are
Moreover, the method has little dynamic range, despite the fact that the number of sites seen in 2-D sections, with knowledge of nucleoplasmic volume. The density of sites seen here (i.e., 1.6 cluster/μm²) corresponds to 6500 extranucleolar sites/nucleus (see also Iborra et al. 1996a; Iborra and Cook 1998; Jackson et al. 1998). This value should be compared with the 75,000 RNA polymerases that are believed to be active within the nucleoplasm (Jackson et al. 1998). Why is the number of sites seen so much smaller than the number of nascent transcripts?

Until recently, it had been assumed that active polymerases were spread randomly throughout euchromatin, so that only a few would appear to form local clusters. Then, using an insensitive detection method for detecting nascent RNA, we might observe only the few clustered transcripts, while missing the majority. However, the results described above make this possibility unlikely. First, the two detection methods (involving light and electron microscopy), which would be expected to have different thresholds of detection, image roughly the same number of sites (Figure 4). Second, no signal above background is detected between sites by either method (Figure 4). This is so, despite one method proving sufficiently sensitive to detect Br-RNA over a 3000-fold range (see above). Moreover, similar results are obtained with intact and permeabilized cells (Jackson et al. 1998), different analogues incorporated in different amounts (e.g., Br-U in vivo, biotin-CTP in vitro (Iborra et al. 1996a; Jackson et al. 1998), and after nuclease digestion or extraction to improve immunolabeling (Iborra et al. 1997; Jackson et al. 1998). If most sites are being detected, this means that each site contains ~14 polymerizing complexes and nascent transcripts. Therefore, we must reevaluate both how polymerases are organized within nuclei and the mechanics of transcription (Iborra et al. 1996b).

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