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(57) Abrégé/Abstract:

Computerized analysis of 2-D gels, both carrier ampholyte (CA) and immobilized pH gradient (IPG) based, of the proteins in tissue from esophageal tumors, reveals proteins which are different in tumor and control tissue.



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PROTEIN MARKERS FOR ESOPHAGEAL CANCER

Background of the Invention

Field of the Invention

5 The present invention relates to proteins which are markers for esophageal cancer.

Esophageal cancer is a deadly disease, and one of the ten most common cancers worldwide. For reasons that are not clear, there has been a rapid increase in the incidence of esophageal adenocarcinoma. The occurrence of a change in the esophagus, referred to as Barrett's mucosa, in
10 which a premalignant metaplastic epithelium replaces the normal squamous epithelium following gastroesophageal reflux, is the major risk factor for this deadly cancer. Reducing the high mortality associated with this cancer will require simplified means of diagnosing Barrett's mucosa and identifying, at an early stage, changes in Barrett's mucosa that indicate progression to cancer.

15 Description of Related Art

At the present time, esophageal cancer is diagnosed primarily by biopsy. Unfortunately, by the time the cancer is diagnosed it is often far advanced. Survival after diagnosis is poor.

Thus, a need exists for the diagnosis of esophageal cancer at an early
20 stage, or even a pre-malignant stage. Markers which correspond to the advance of the illness may be used to monitor therapeutic regimens.

Summary of the Invention

By comparison of 2-D gels showing proteins from normal esophagus, normal gastric mucosa, Barrett's mucosa, and esophageal cancer, a set of
25 proteins have been identified in the different source tissues. These proteins provide information on the pathogenesis of esophageal cancer, and may have utility as markers to monitor therapeutic regimens.

The proteins can also be purified and used as immunogens to generate antibodies which can be used as diagnostic reagents. Antibodies which
30 specifically bind the proteins can be used in an immunoassay of body fluids to detect the proteins, thereby providing diagnostic information and/or information to monitor therapeutic regimens. The body fluids used in the

immunoassay method may be samples of blood or serum, urine, saliva, tears, or other body fluids.

In addition, the proteins and products derived therefrom have therapeutic applications.

5 Brief Description of the Drawings

Figure 1 shows an entire carrier ampholyte (CA) gel with many important spots labeled.

Figure 2 shows the region around spot 102 for CA gels from 12 samples.

10 Figure 3 shows the region around spots 109-111 for CA gels from 12 samples.

Figure 4 shows the region around spot 61 for CA gels from 12 samples.

Figure 5 shows the region around spot 112 for CA gels from 12 samples.

15 Figure 6 shows the region around spots 115 and 117 for CA gels from 12 samples.

Figure 7 shows the region around spot 118 for CA gels from 12 samples.

20 Figure 8 shows the region around spots 42, 44, and 120 for CA gels from 12 samples.

Figure 9 shows the region around spot 121 for CA gels from 4 samples.

Figure 10 shows the region around spots 122, 123, and 124 for CA gels from 12 samples.

Figure 11 shows one quadrant of a gel.

25 Figure 12 shows one quadrant of a gel.

Figure 13 shows the region around spot PCNA for CA gels from 12 samples.

Figure 14 shows the region around spot 29 for CA gels from 12 samples.

30 Figure 15 shows the region around spot 31 for CA gels from 12 samples.

Figure 16 shows the region around spot 42 for CA gels from 12 samples.

Figure 17 shows the region around spot 44 for CA gels from 12 samples.

5 Figure 18 shows the region around spot 5 for CA gels from 12 samples.

Figure 19 shows one quadrant of an immobilized pH gradient (IPG) gel.

Figure 20 shows one quadrant of an IPG gel.

Figure 21 shows the region around spot 29 for IPG gels from 10 samples.

10 Figure 22 shows the region around spots 15, 51, and 53 for IPG gels from 10 samples.

Figure 23 shows the region around spot 56 for IPG gels from 10 samples.

15 Figure 24 shows the region around spot 57 for IPG gels from 10 samples.

Figure 25 shows one quadrant of an IPG gel.

Detailed Description of the Invention

Example 1

Carrier ampholyte (CA) based 2-D gels of esophageal cancer.

20 Tissue from 27 patients was obtained for 2-D analysis from tumor tissue and normal esophagus. For patients in whom the tumor occurred in the context of Barrett's mucosa, Barrett's tissue was also obtained. (An interesting pathological feature of Barrett's mucosa is that it resembles gastrointestinal type of tissue rather than esophageal tissue, where it is found. The lower end
25 of the esophagus resembles gastric mucosa rather than true esophageal tissue so that tumors that arise in that location need to be compared to normal esophagus as well as gastric tissue as control.) For tumors that occurred at the lower end of the esophagus, normal gastric mucosa was also obtained. All specimens were obtained at the time of initial surgery. Therefore, for every
30 patient two control tissues were obtained, in addition to the cancer: normal

esophagus and Barrett's for half of the patients, and normal esophagus and stomach for the other half.

Carrier ampholyte-based 2-D gels that cover the pH range of approximately 3.5-10.0 were prepared for all specimens.

5 Tissue was solubilized by addition of lysis buffer consisting of (per liter) 8 M urea, 20 ml of Nonidet P-40 surfactant, 20 ml of ampholytes (pH 3.5-10), 20 ml of 2-mercaptoethanol, and 0.2 mM of phenylmethylsulfonyl fluoride in distilled deionized water. Approximately 30 μ l aliquots containing 70 μ g of protein were loaded on individual gels.

10 Because isoelectric focusing is sensitive to charge modification, it is important to minimize protein alternations (e.g., proteolysis, deamidation of glutamine and asparagine, oxidation of cystine to cystic acid, carbamylation) that can result from improper sample preparation. Once solubilized, samples may be stored frozen at -80°C for short periods (<1 month) without significant
15 protein modification).

2-D PAGE was done as previously described (Strahler et al, Journal of Clinical Investigation, 85:200-207, 1990). In most cases aliquots were immediately applied onto isofocusing gels. First-dimension gels contained 50 ml of ampholytes per liter (pH 3.5-10). Isofocusing was done at 1,200 V for
20 16 h and 1,500 V for the last 2 h. 20 gels were run simultaneously. For the second-dimension separation, an acrylamide gradient of 11.4-14.0 g/dl was used. Protein spots in gels were visualized by the silver-staining technique of Merril et al. (Merril et al, Science, 211:1437-1438, 1981).

Example 2

25 Immobilized pH gradient (IPG) 2-D gels of esophageal cancer

In addition to generating 2-D patterns that were carrier ampholyte-based, a second set of patterns using immobilized pH gradients were generated as a complementary set.

30 Samples were prepared as for the CA gels discussed in Example 1. For first dimension separation an immobilized pH gradient covering the separation

range of pH 4-10. The second dimension is the same as for the CA gels of Example 1.

5 IPG gels are prepared using derivatives of acrylamide having carboxyl or tertiary amino groups with specific pK values. A linear pH gradient is prepared from a dense, acidic solution and a light, basic solution using a two-chamber microgradient former. The pH gradient is stabilized during polymerization of the Immobiline®-acryl-amide-bisacrylamide matrix by a co-linear gradient of glycerol. Formulations of buffering Immobiline mixtures with titrating Immobiline for the pH limit solutions for narrow pH gradients (1 pH
10 unit) or for broad pH gradients (>1 pH unit, up to 6 pH units) (Gianazza et al, Electrophoresis 6:113 (1985) and LKB application Note 324 (1984)) have been published.

The second dimension separates proteins on the basis of molecular weight in an SDS gel. An 11.5 to 14% T (2.6% cross-linking) acrylamide
15 gradient provides effective separation of proteins of mass from 15,000 to 100,000. Proteins outside this range are less well resolved. Proteins with molecular weight less than 10,000 Da electrophorese close to the dye front and are not resolved.

Example 3

20 Computer assisted analysis of 2-D gels

Each gel was scanned in a 1024 X 1024 pixel format, where each pixel can have one of 256 possible values representing different degrees of intensity. Spot lists for study images are matched to spot lists of master images so that the result is a hierarchy of matched protein spots. The purpose
25 of the matching is to link the same polypeptide spot through the hierarchy to allow assessment of its present, quantitative variation and specificity, as described in Strahler et al, 1990 (J. Clin. Invest. Vol.85, pp200-207). For comparison, between gels, of the amount of individual proteins, an adjustment process is utilized. The integrated intensity of detected polypeptides,
30 measured in units of optical density per square millimeter, is adjusted relative to the intensity of reference polypeptides that are ubiquitously expressed. The

adjustment is made to compensate for any variation between gels due to protein loading or staining.

Example 4

Proteins that are overexpressed in Barrett's dysplasia compared to normal tissue.

The number of spot differences between Barrett's and normal esophagus was enormous. It was very clear that Barrett's samples were much more similar to gastric mucosa samples, so spots that were larger in Barrett's compared to Gastric mucosa were identified.

Some of the spots present in Barrett's and not in gastric tissue were present in normal esophagus, and these are probably less interesting. They appear at the bottom beginning with spot 6. It should be noted that the spots that are bigger in Barrett's are also big in esophageal adenocarcinoma, since the two sample types represent essentially the same tissue but differ with respect to malignancy status.

Spot 102 is bigger in Barrett's than in normal tissues analyzed.

The large spots 109,110,111 are nearly always present in larger amounts in Barrett's than in either gastric mucosa or normal esophagus. They are also large in esophageal adenocarcinoma.

Spot 33 is always a pretty big spot in Barrett's, and occasionally is about the same in gastric mucosa, but often it is smaller. It would not deserve mention, except that it appears to be larger in esophageal adenocarcinoma.

Spot 61 is nearly always bigger in Barrett's. A spot just to the right is usually larger in gastric mucosa.

Spot 112 is larger in most Barrett's

Spot 113 is larger in Barrett's, but also present as an obvious spot in gastric mucosa. (the spot just to the right of 113 is large in gastric mucosa).

Spot 117 is potentially interesting. It is difficult to find in many gastric mucosas because it is in an area of many small spots.

Spot 115 looks pretty good and is near 117.

Spot 118 is usually bigger in Barrett's. It is quite basic and therefore difficult to compare in all gels.

Spot 44 looks good, but has already been found as an interesting spot because it seems bigger in esophageal adenocarcinoma.

5 Spot 120 is particularly large in Barrett's and esophageal adenocarcinoma.

Spot 121 is an enormous, gray, very basic, 18kD spot in Barrett's and esophageal adenocarcinoma. It is so far to the right on the gel that it is sometimes tough to figure out if it is there or off the gel.

10 Spot 122 is bigger in Barrett's.

Spot 123, very low and acidic, is part of a family of spots bigger in Barrett's.

Spot 124 looks good. It is possible that it is a phosphorylated P18 ("c").

15 Spots 6, 7, and 8 are bigger in Barrett's, but these are HUGE spots in normal esophageal.

Spot 116 is usually bigger in Barrett's but appears large in the esophagus.

HSP27 spots are bigger in Barrett's usually, but they are even larger in normal esophagus.

20 Spot 119 looked exciting, but it is a huge spot from the esophagus.

Summary of spots across Gastric Mucosa (GM), Cancer of the Cardia (CC), Barrett's (B), and Esophageal Adenocarcinoma (EA).

Key:

U= interesting and up

25 i= somewhat interesting (up) but not as convincing as other spots.

n= not so interesting. For EA vs B it usually means the spots stay the same size (bigger than GM).

D = would mean down, but it is not needed for these spots.

30 The first 5 spots were first noticed comparing EA vs B, the remainder from comparing B vs GM. (Perhaps 115 can be noticed in EA vs B as well.)

8

	<u>Spot#</u>	<u>B vs GM</u>	<u>EA vs B</u>	<u>CC vs GM</u>	<u>pH</u>	<u>MW</u>
5	5	n	U	n	4.4	57
	29	i	U	i	6.1	42
	31	n	U	n	5.7	33
	42	n	U	n	6.2	25
	44	U	U	U	5.9	27
	33	U	n	n		
the typical pattern: bigger in B, EA than GM, CC						
10	61	U	n	i	4.4	58
	113	U	n	n	4.4	41
	102	U	n	n	5.7	51
	109	U	n	i	6.0	48
	110	U	n	i	6.1	49
15	111	U	n	i	6.2	50
	112	U	n	n	6.3	53
	117	U	n	i	6.1	41
	115	U	i	U	6.1	39
(even larger in EA than B, but not outstanding)						
20	113	U	n	n	4.4	41
	120	U	n	n	5.8	25
	122	U	n	n	5.2	17
	123	U	n	i	5.0	14
	124	U	n	n	5.6	19
25	121	U			6.8	17

Barrett's spots that can be confidently identified in IPGs:

The comparison of Barrett's to gastric mucosa and normal esophagus for the same patients described above identified some 15 spots that were thought to be increased in Barretts, using gels prepared with isoelectric focusing (IEF) in the first dimension. All of these except for one were present in equal or increased amounts in esophageal adenocarcinoma samples.

Efforts have been made to identify these same protein spots in Immobilized pH gradient (IPG) gels using as guides the spot location on IEFs, spot color using the same silver staining protocol, and whether a similar difference between Barrett's and control material (normal esophagus, gastric mucosa) was observed in IPGs.

Nine of the spots were confidently identified. They had spot numbers 113, 109-111 (a group of spots counted as a single protein), 117, 118, 120, 122, 123, 124, and 44 (which is among the spots reported as being larger in esophageal adenocarcinoma).

Spots 61 and 121 were also confidently identified. The remaining four protein spots (38, 102, 112, 115) were tentatively identified in IPGs.

Changes observed in esophageal adenocarcinoma

This component of the study was initiated by analyzing the patterns of five patients that appeared to be the most outstanding. This is a comparison of Barrett's versus adenocarcinoma. The comparison led to the following findings:

Spot 5	looks increased in EA,
Spot 13	is increased in EA, I
Spot 25	is up in most EA for which it there is good basic-side pattern.
Spot 29	is up in EA in most patients, but is small and tough.
Spot 30	is up in EA for most samples.
Spot 31	is just left of 30, up in EA in all except one patient where it is down.
Spot 32	is up in EA in most patients
Spot 33	is up in EA in most patients
Spot 35	is up in EA, but tough. Most clearly up in a couple of patients.
Spot 38	is up in EA in every sample. It may be PCNA.
Spot 40	is up in EA in two patients.
Spot 41	is way up in EA in some patients. It also appears in esophagus though.
Spot 42	is up in EA. One patient does not show it.

Spot 44 is up in EA in most patients.

Spot 47 It looks rather good.

Spot 50 is up in EA, very acidic, and tough.

Spot 52 is up in EA in all except one patient.

5 Subsequently other gels were reviewed for the above spot differences. All the gels were analyzed but the data was tabulated for only a subset of the clearest gels.

10 The table below shows only a subset of the spots above, in that it includes only the best spot differences. Asterisks appear next to those that were thought to be the most interesting among these.

Symbols in the table mean:

+,++ = increases

-,-- = decreases,

0 = no spot,

15 ? = difficult area/tough to judge,

"s" = little if any difference in spot size.

Patients are identified by three letters.

spot	Wil	Ray	Har	Sta	Sco	Bal	Ing	Pal	Ben	Rea	Ril
P18	++	s	+	+	s	+	s	-	+	+	+
20 NDPK	++	s	+	s	s	s	+	-	+	+	+
38=											
PCNA	++	s	s	s	+	s	++	+	+	+	+
5*	+	+	s	+	s	s	+	+	+	?	++
small but often nearly qualitative diff											
25 13	---	s	+	s	--	+	+	s	s	+	
near actin. not always up.											
29*	0	0	s	+	+	0	+	0	+	++	++
small but often near qualitative											
31*	++	0	s	+	+	+	+	++	++	+	
30 A good spot	never very large though.										
35	+	0	0	+	0	0	?	?	?	+	+

might be good, but small and difficult.

42* ++ + + ++ s ? + ++ ++ ++

Good, sometimes big. Just up and right of "L2" hsp27 form.

44* + + s + + s/+ - s + + +

5 Good, sometimes big always present in Barrett's though.

47 + ? s + + ? - + + + ++

Interesting. A rather streaky, rather basic spot. Hard, not that convincing.

50 ? 0 + + s + s s + + +

10 A very low MW, very acidic protein. Fuzzy. Perhaps partly a function of better sample quality for the tumor samples.

57 ++ s ? + + - - + + + +

A very low MW spot, sometimes large, but down in some.

IPG gels comparing esophageal adenocarcinoma and Barrett's samples.

Initially acidic-loaded IPG's (immobilized pH gradient gels) were run for three patients (cOO71-80) and basic-loaded IPG's for three different patients (cLO25-34) and analyzed separately. As in previous experiments, there are many unique differences for each pair of samples from an individual, some due to varying amounts of various sample impurities, others perhaps idiosyncratic alterations which are true differences but are not shared among most tumors. The difficulty is therefore in finding differences shared among most tumors.

Figure 19 shows 12 spots that were larger in tumors compared to Barrett's in the acidic-loaded gels, for which the protein's identities are not known. (The known spots P18, and NDPKA also appeared larger in the tumors of these patients.). Some of these gels were rather light, so that the confidence in this set was not as great as for the basic-loaded gels, nor is the Est of candidate spots as large.

Figure 20 shows a much larger set of candidate spots thought to be larger in the basic-loaded IPG's. In fact there were so many differences that it caused some concern that a special subset of patients had been selected. The numbering system is consistent with the spot numbering in the acidic-

loaded gels so that spots 15, 18, and 19 are the same spots as indicated in the first figure, however it is not always easy to relate spots between the two sets of gels loaded at opposite ends of the IPG strip.

Two new sets of basic-loaded IPG's were made from which the three
5 best pairs (tumor, Barrett's) of patterns were selected in order to determine which candidate spots exhibited the most reproducible spot changes. From this review there appear to be six spots which are most worth pursuing for sequencing. That these spots are more worthy than some of the others is partially colored by their intensity in the tumors, as this relates to how easily
10 they might be purified and sequenced. Also, in the case of spots 15 and 51 the fact that these spots were previously identified as interesting differences in IEF gels helped keep them on the list.

There are figures containing close-up sections of gels for three patients from one run, and two more from a second run (slightly different conditions),
15 with Barrett's sample on the left and tumor sample on the right.

Spot 29 (approximately 35 kD) is reddish colored.

Spot 51(28kD) has consistently been larger in tumor. On some runs the tumors have given large spots while sometimes it is not as big, suggesting that the protein may be difficult to solubilize.

20 Spot 15 (26kD) has been a very consistent difference, but may be challenging to sequence because the spot is often not very large.

Spot 53 (24kD) has some overlap with another spot which may or may not be related.

Spot 56 (20kD) actual appears as a pair of spots which do seem related, as
25 their sizes appear to be correlated.

Spot 57 (10kD?) is a reddish, very low molecular weight spot. It does not show up very well in the bottom 2 pairs of gels in the figure because it is "in the dye front" a bit, but the difference is apparent on the actual gels.

Most interesting spots in Esophageal Adenocarcinoma compared to Barrett's

30 It follows that studies were performed using two separate methods of first dimension separation, namely isoelectric focusing gels (IEFs) and

Immobilized pH gradient gels (IPGs). We had first singled out 5 spots (IEF5, IEF15, IEF29, IEF31, IEF51) as the most consistent differences in IEF samples, where we searched for spots that were absent or very nearly absent in tissues other than the esophageal adenocarcinoma (Barrett's, gastric mucosa, normal esophagus). Subsequently in studies of IPGs, 6 very interesting spots were identified which were larger in the esophageal adenocarcinoma samples (IPG29, IPG56, IPG57, IPG53, IPG51, IPG15, the last two being identical to spots IEF42 and IEF44 respectively). Spots IEF5, IEF29, and IEF31 were tentatively identified in the IPG gels.

10 The table below summarizes which spots were increased (in both types
of gels) in the esophageal adenocarcinoma compared to Barrett's. "+"
15 indicates clear increase, "-" indicates no difference, and "?" implies difficulty
scoring the spot, usually due to being obscured by another large spot, or in the
case of spot IPG57 because the spot was not well separated from the dyefront
(bottom) of the second dimension gel.

		patient	gel	IPG29	IPG56	IPG57	IPG53	IPG51	IPG15	IEF5	IEF29	IEF31
				type					=IEF42=IEF44			
		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
20	1 Bal	IPG	+	+	+	+	+	-				
		IEF	+	+	+	+	-	+	-	-	+	
	2 Ben	IPG	+	+	+	-	-	+				
		IEF	+	+	+	+	+	+	+	+	+	
	3 Sta	IPG	+	+	+	+	+	+				
		IEF	+	+	+	+	+	+	+	+	+	
25	4 Rea	IPG	+	+	+	+	+	-				
		IEF	+	+	+	+	+	+	?	+	+	
	5 Sco	IPG	+	+	?	-	+	-				
		IEF	-	+	+	-	-	+	-	+	+	
	6 Wil	IPG	+	+	+	+	+	+				

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		14									
5		IEF	+	+	+	+	+	+	+	-	+
	7 Pal	IPG	-	+	+	-	-	-			
		IEF	-	+	+	+	+	-	+	-	+
	8 Har	IPG	+	+	?	-	+	+			
		IEF	+	+	+	-	+	-	-	-	-
10	9 Bit	IPG	+	+	+	+	-	+			
		IEF	+	+	+	+	-	+	?	?	?
	patient	gel	IPG29	IPG56	IPG57	IPG53	IPG51	IPG15	IEF5	IEF29	IEF31
			type	=IEF42=IEF44							
	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
15	10 Lab	IPG	+	-	+	+	+	-			
		IEF	+	-	+	+	+	-	?	?	?
	11 Duc	IPG	+	+	?	-	+	+			
		IEF	?	+	+	+	+	+	+	+	+
	12 Ray										
20		IEF	-	-	?	-	+	+	+	-	-
	13 Ril										
		IEF	+	+	?	+	+	+	+	+	+
	Molecular										
	Weight kD		37	19	10	26	25	27	57	42	33
		pH	4.7	4.3	4.6	6.4	6.2	5.9	4.4	6.1	5.7

Spot 56 actually appears as a pair of spots which do seem related, as their sizes appear to be correlated.

25 Example 5
Analysis of Secreted Proteins

Gels of secreted proteins were prepared from fresh tissue pieces from two new patients for normal esophagus, gastric mucosa, and Barrett's, as well as control gels separating proteins in the media used to culture the cells. These patterns of "secreted proteins" mixed with media proteins (serum) are promising. However, the limitation here is that this study requires fresh samples and therefore the number of samples is bound to be small. The results to date are that a group of spots numbered 109-111, which were found to be increased in Barrett's relative to normal esophagus and gastric mucosa, are quite large in the Barrett's "secreted proteins" samples compared to control samples.

Example 6

Sequencing

There are a total of at least some twenty spots that are of interest to sequence and identify. The spots are eluted from the gels and subjected to sequence analysis.

A number of potential markers described above on the basis of their estimated isoelectric point (pI) and molecular weight, have been further characterized with respect to their N-terminal sequence. This information has led to the identification of some of the proteins based on their matching with protein databases and to novel sequences for others. The data is shown below.

Spots which have been sequenced

- | | |
|--------------|---|
| SEQ ID NO. 1 | 101, MW 52 kD, pH 5.8, cytokeratin 8, cleaved
based on the sequence T?GPRAF??R |
| | 111, MW 50 kD, pH 6.2, albumin, cleaved |
| | 109-111 are a train of related spots. 11 identified as a cleaved albumin based on sequence |
| SEQ ID NO. 2 | 120, MW 25 kD, pH 5.8, albumin, cleaved
also appears to be a cleaved serum albumin based on the sequence DAHKSEV |
| SEQ ID NO. 3 | 124, MW 19 kD, pH 5.6, possibly related to collagen |

sequence XXAPLTATAP. No match in data base but APLTAT matches part of collagen

29(IPG), MW 37 kD, pH 4.7, HS72 or HS7C human, cleaved

sequence of GSTRIPKIQKLLQD, which is cleaved HS72 or HS7C human

SEQ ID NO. 5 42, MW 25 kD, pH 6.2, actin capping protein, cleaved

gave sequence QVKGKKNIRATE, which is cleaved actin capping protein

SEQ ID NO. 6 53(IPG), MW 26 kD, pH 6.4, triose phosphate isomerase, cleaved

appears to be triose phosphate isomerase based on sequence GNWKMNQR

SEQ ID NO. 7 (and a minor sequence VGGNWKMN)

SEQ ID NO. 8 56(IPG), MW 19 kD, pH 4.3, no match for sequence

gave sequence (ASQ)VFV(FG)RM?MK but nothing matches in the data base and may be novel

SEQ ID NO. 9 14, MW 50 kD, pH 5.8, moesin or ezrin

internal sequence of moesin or ezrin based on sequence of (LHD)-MGNHELYM

SEQ ID NO. 10 139, MW 20 kD, pH 4.4, amyloid beta (A4) homolog 2 precursor

may be cleaved amyloid beta (A4) homolog 2 precursor based on the sequence MYPELQV(?)D

SEQ ID NO. 11 157, MW 52 kD, pH 4.2, no match for sequence

gave sequence K(?)HKGE(?)N(?)LLRAQP(small)EL. There are no data bases matches for this sequence

SEQ ID NO. 12 164-166, MW 46 kD, pH 4.4, vimetin, cleaved

a train of related spots. Appears to be cleaved vimetin based on the sequence SSVPGVRLLQ

SEQ ID NO. 13 92, MW 5 kD, pH 5.1, possibly cytokeratin 17

based on amino acid analysis this was thought to be cytokeratin 17. Sequence

SEQ ID NO. 14 analysis gave a weak signal of XTXILKFTL and ck17 has N-terminal sequence TTSIRQFTSS, some of which lines up

143, MW 56 kD, pH 5.6, possibly glutathione synthetase

possible glutathione synthetase but the data from amino acid analysis is not strong enough to be certain.

Example 7Antibody production

5 The proteins eluted from the gels as in Example 6, or peptide fragments thereof, may be used as immunogens for the production of antibodies. The antibodies may be polyclonal antibodies or may be monoclonal antibodies. The antibodies are made by methods known to those skilled in the art. Antigen-binding fragments of the antibodies are prepared by methods known to those skilled in the art. Antibodies or antibody fragments with very high affinity and specificity may be used for immunological tests for markers of esophageal cancer.

10 Antibodies or antibody fragments with very high affinity and specificity are used to test body fluids for the presence of the proteins identified above. The body fluids tested may be blood or serum, urine, saliva, tears or other body fluids. The immunoassay may be of any type known to those skilled in the art. The assay may be homogeneous or heterogeneous. The assay may be used with automated machines, or may be functional in lower technology environments.

15 Such a test provides a simple and rapid test to aid in diagnosis of these serious diseases.

20 Example 8In vivo applications

25 The antibodies or antibody fragments produced in Example 7 may be conjugated with a radioactive tag and injected into a patient. With appropriate imaging techniques the tumor can be located using the radioactively conjugated antibody. If the amount of radioactivity attached to the antibody is increased considerably, or the antibody or antibody fragment is conjugated to a toxin, the conjugate can be used to kill tumor cells *in vivo*. The antibody or fragment provides the targeting function, and the toxin or radioactivity kills the cells which are targeted by the antibody.

30 Example 9Gene Therapy

The gene corresponding to tumor specific proteins identified by the method of the present invention may be isolated and identified by techniques known to those skilled in the art. The gene can then be inactivated by molecular biological techniques and replaced into the body by gene therapy.

5 Alternatively, anti-sense molecules can be made to genes of the tumor specific markers, and the anti-sense molecules can be used as therapeutics. By either of the above methods known to those skilled in the art, the tumor specific gene expression is decreased.

SEQUENCE LISTING

<110> Regents of the University of Michigan

<120> Protein Markers for Esophageal Cancer

<130> 58146/00003

<140> PCT/US97/08656

<141> 1997-05-29

<150> 60/018,659

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<170> PatentIn version 3.5

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<223> "X" refers to any amino acid

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 Page 2

phosphate isomerase, cleaved

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1 5 10

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method of diagnosing or monitoring *in vitro* the progression of esophageal cancer disease in a human patient suffering from an esophageal tumour comprising an adenocarcinoma, the method comprising:

a) determining the amount of at least one human protein which is over-expressed in esophageal tumours comprising adenocarcinomas, compared to normal esophageal tissues, in a sample from the patient, the protein giving on 2-D gel electrophoresis of esophageal tumour tissue

an N-terminal amino acid sequence:

(1) Gly Ser Thr Arg Ile Pro Lys Ile Gln Lys Leu Leu Gln Asp and molecular weight in the gel of 37 kD;

an N-terminal amino acid sequence:

(2) Gln Val Lys Gly Lys Lys Asn Ile Arg Ala Thr Glu and molecular weight in the gel of 25 kD;

N-terminal amino acid sequences:

(3a) Gly Asn Trp Lys Met Asn Gln Arg and (3b) Val Gly Gly Asn Trp Lys Met Asn and molecular weight in the gel of 26 kD;

or an N-terminal amino acid sequence:

(4) Xaa Val Phe Val Xaa Arg Xaa Met Lys,

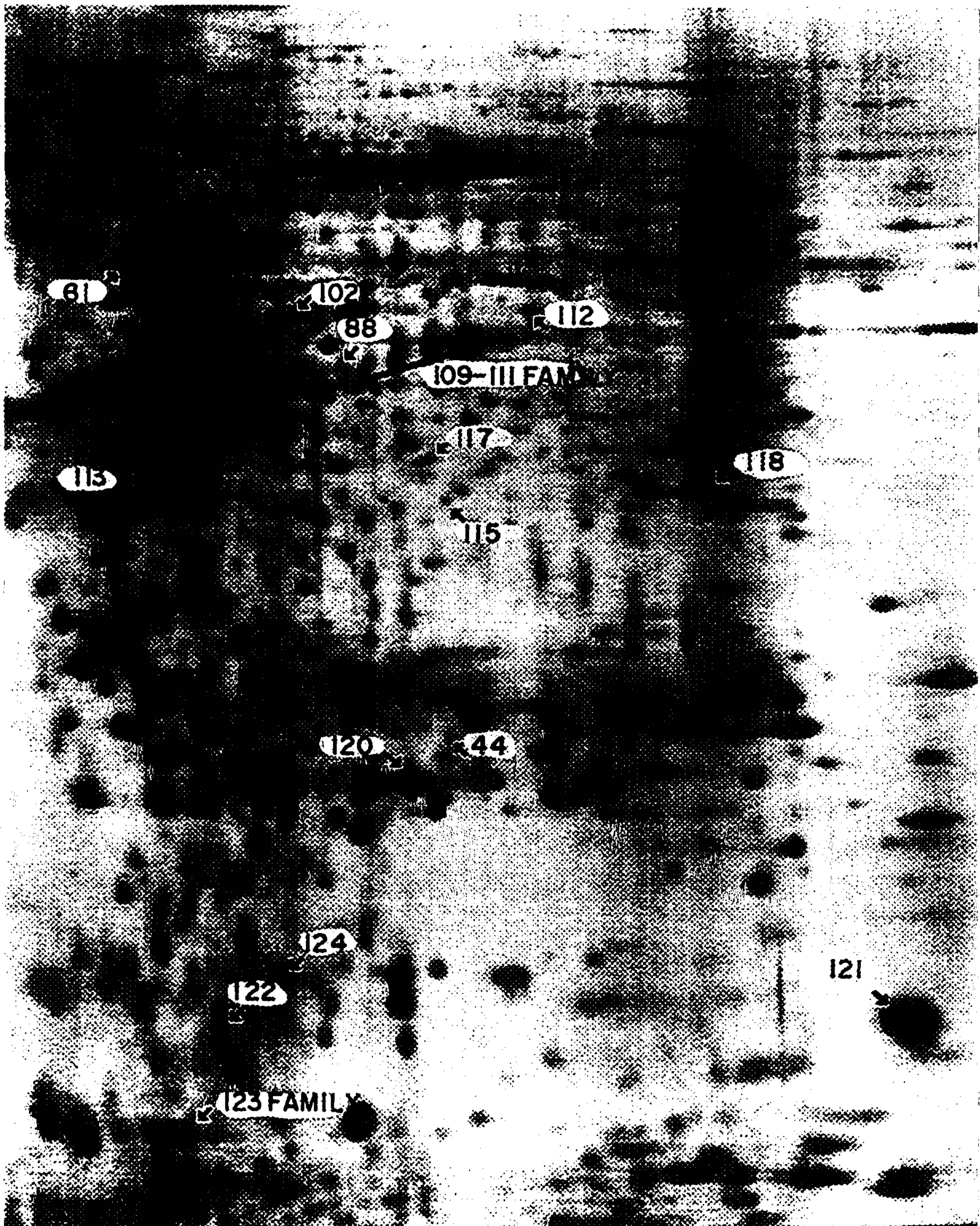
where Xaa is an unknown or incompletely determined amino acid, the first Xaa being Ala, Ser or Gln, the second Xaa being Phe or Gly and the third unknown and a molecular weight in the gel of 19 kD; and

b) correlating the amount of the protein with the presence or stage of the disease.

2. The method according to Claim 1, wherein the determination comprises an immunological assay.

3. The method according to Claim 1, wherein the determination comprises 2-D gel electrophoresis.
4. A conjugated antibody or antibody fragment to a protein defined in Claim 1 with (a) a radioactive tag, for use in an *in vivo* imaging technique to locate a tumour within a human patient or (b) a toxin, for use in killing tumour cells within a human patient.

1/4 3

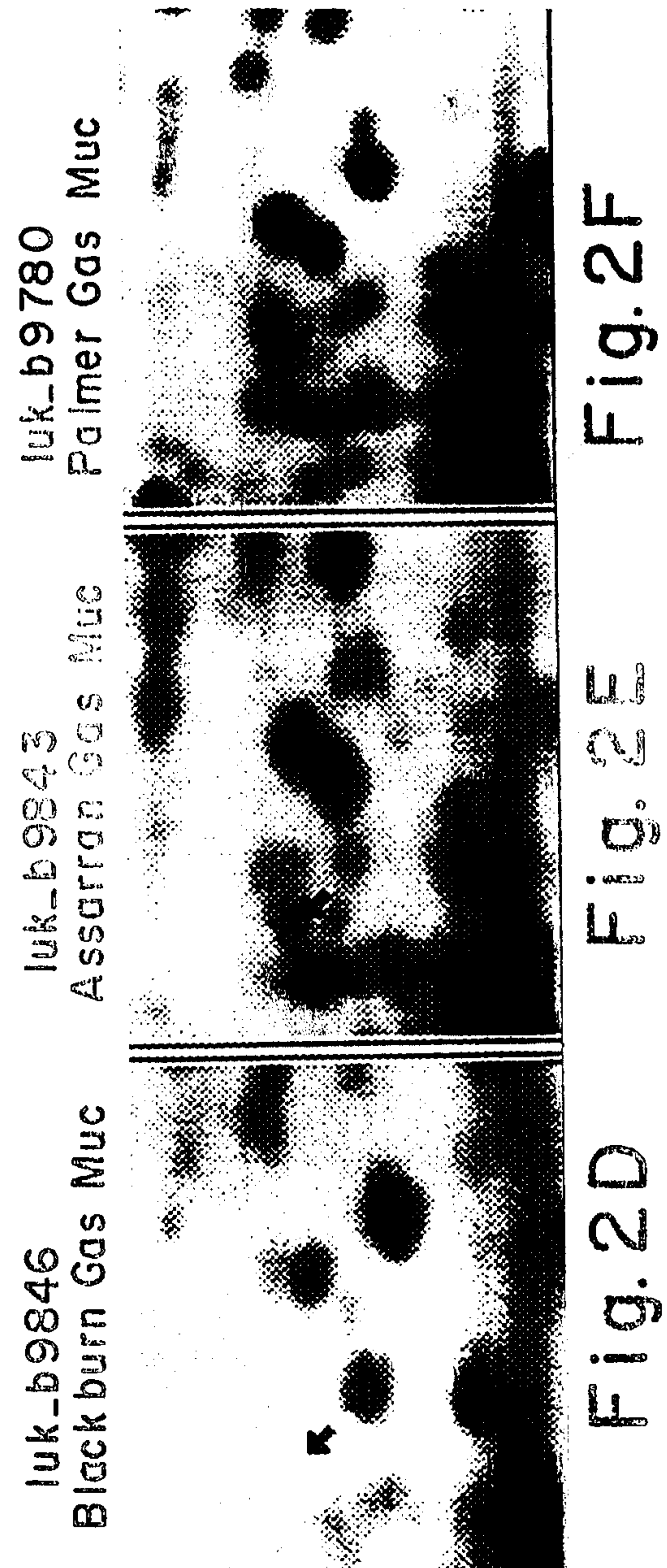


b9778 Palmer, barretts

Fig. 1

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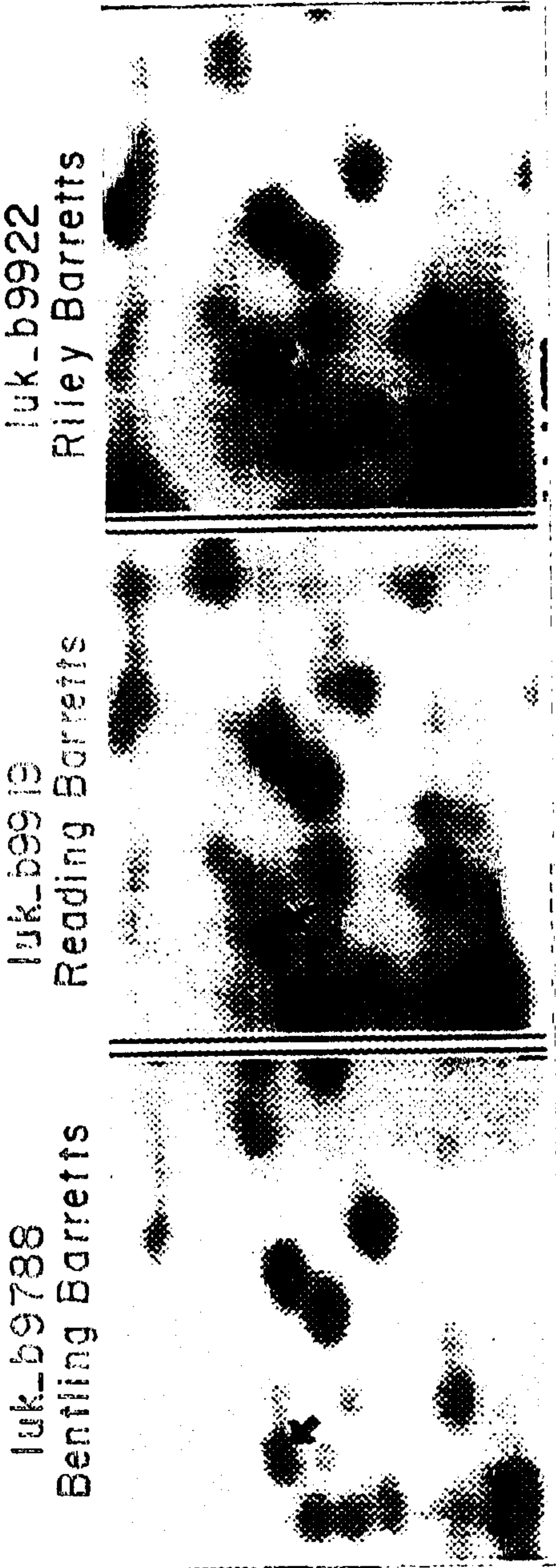


Fig. 2G Fig. 2H Fig. 2I

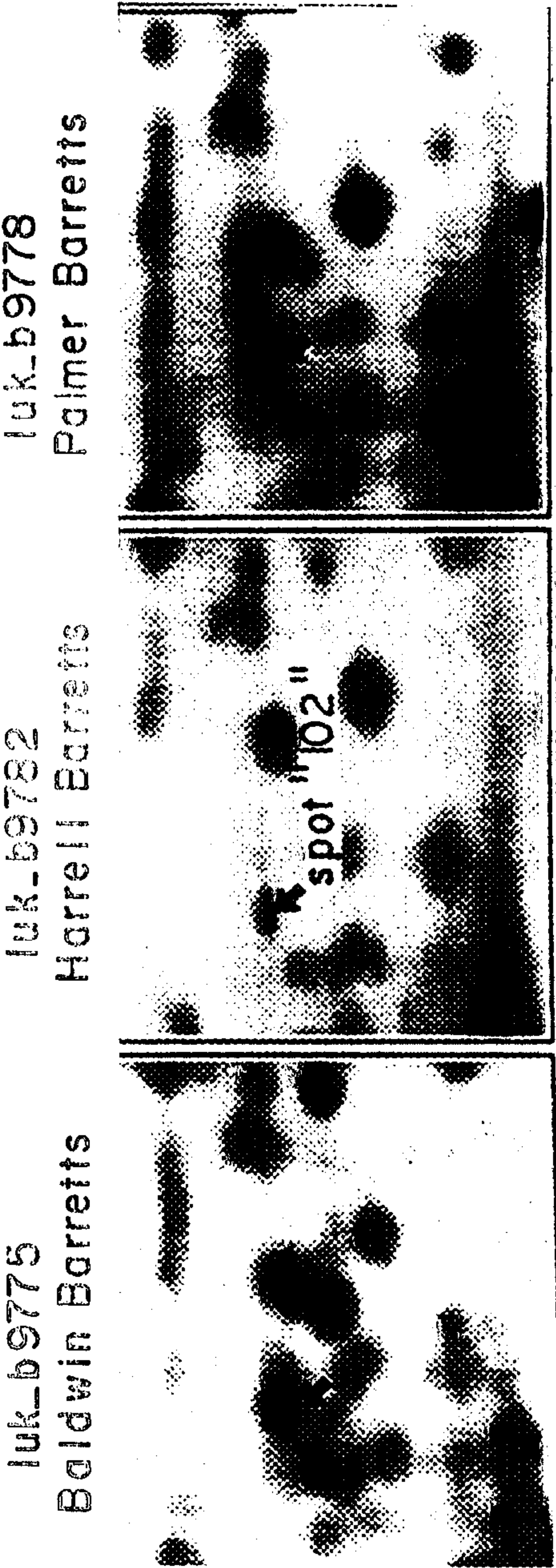


Fig. 2J Fig. 2K Fig. 2L

4 / 43



Fig. 3A Fig. 3B Fig. 3C

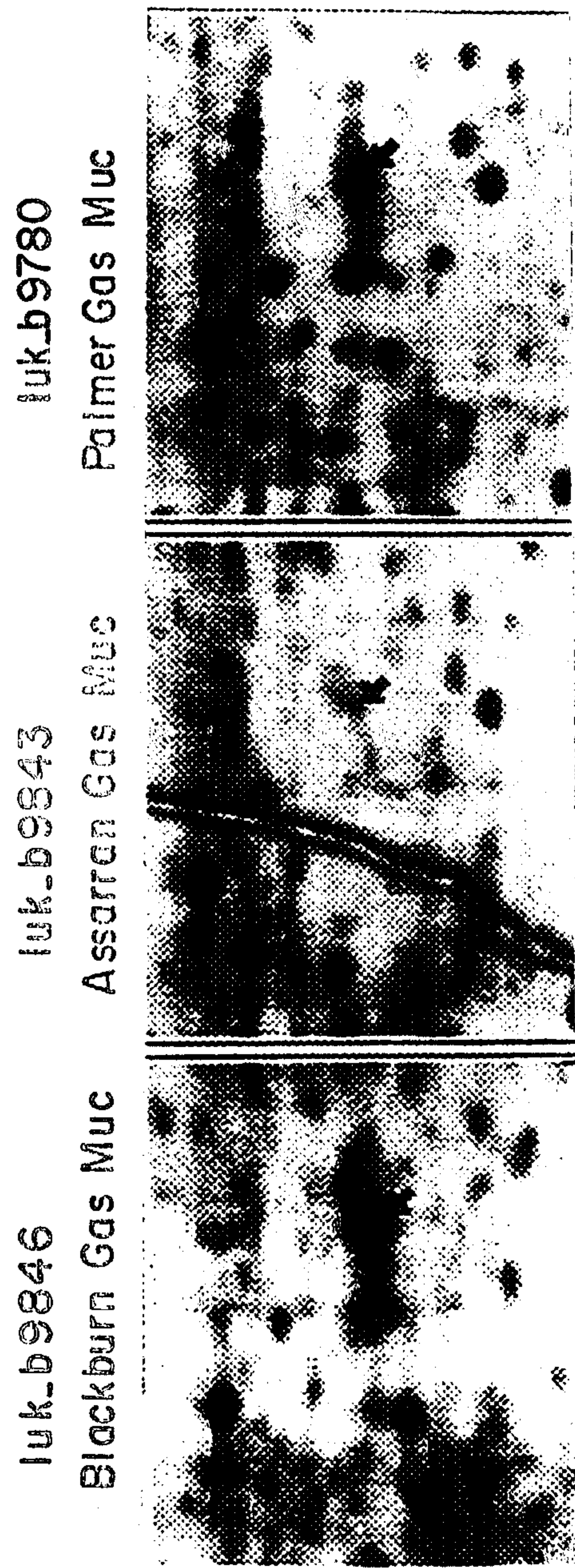


Fig. 3D Fig. 3E Fig. 3F

5/43

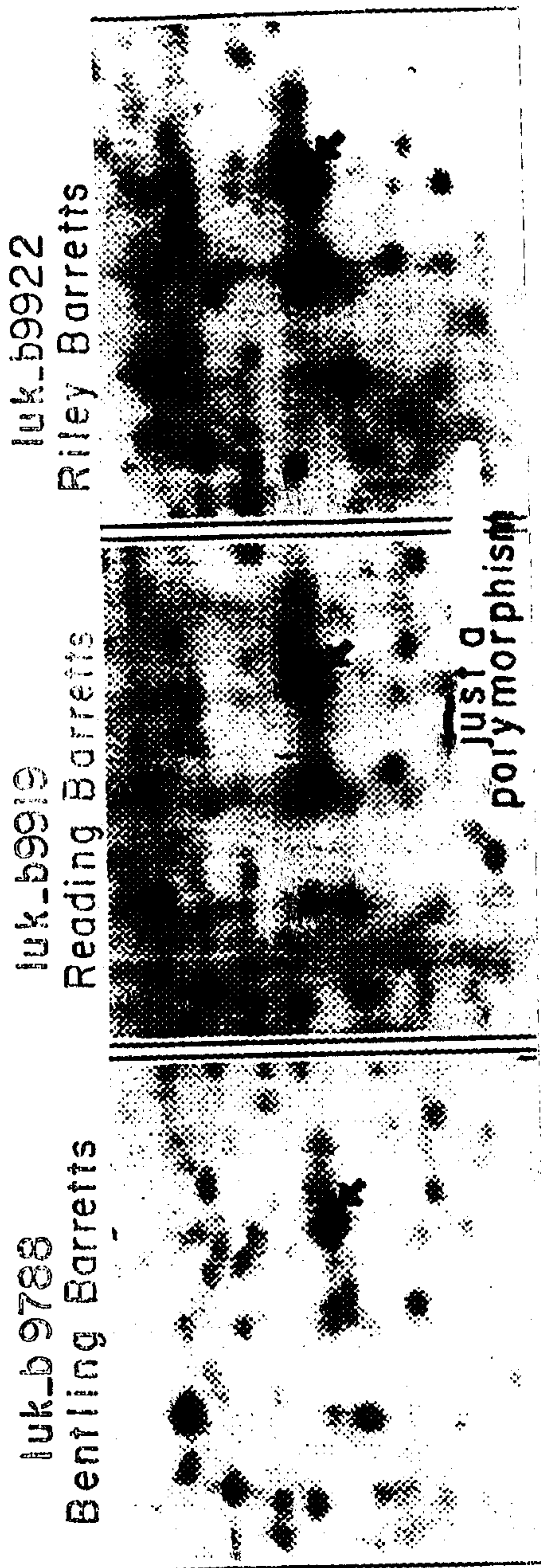


Fig. 3G

Fig. 3H

Fig. 3I

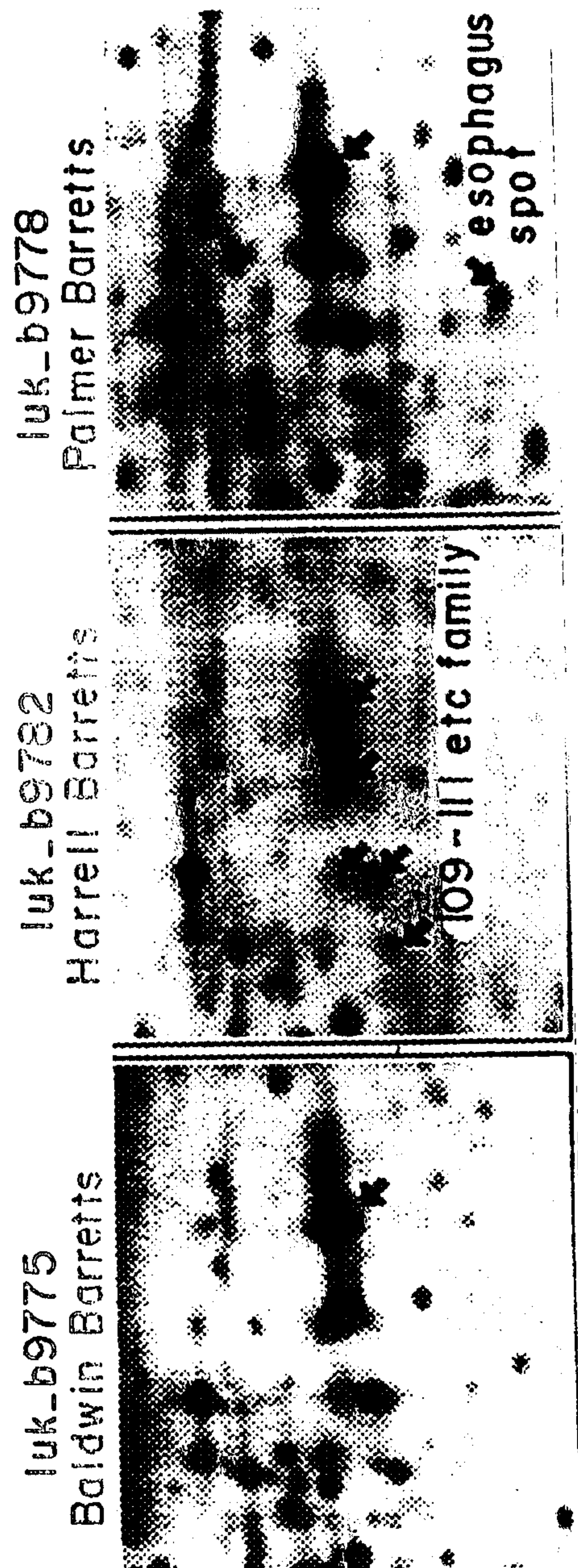


Fig. 3J

Fig. 3K

Fig. 3L

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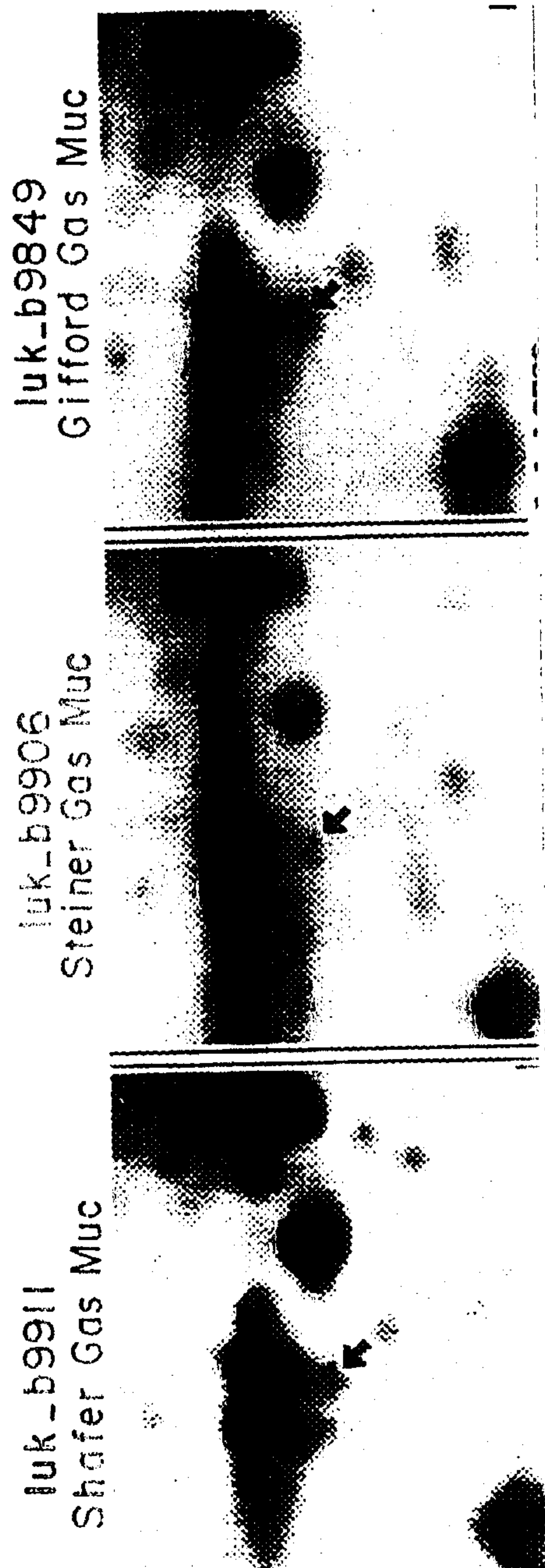


Fig. 4A

Fig. 4B

Fig. 4C



Fig. 4D

Fig. 4E

Fig. 4F

luk_b9788
Bentling Barretts



luk_b9919
Reading Barretts



luk_b9922
Riley Barretts



Fig. 4G

Fig. 4H

Fig. 4I

luk_b9775
Baldwin Barretts



luk_b9782
Harrell Barretts



luk_b9778
Palmer Barretts



Fig. 4J

Fig. 4K

Fig. 4L

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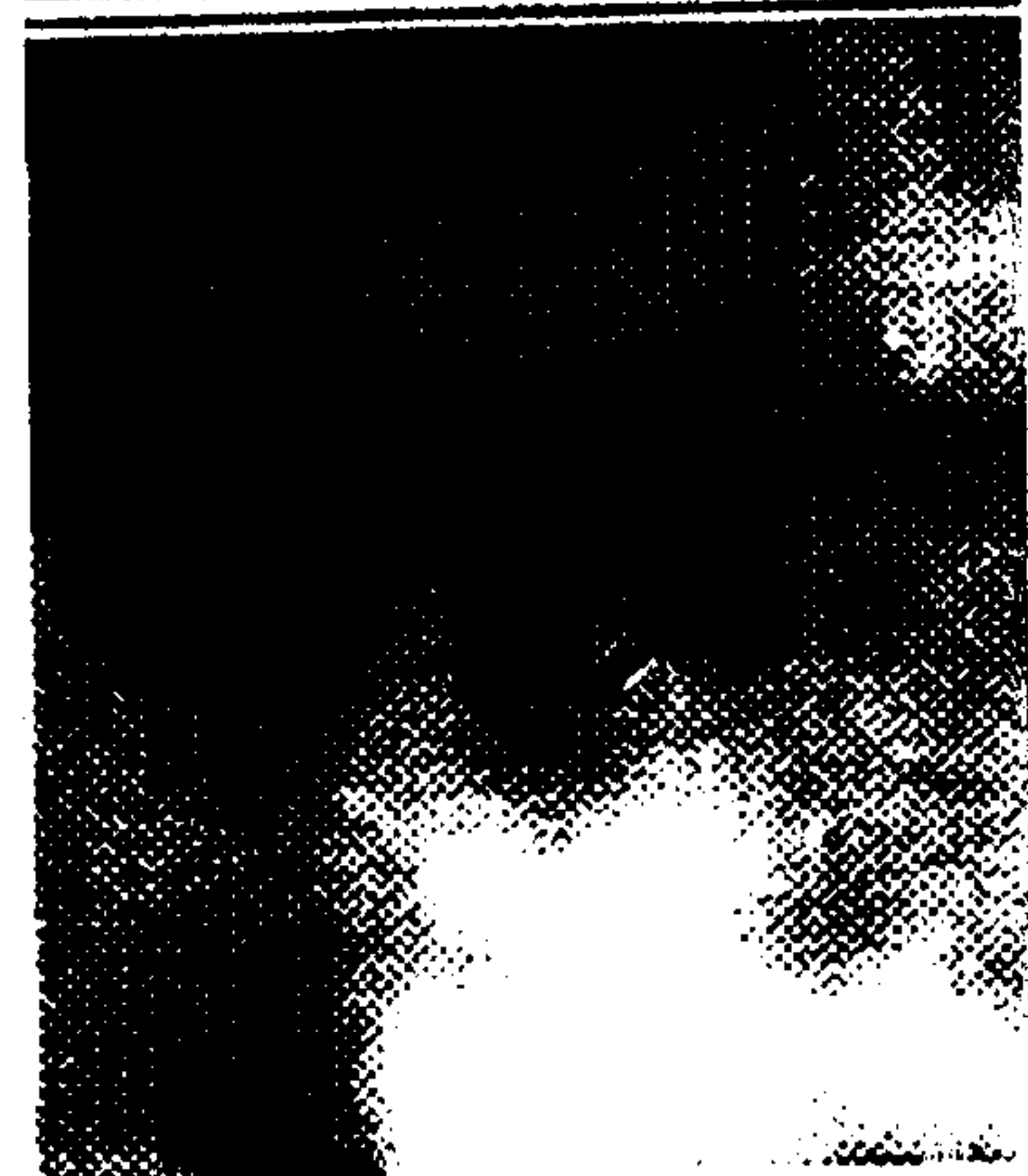
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Steiner Gas Mucluk_b9849
Gifford Gas Muc

Fig. 5A

Fig. 5B

Fig. 5C

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Blackburn Gas Mucluk_b9843
Assarran Gas Mucluk_b9780
Palmer Gas Muc

Fig. 5D

Fig. 5E

Fig. 5F

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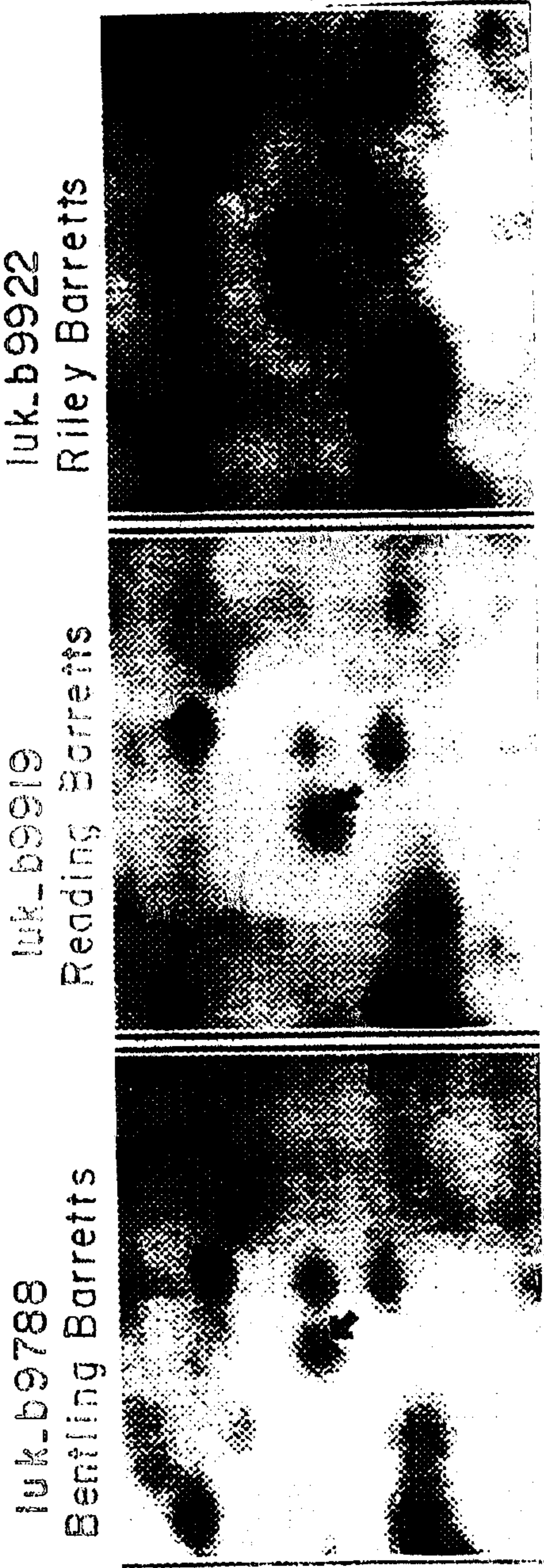


Fig. 5I

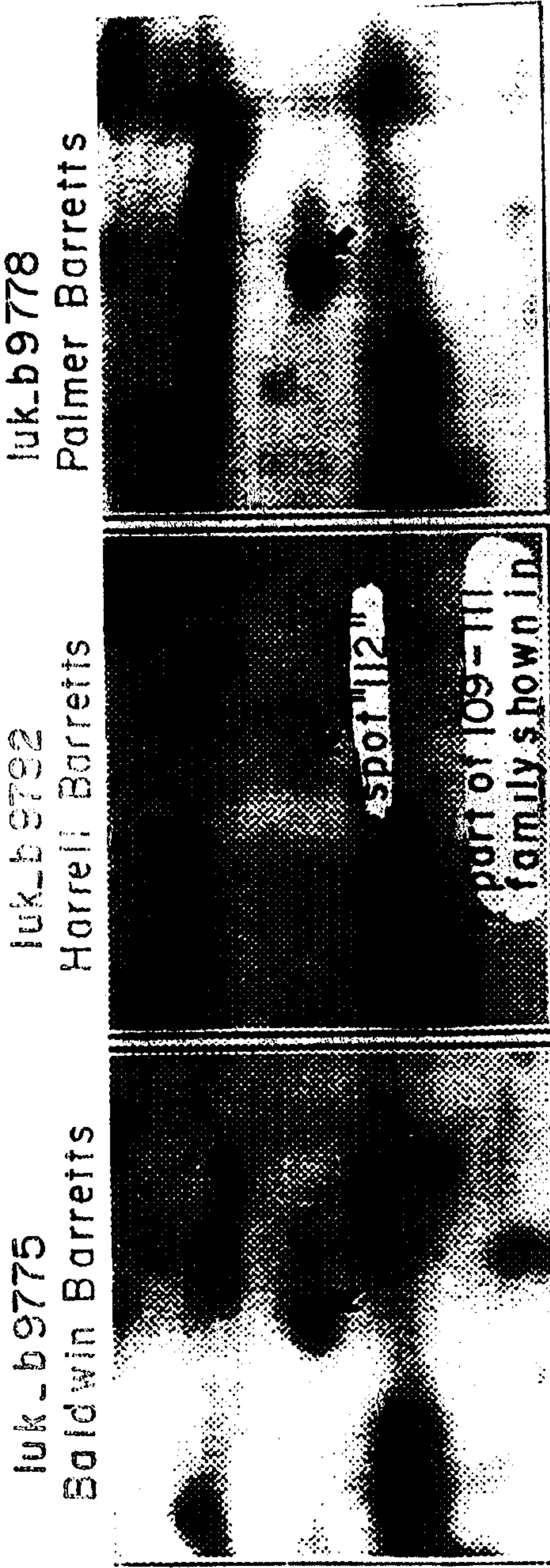


Fig. 5L

another figure
Fig. 5K

Fig. 5J

10/43

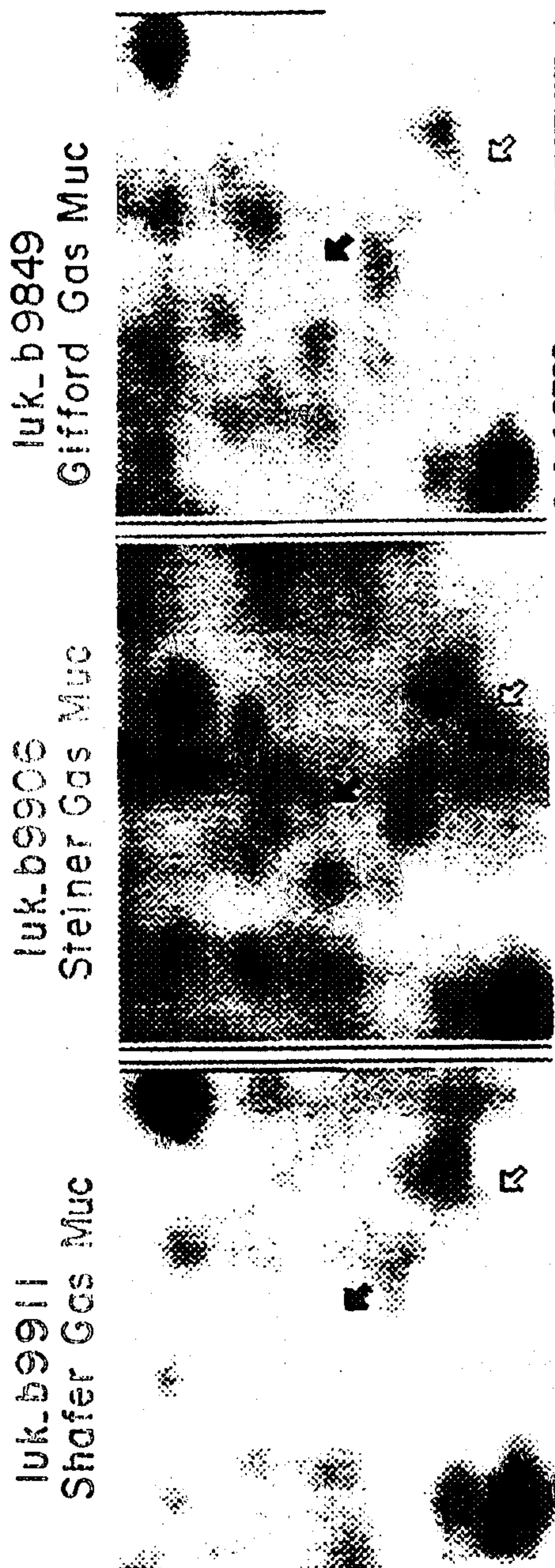


Fig. 6A

Fig. 6B

Fig. 6C

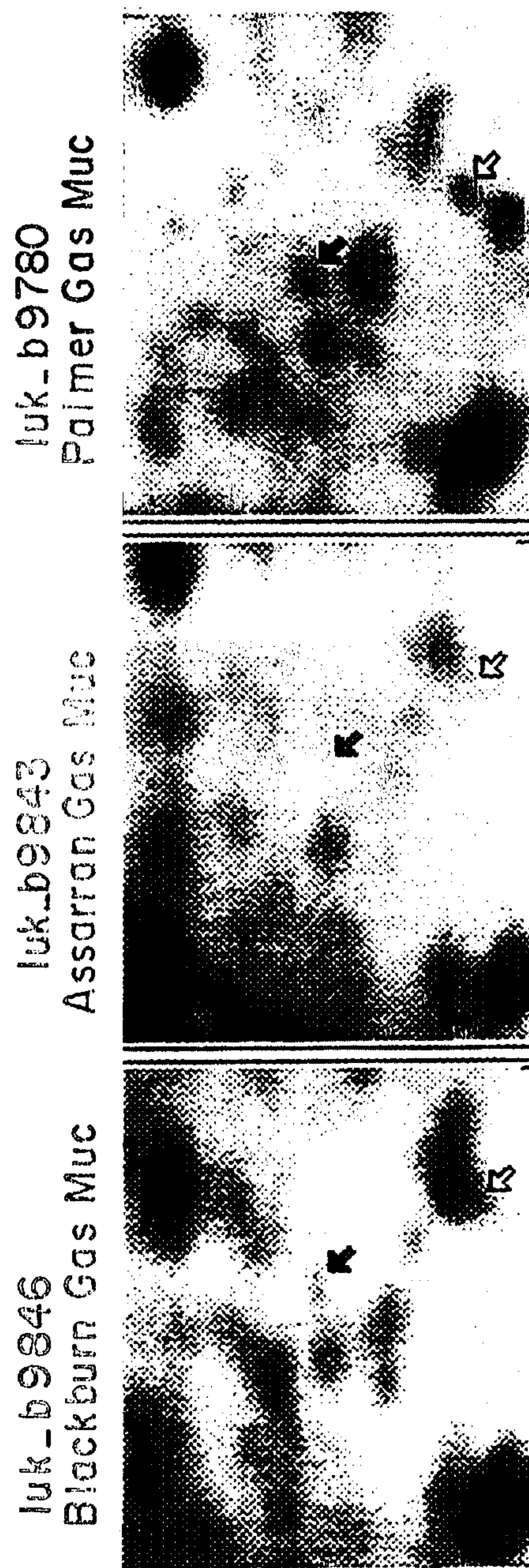


Fig. 6D

Fig. 6E

Fig. 6F

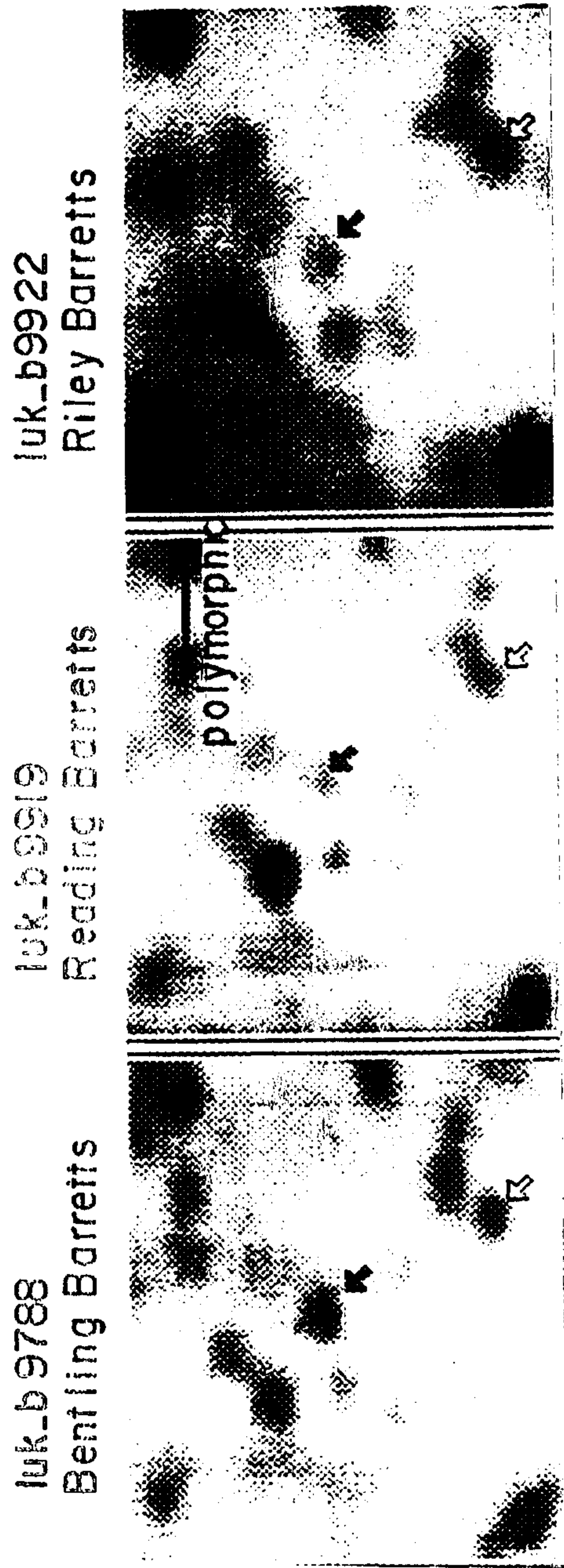


Fig. 6G Fig. 6H Fig. 6I



Fig. 6J Fig. 6K Fig. 6L



Fig. 7A Fig. 7B Fig. 7C



Fig. 7D Fig. 7E Fig. 7F

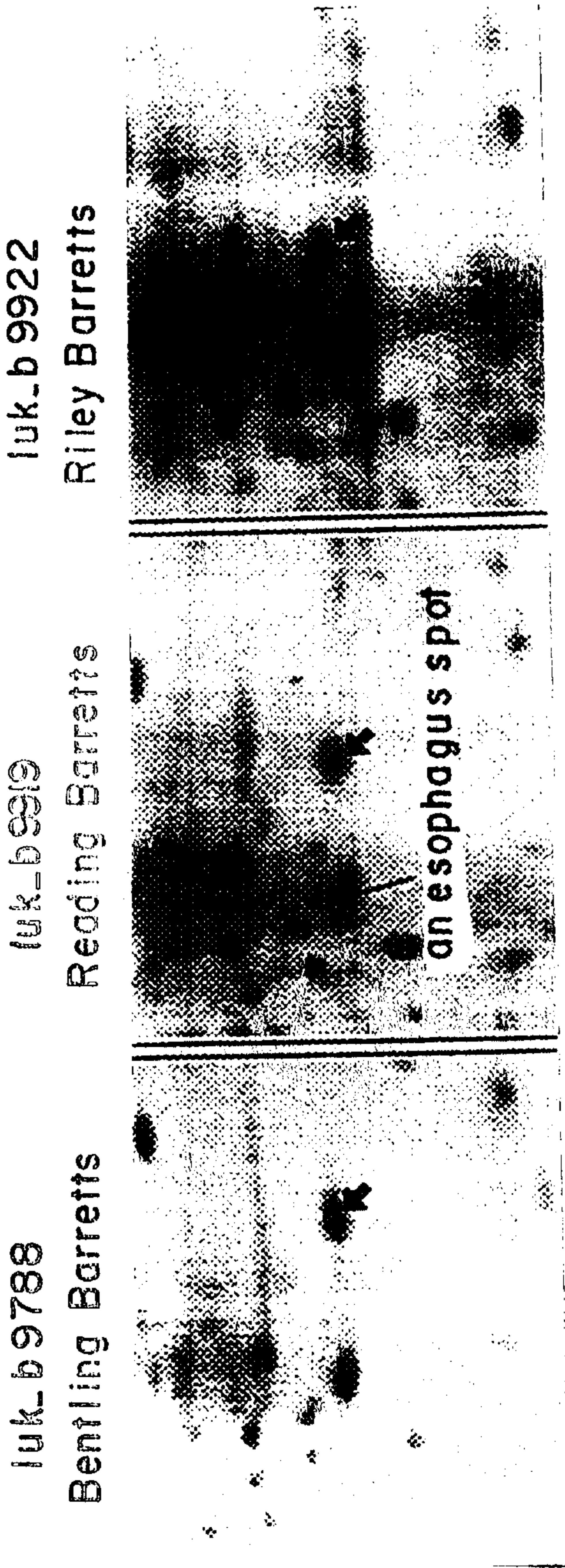


Fig. 7G Fig. 7H Fig. 7I

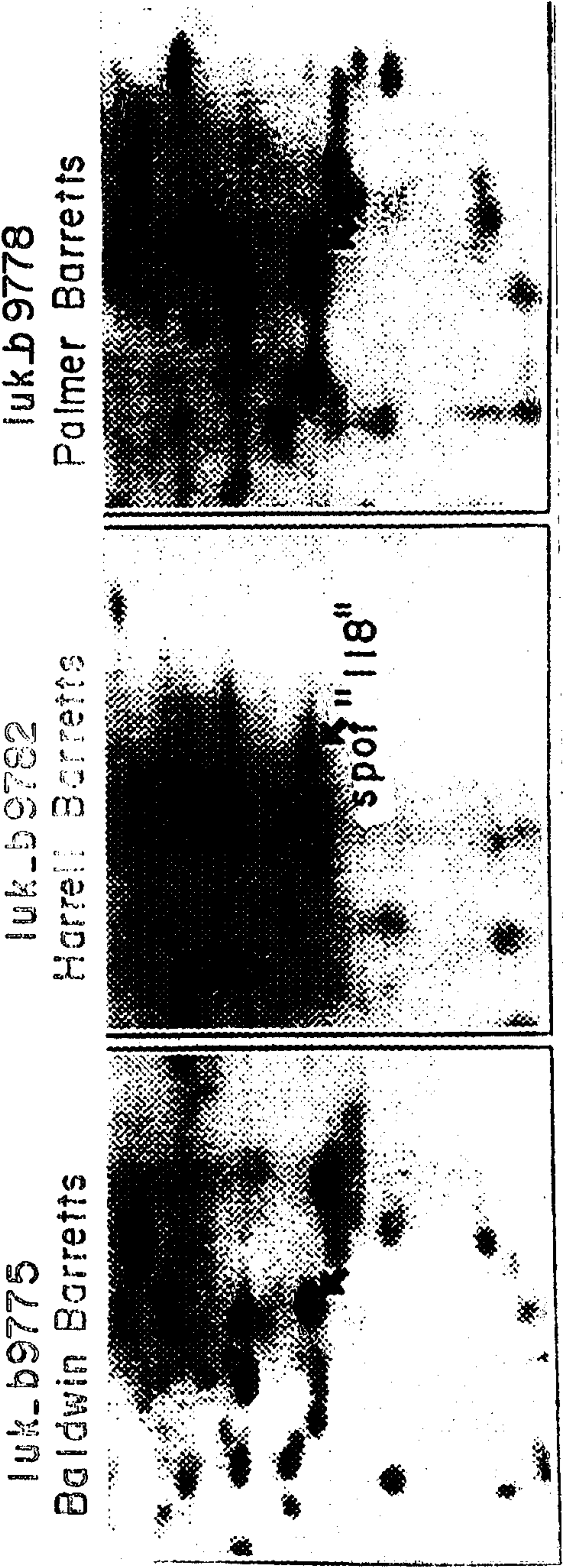


Fig. 7J Fig. 7K Fig. 7L

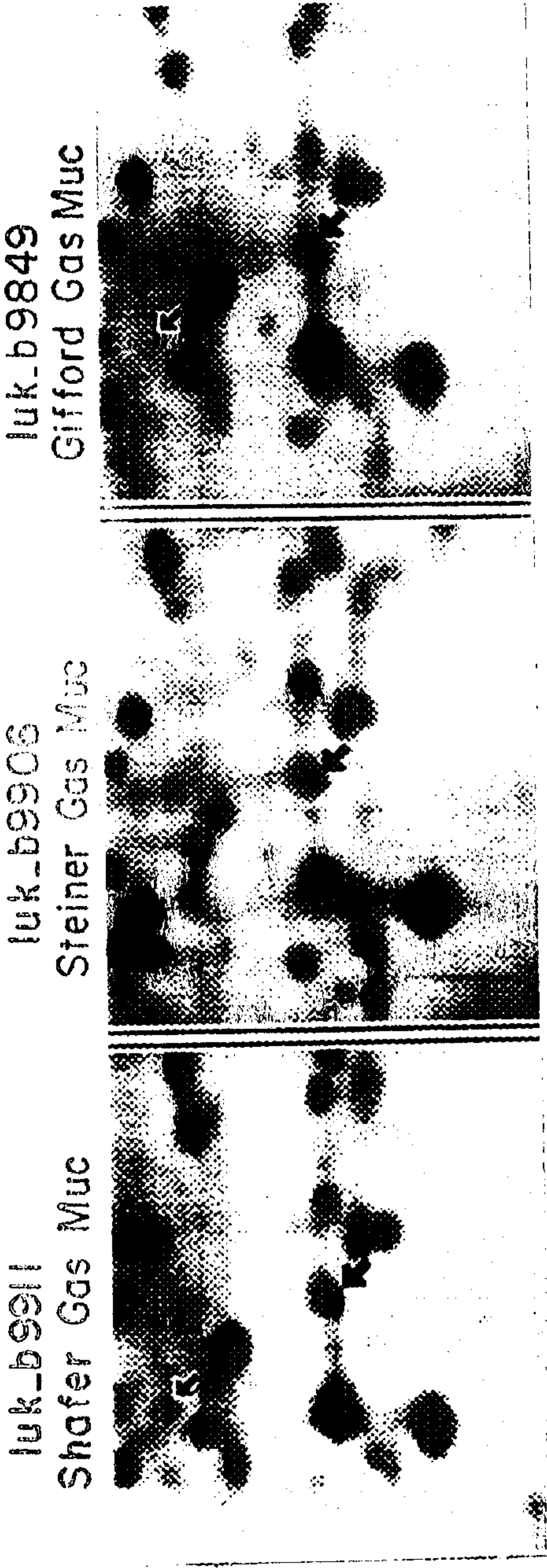


Fig. 8A

Fig. 8B

Fig. 8C

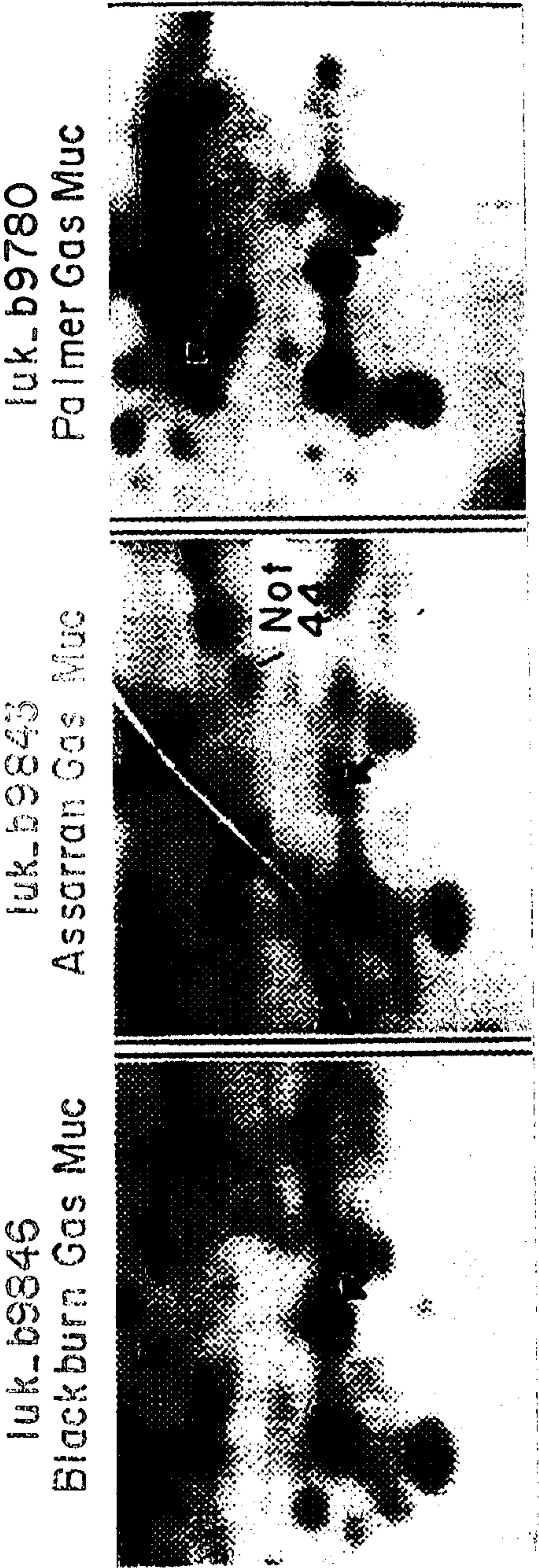


Fig. 8D

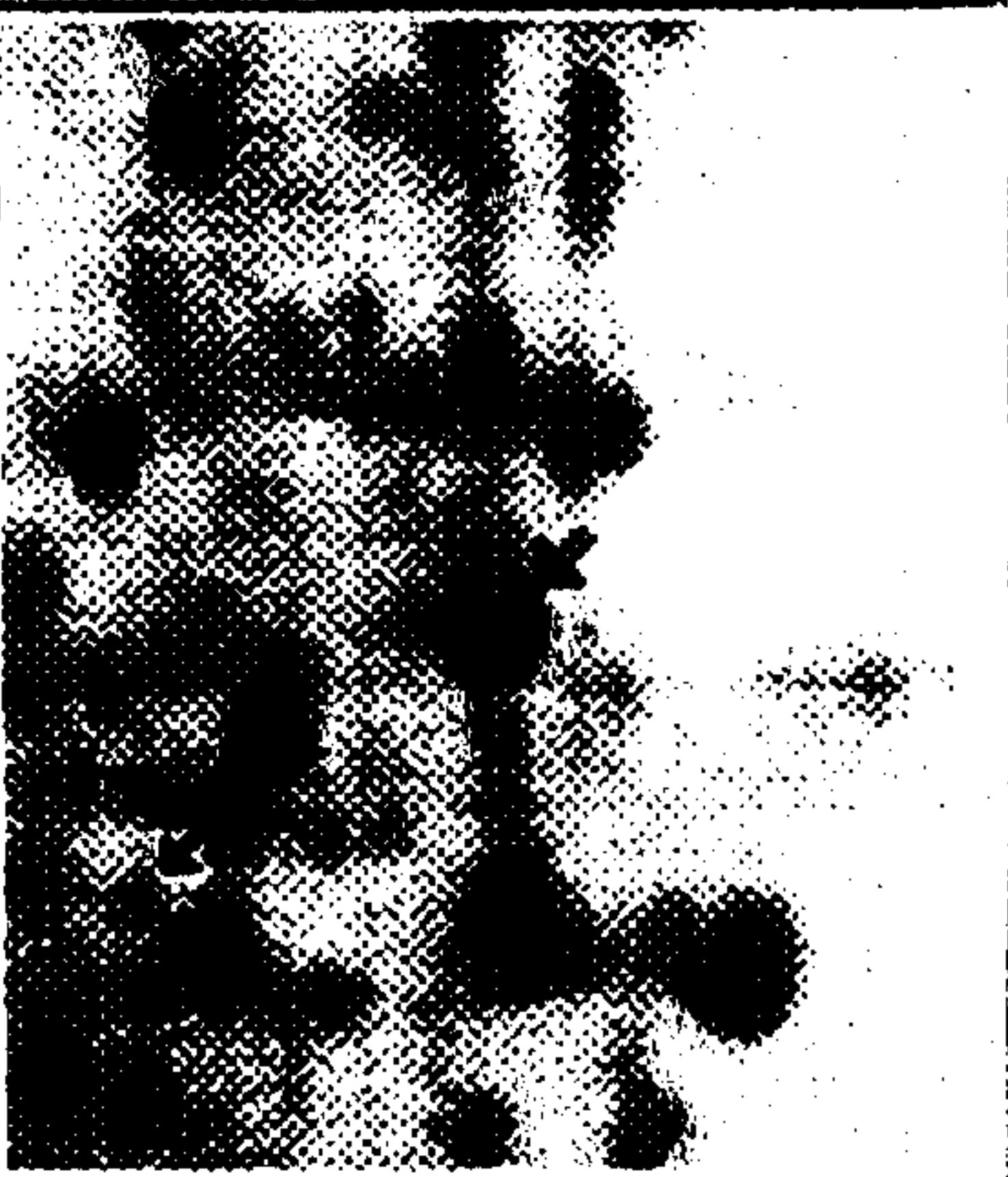
Fig. 8E

Fig. 8F

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Riley Barretts



luk_b9919
Reading Barretts



luk_b9788
Bentling Barretts

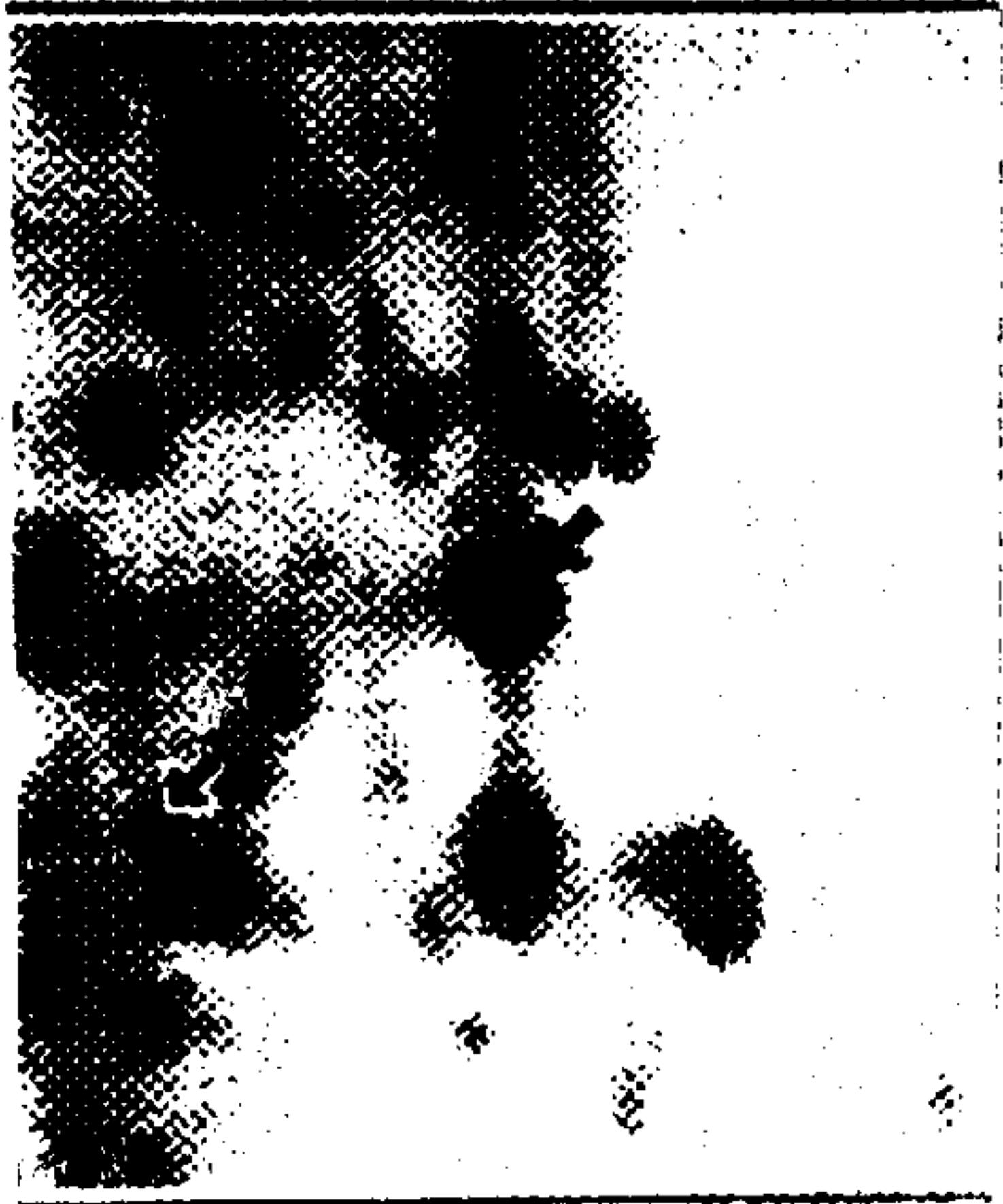


Fig. 8I

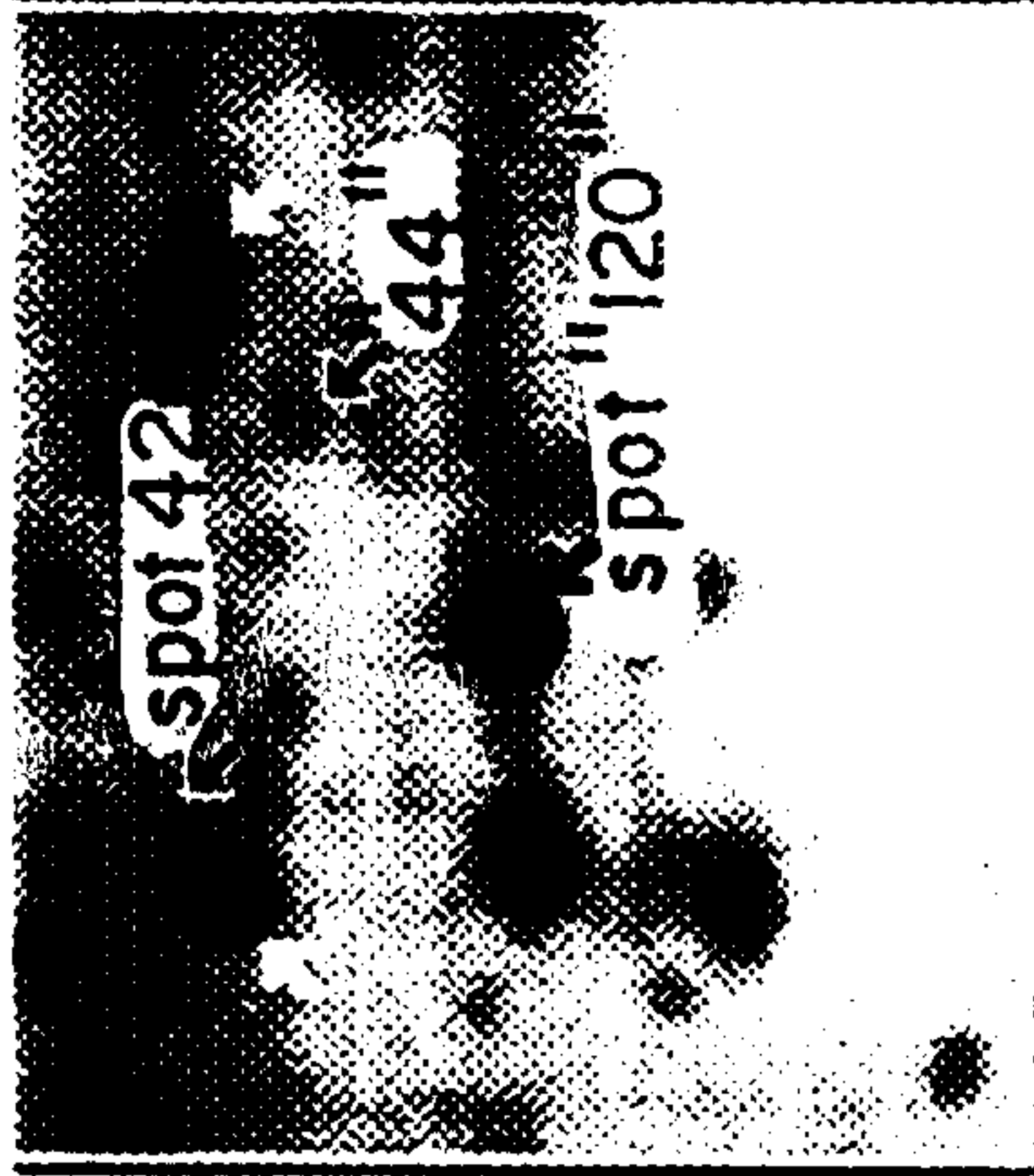
Fig. 8H

Fig. 8G

luk_b9778
Palmer Barretts



luk_b9792
Harrell Barretts



luk_b9775
Baldwin Barretts



WHITE ARROWS
POINT TO 2 OF
THE HSP27's

Fig. 8L

Fig. 8K

Fig. 8J

16/43

luk_b9777 / gel.Rx 838 y
467g 184 OD

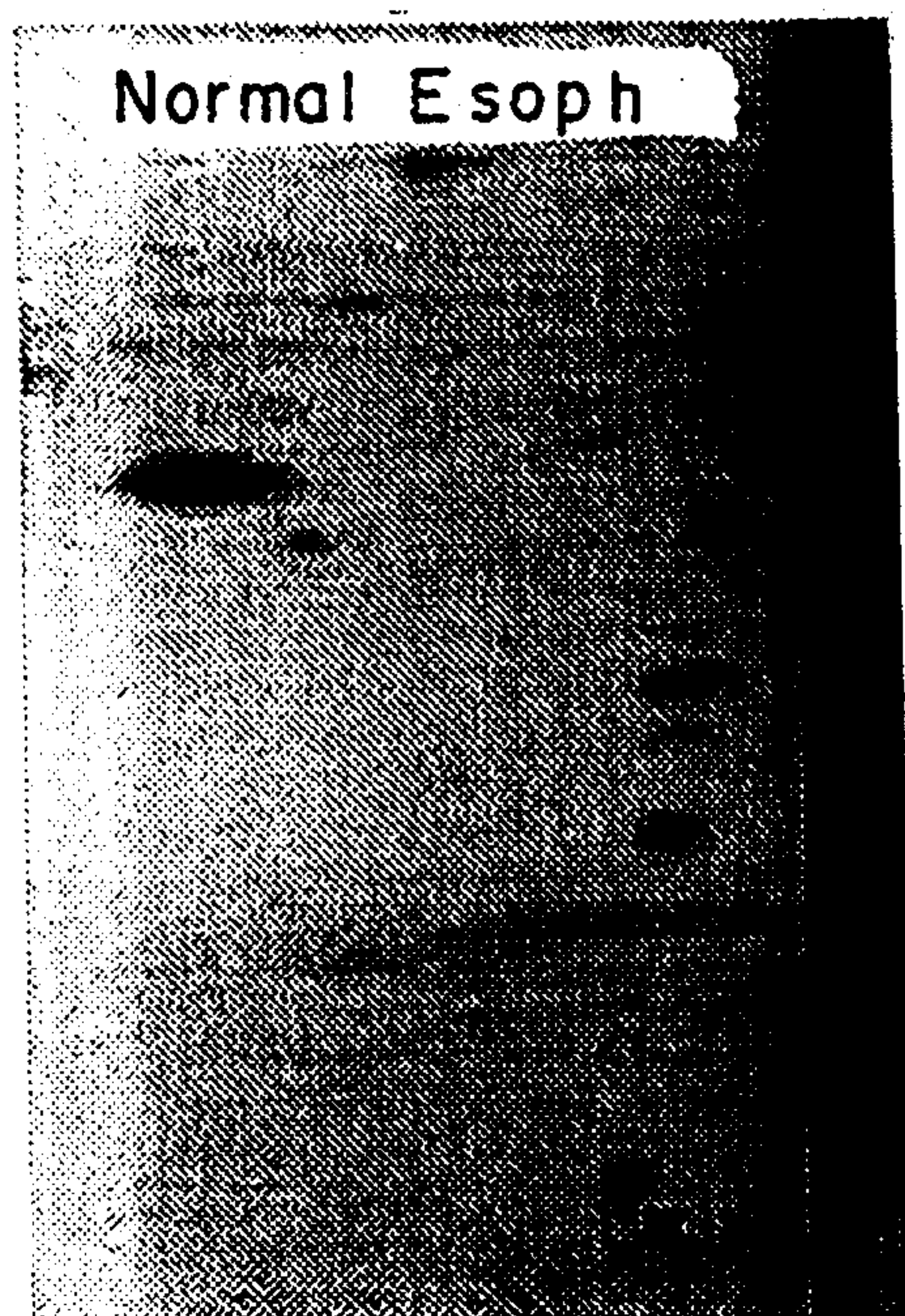


Fig. 9A

Fig. 9B

luk_b9780 / gel.Rx 913 y
695g 216 OD



luk_b9778 / gel.Rx 690 y 455 g
149 OD

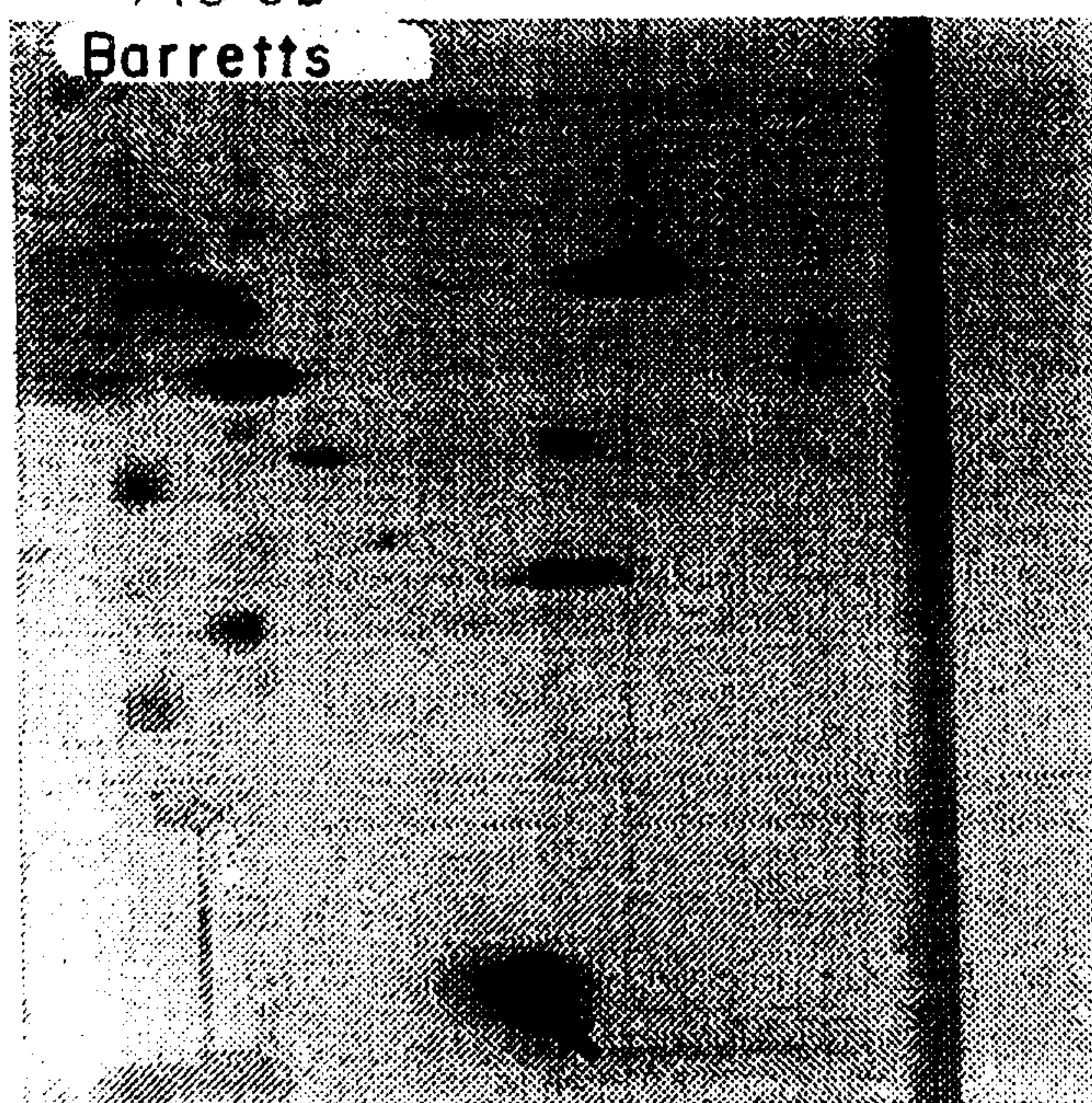
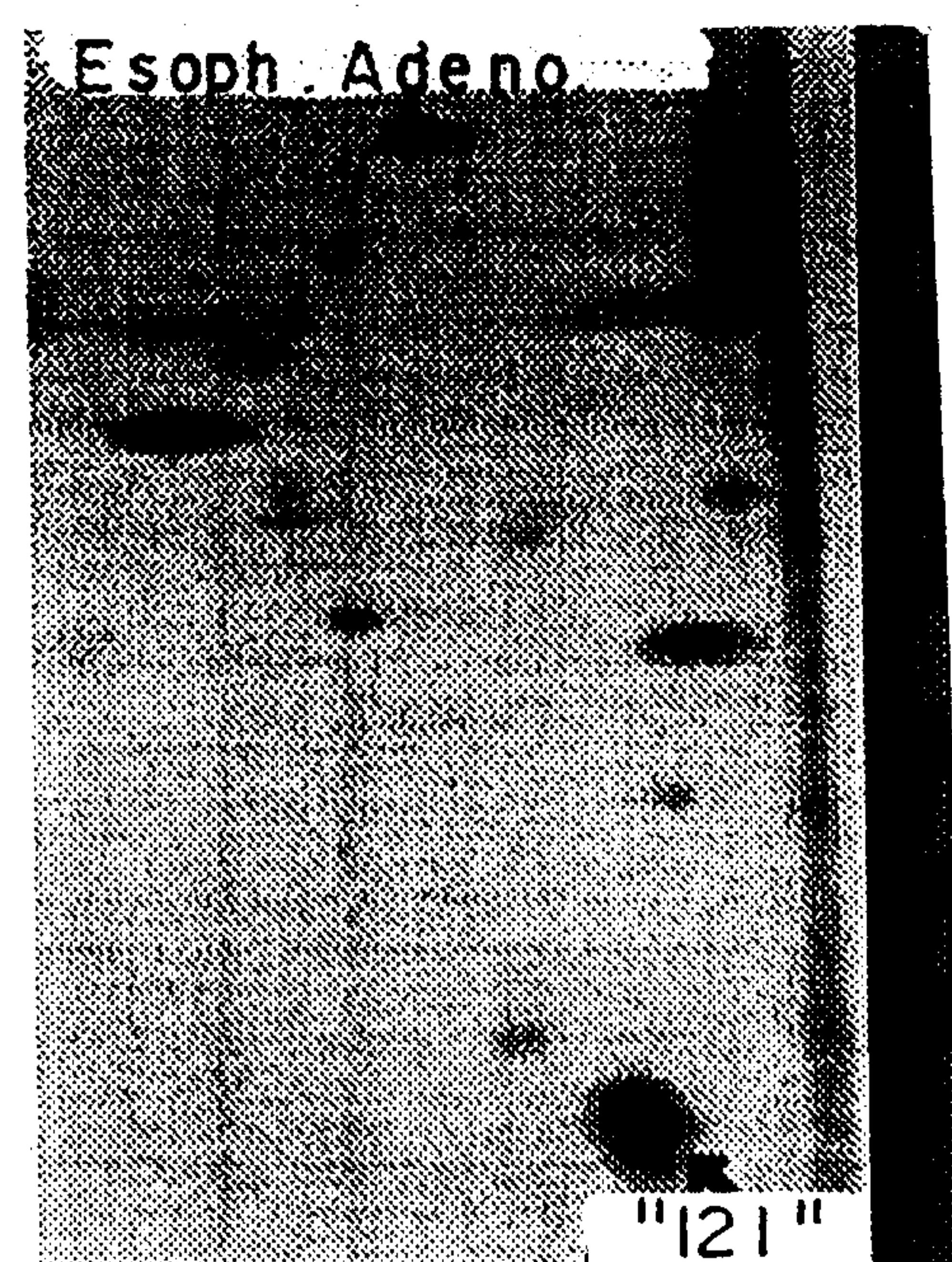


Fig. 9C

Fig. 9D

luk_b9779 / gel.Rx 957 y
784 g 180 OD



17/43

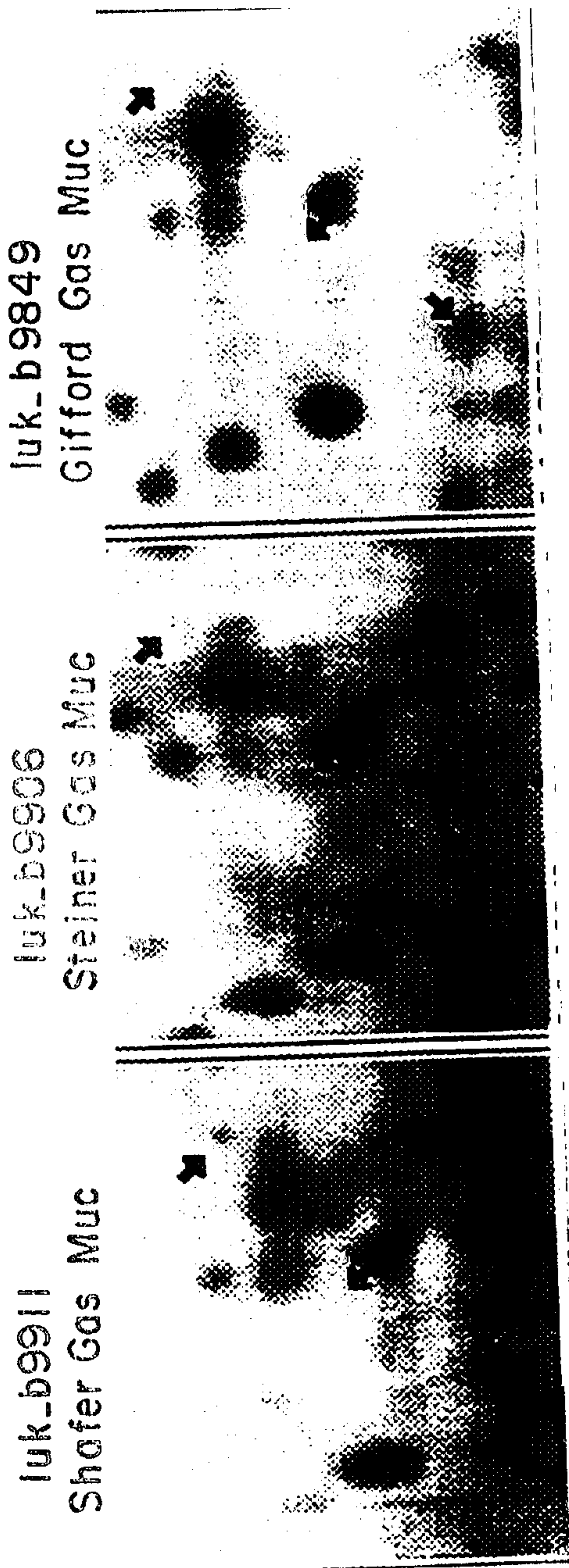


Fig. 10A Fig. 10B Fig. 10C

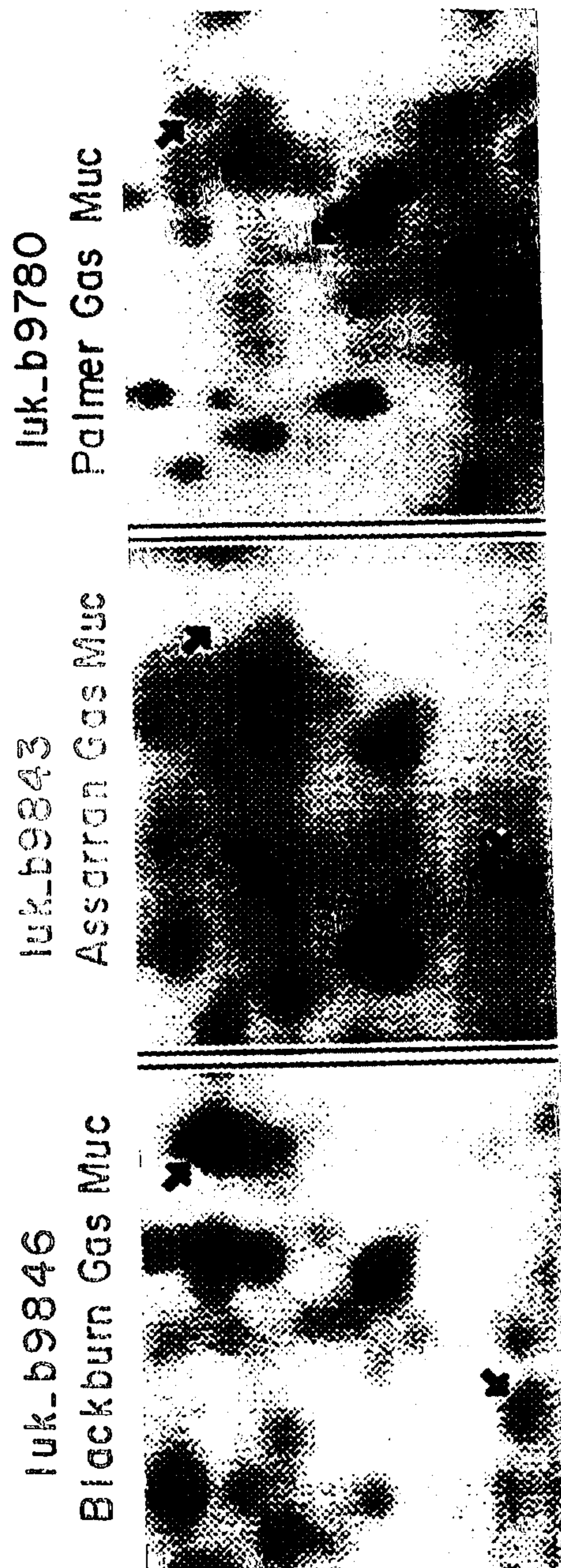


Fig. 10D Fig. 10E Fig. 10F

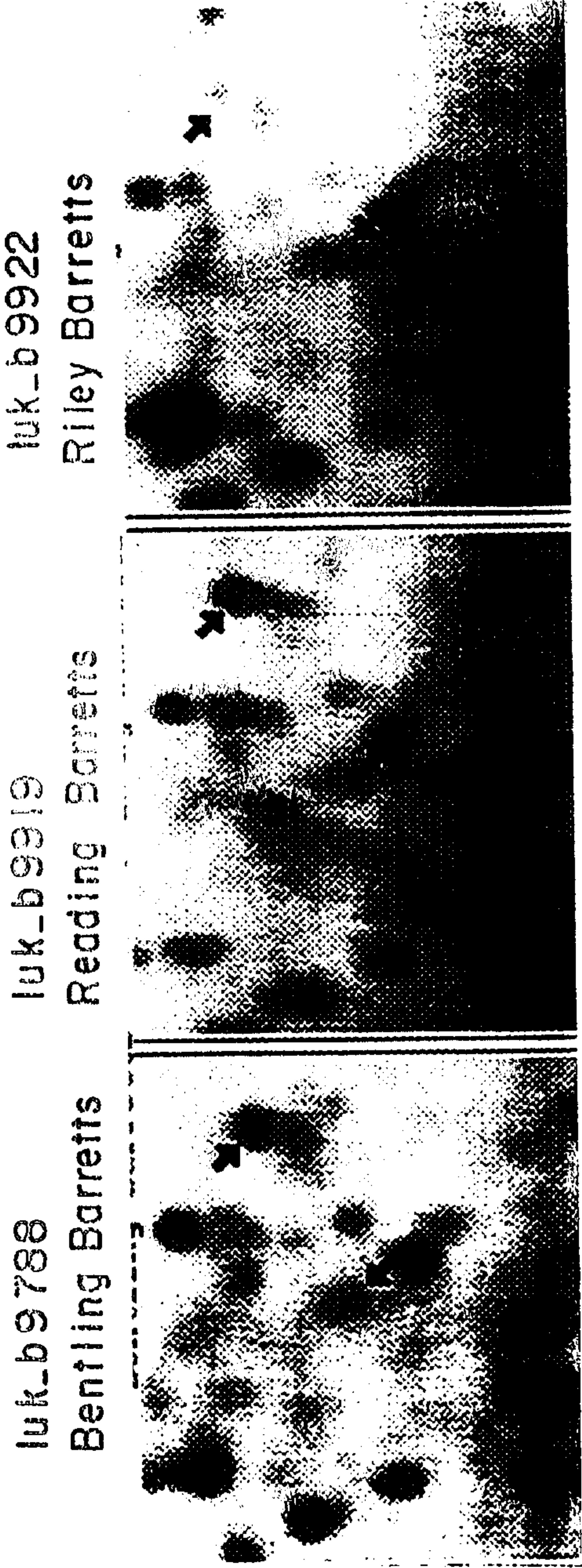


Fig. 10G Fig. 10H Fig. 10I

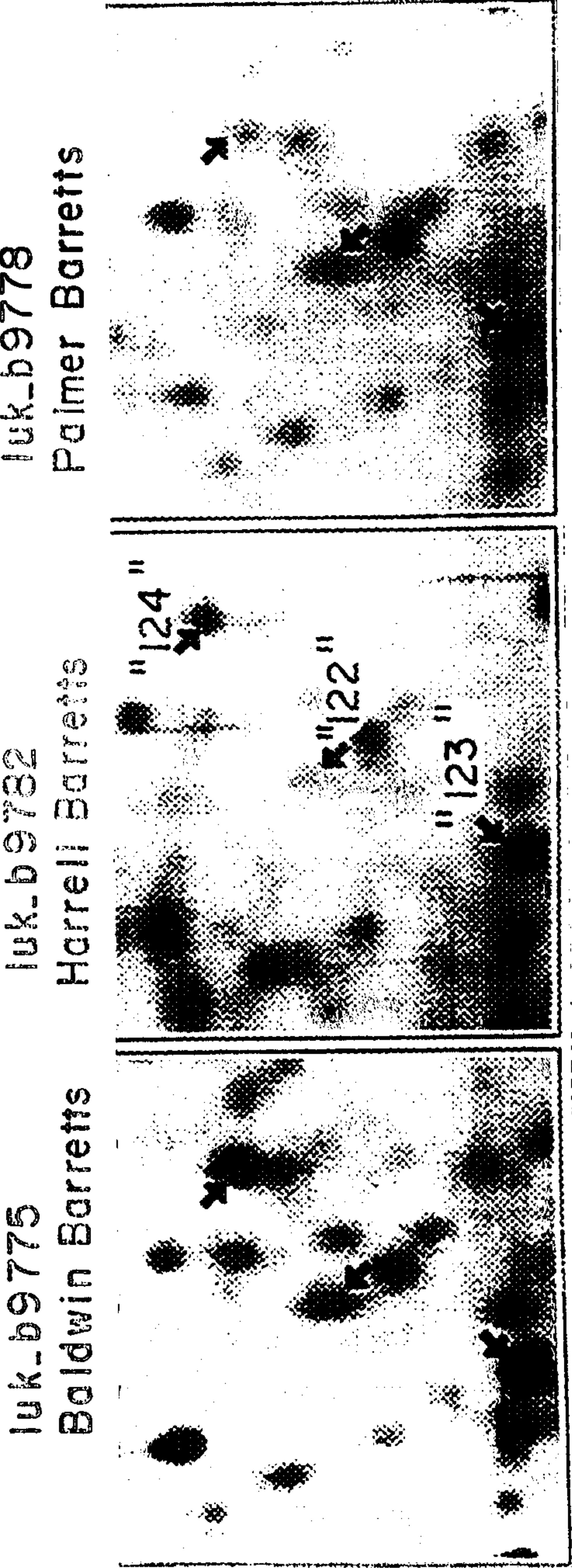


Fig. 10J Fig. 10K Fig. 10L

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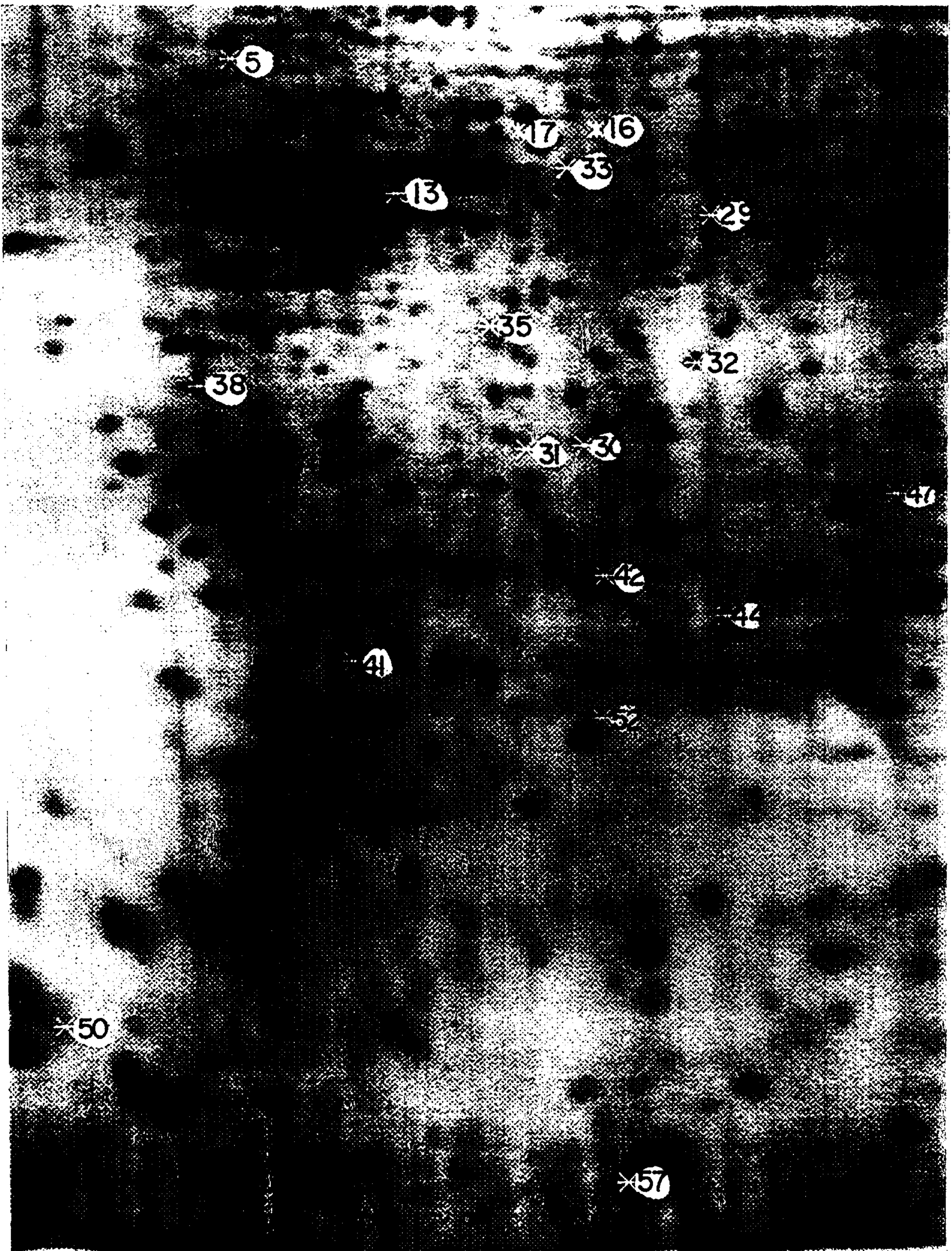


Fig. 11

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b9923 Riley Eso. Adeno.

Fig. 12

SUBSTITUTE SHEET (RULE 26)

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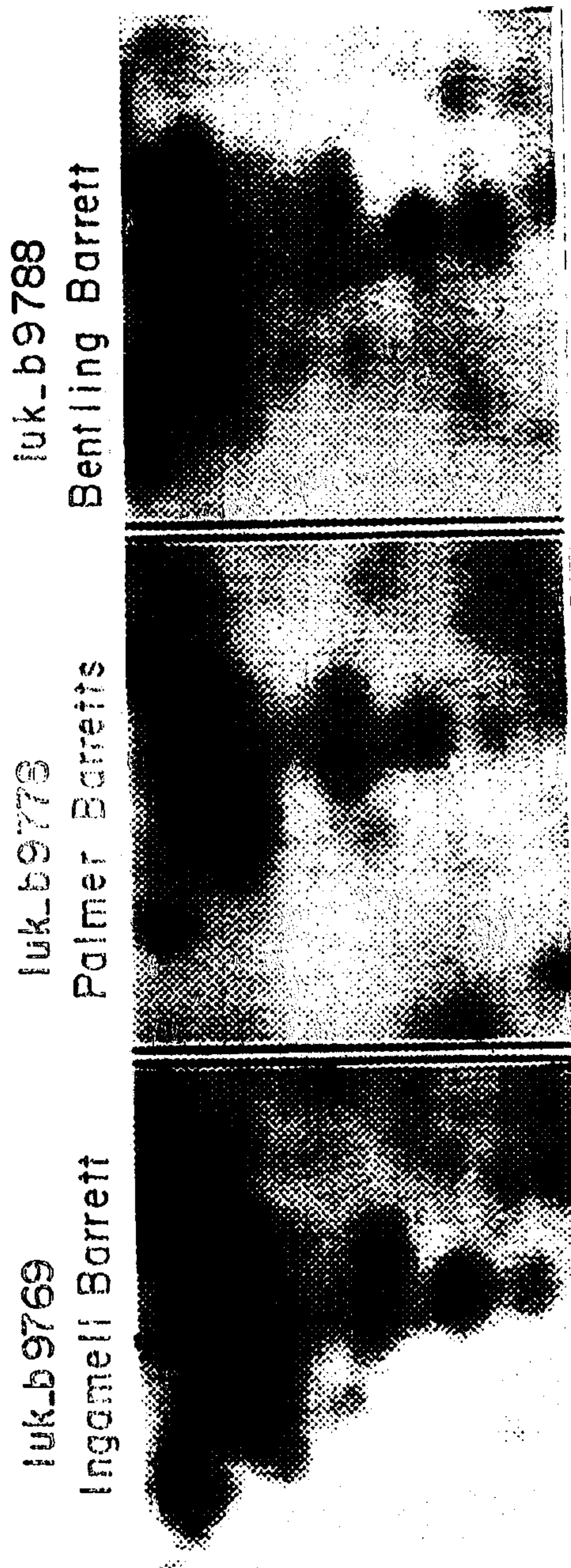


Fig. 13A

Fig. 13B

Fig. 13C

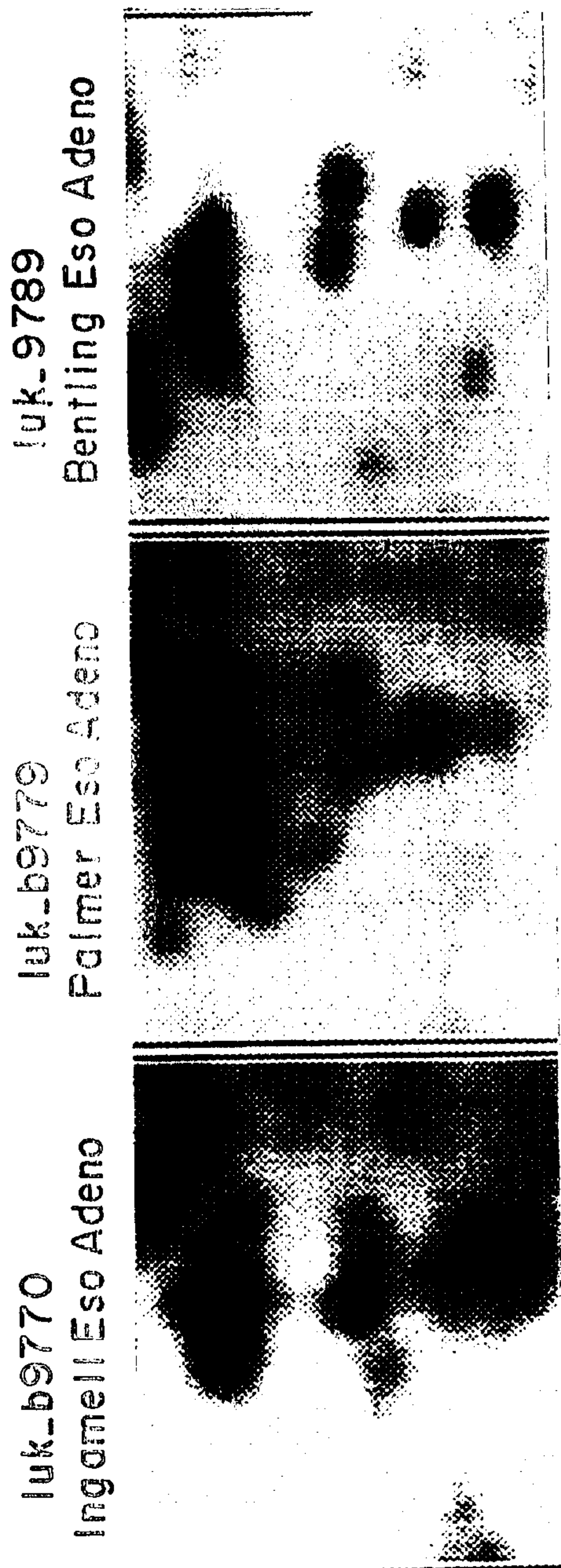


Fig. 13D

Fig. 13E

Fig. 13F

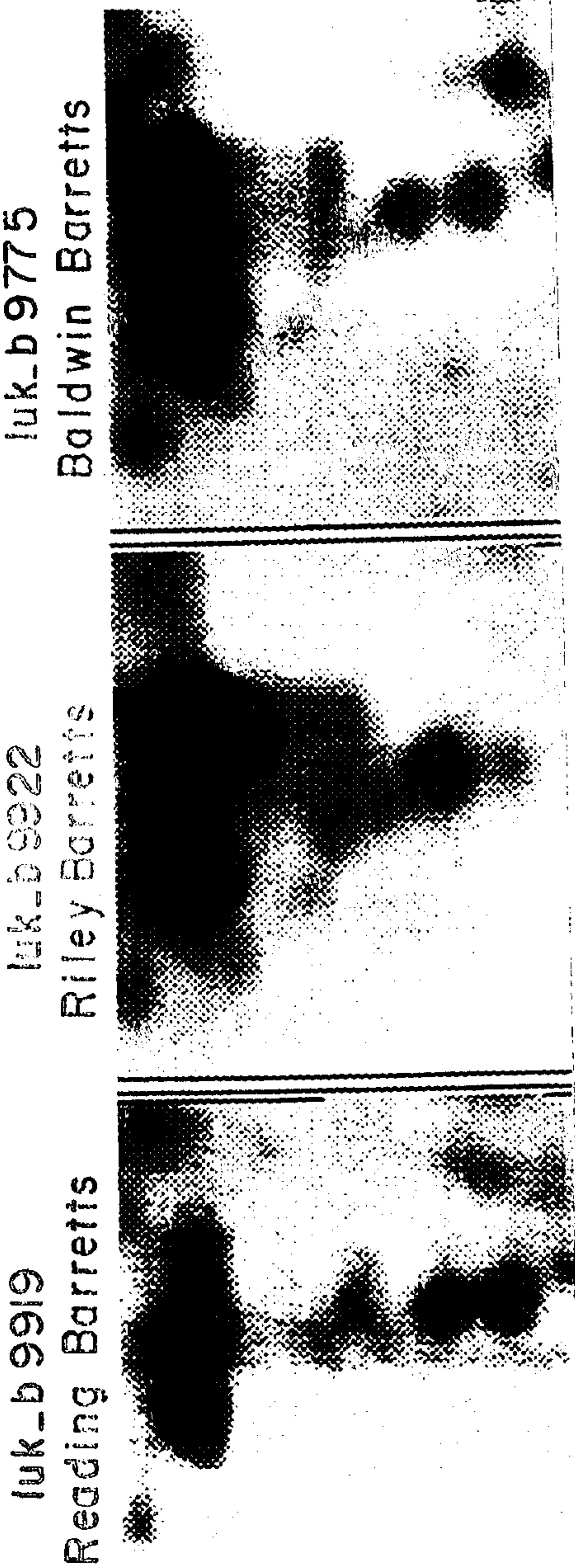


Fig. 13G Fig. 13H Fig. 13I

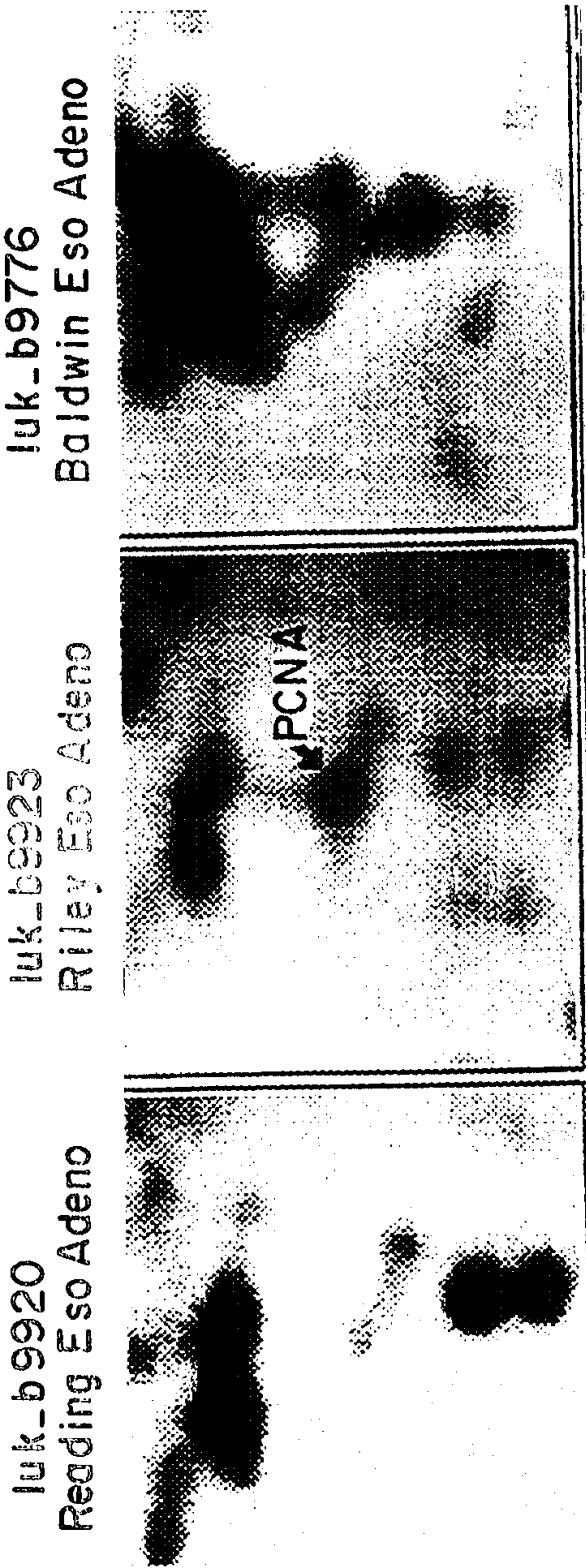


Fig. 13J Fig. 13K Fig. 13L

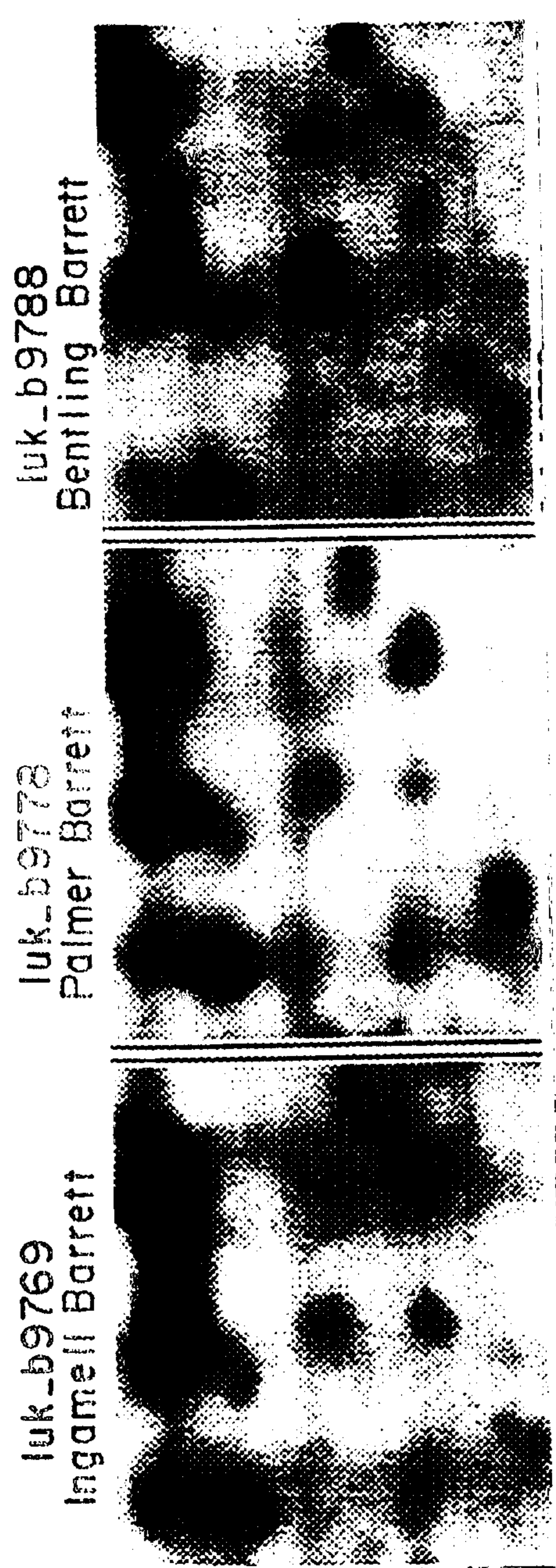


Fig. 14A Fig. 14B Fig. 14C

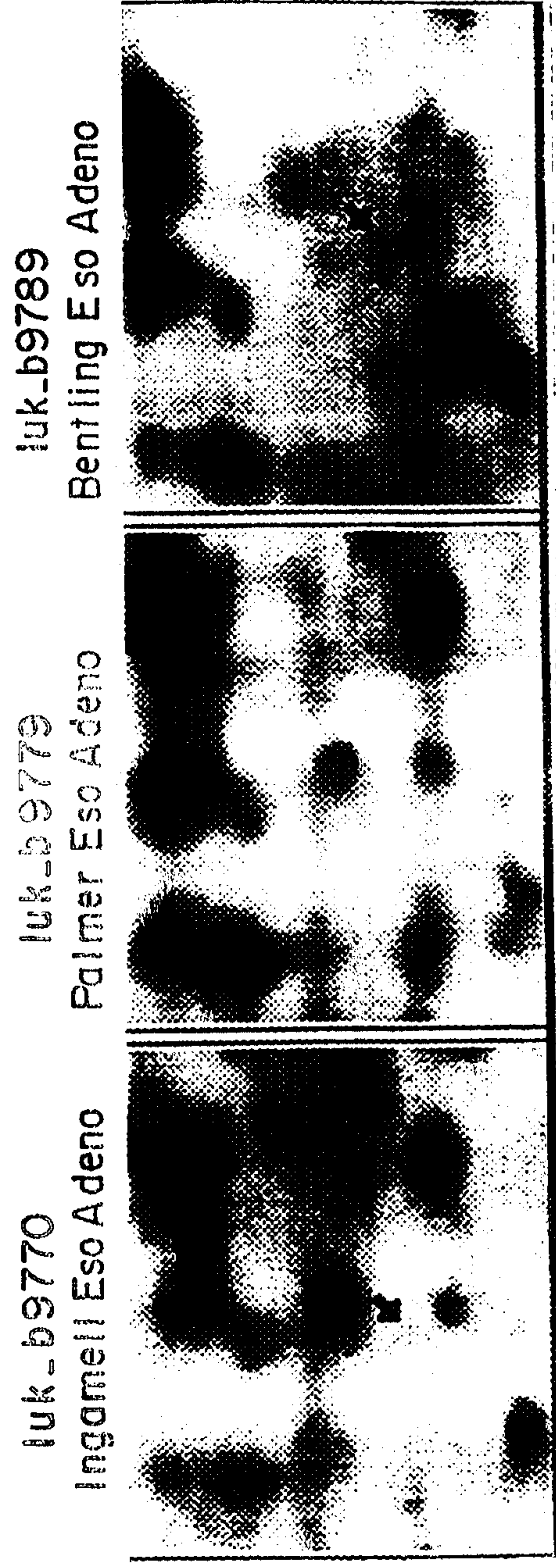


Fig. 14D Fig. 14E Fig. 14F

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Fig. 14G

Fig. 14H

Fig. 14I

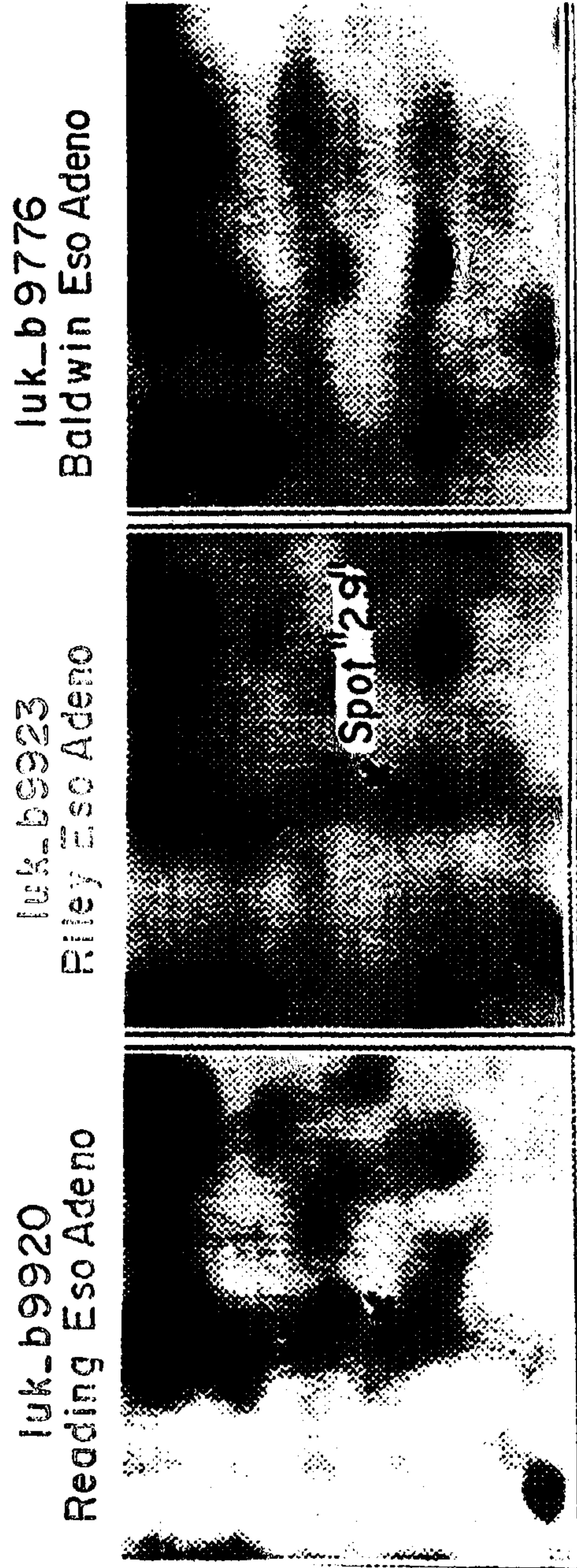


Fig. 14J

Fig. 14K

Fig. 14L

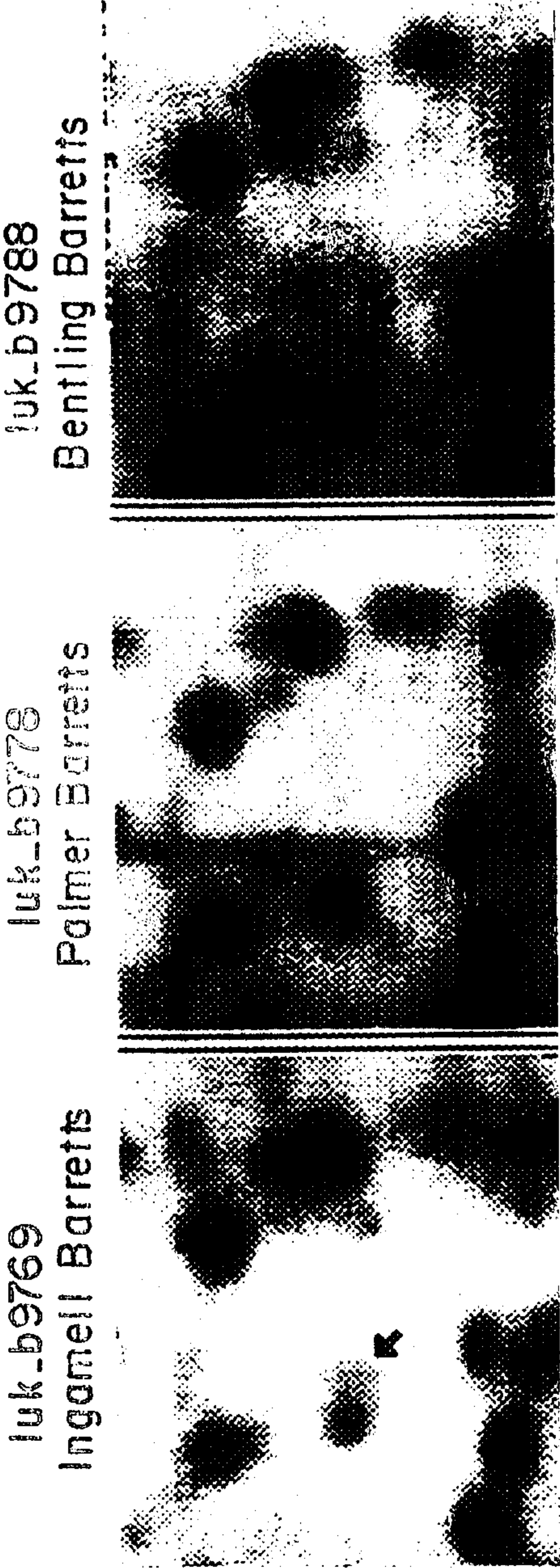


Fig. 15A Fig. 15B Fig. 15C

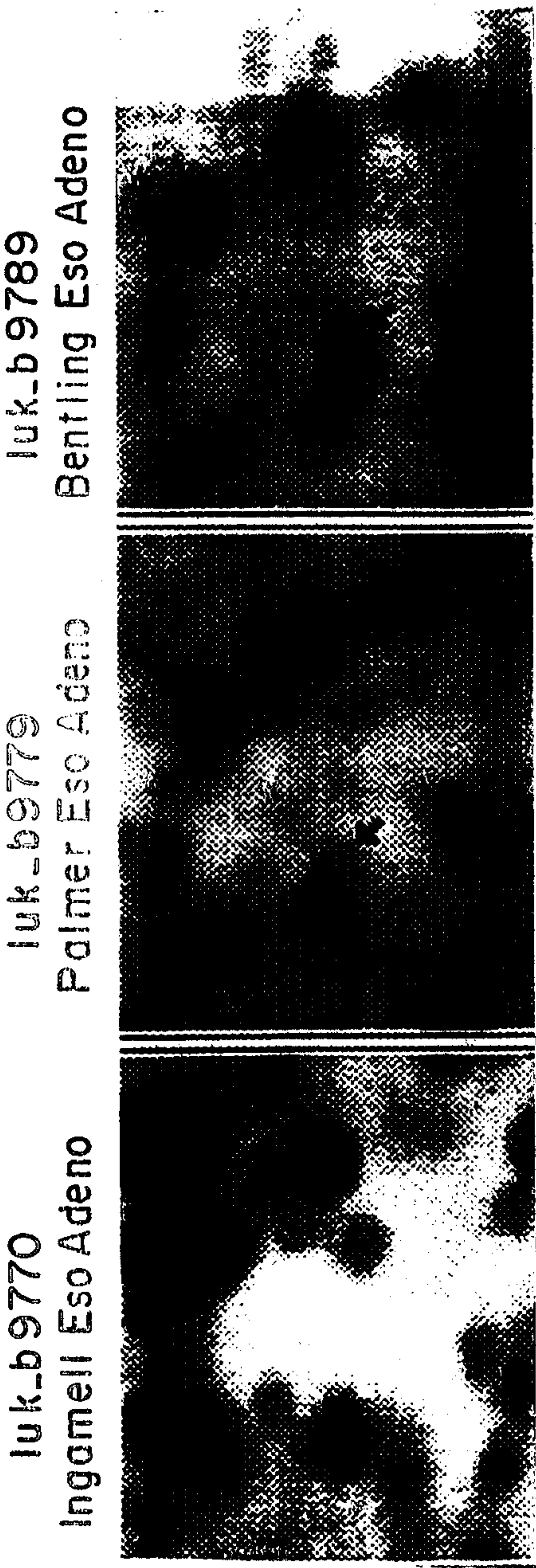


Fig. 15D Fig. 15E Fig. 15F

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Reading Barretts



luk_b9922
Riley Barretts



luk_b9775
Baldwin Barretts



Fig. 15G

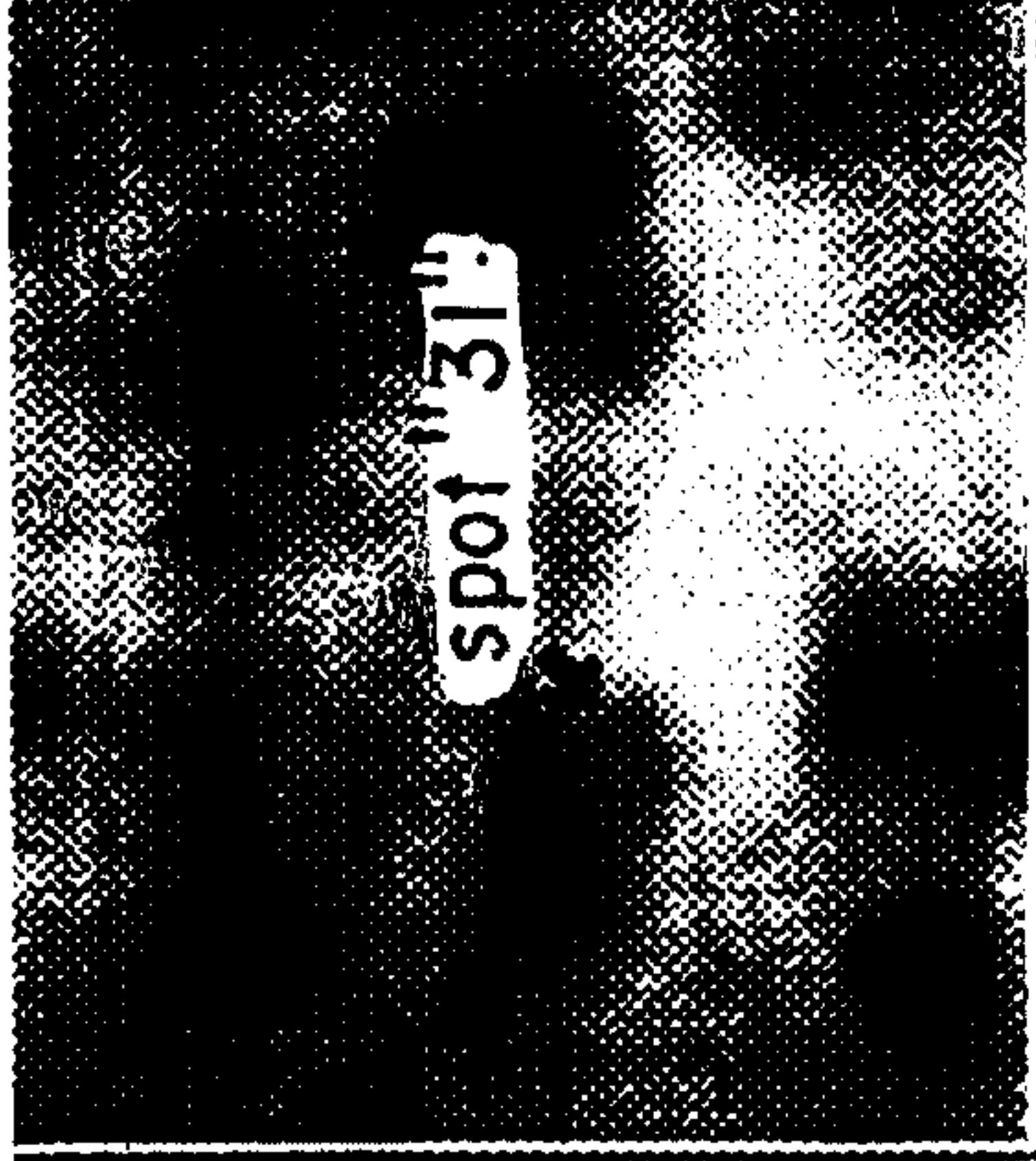
Fig. 15H

Fig. 15I

luk_b9920
Reading Eso Adeno



luk_b9923
Riley Eso Adeno



luk_b9776
Baldwin Eso Adeno



Fig. 15J

Fig. 15K

Fig. 15L

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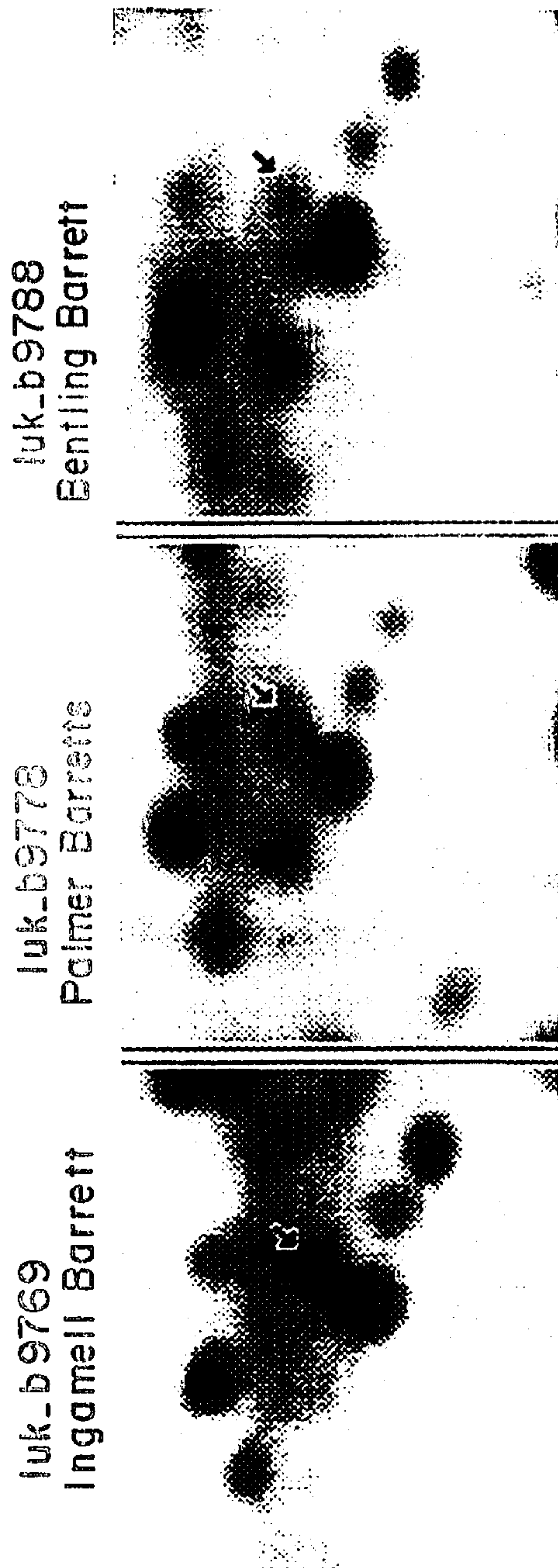


Fig. 16A

Fig. 16B

Fig. 16C

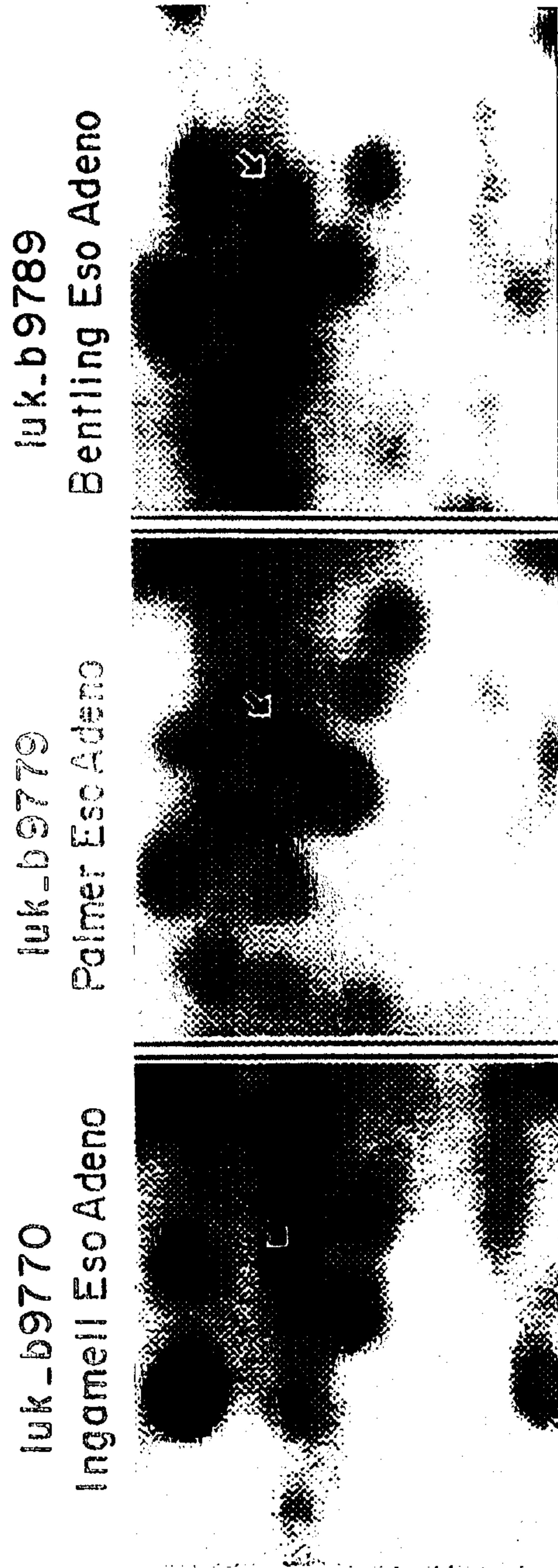


Fig. 16D

Fig. 16E

Fig. 16F

luk_b9919
Reading Barretts

luk_b9922
Riley Barretts

luk_b9775
Baldwin Barretts



Fig. 16G

Fig. 16H

Fig. 16I

luk_b9920
Reading Eso Adeno

luk_b9923
Riley Eso Adeno

luk_b9776
Baldwin Eso Adeno

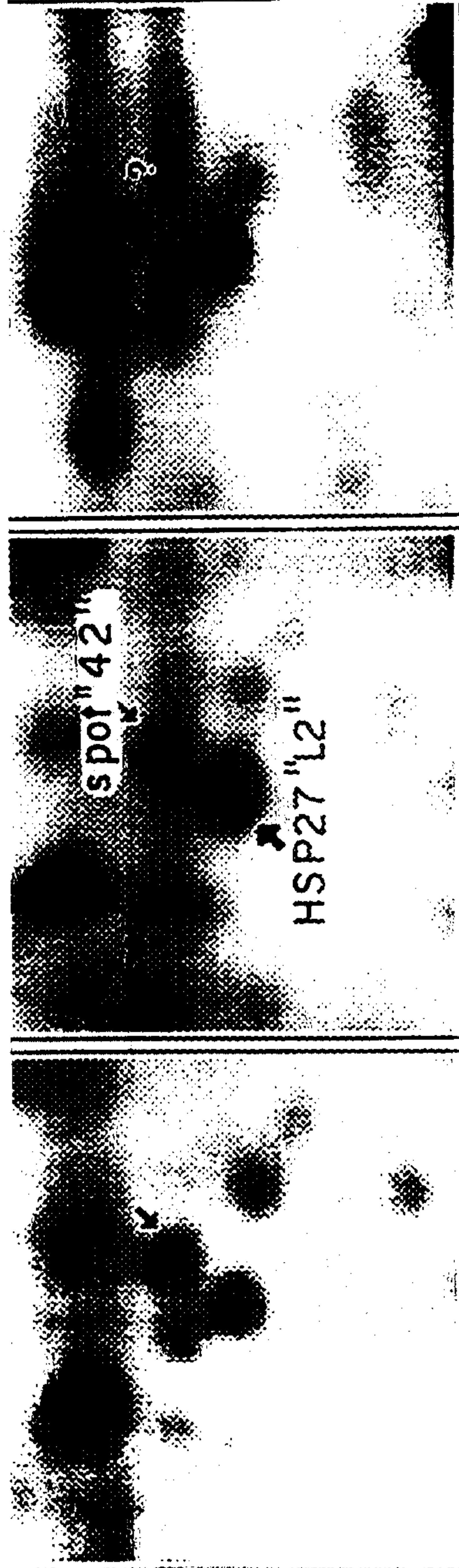


Fig. 16J

Fig. 16K

Fig. 16L

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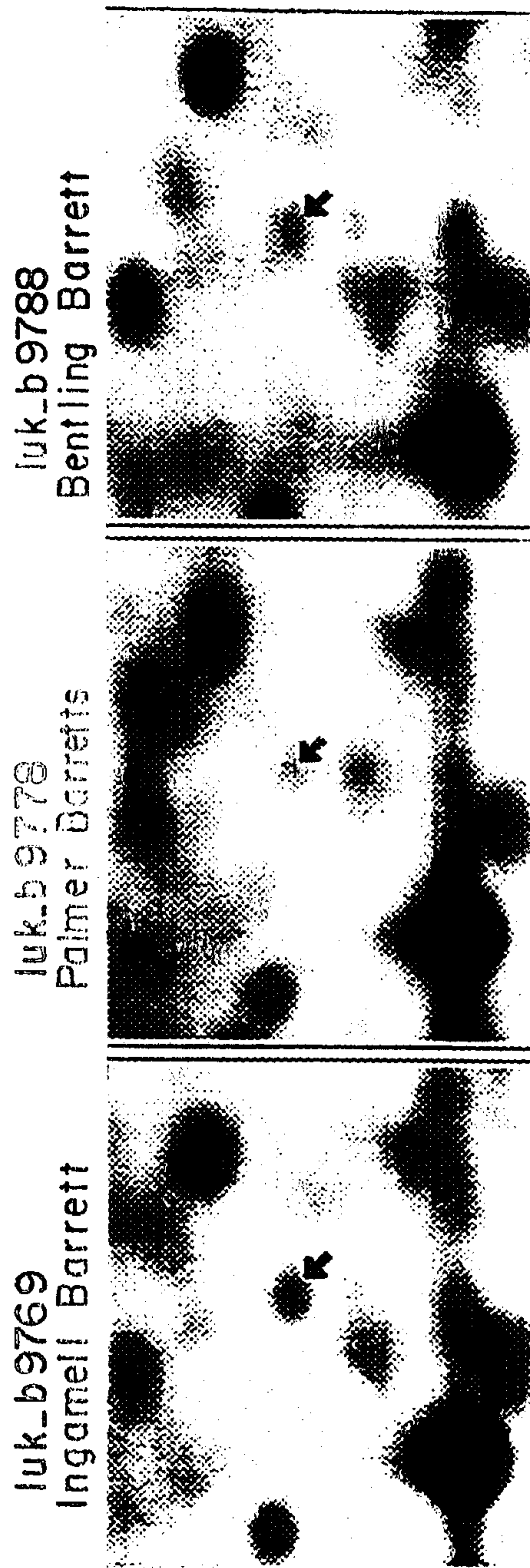


Fig. 17A

Fig. 17B

Fig. 17C

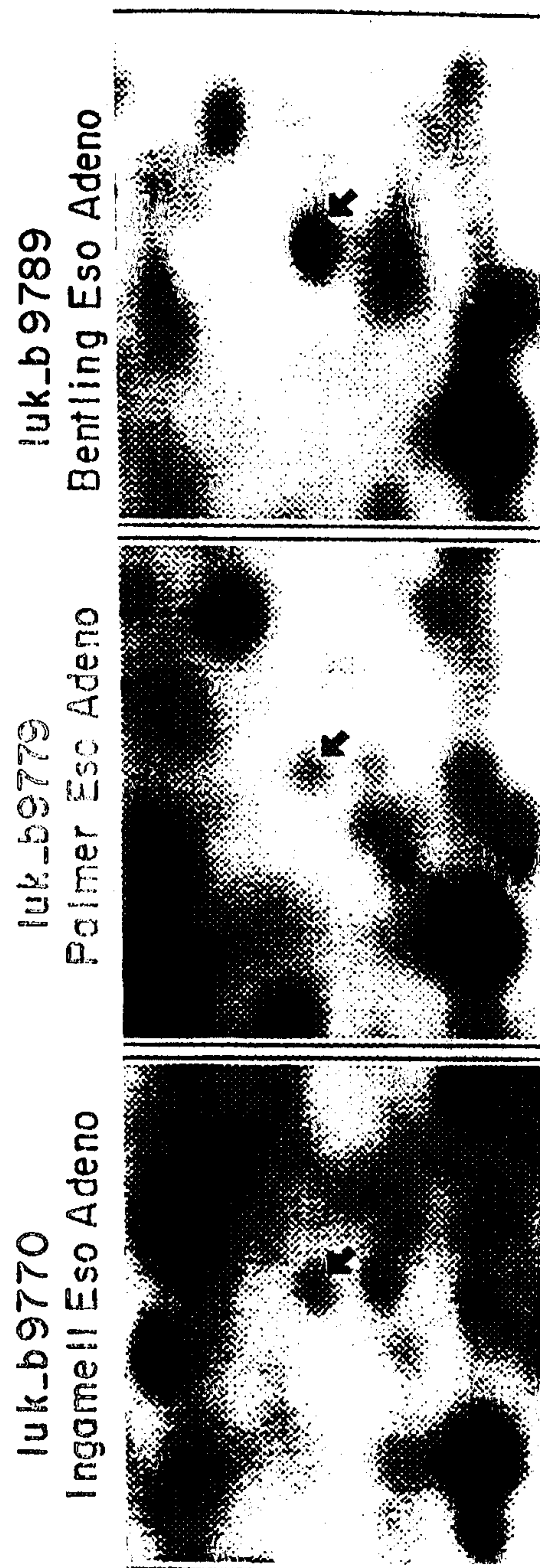


Fig. 17D

Fig. 17E

Fig. 17F

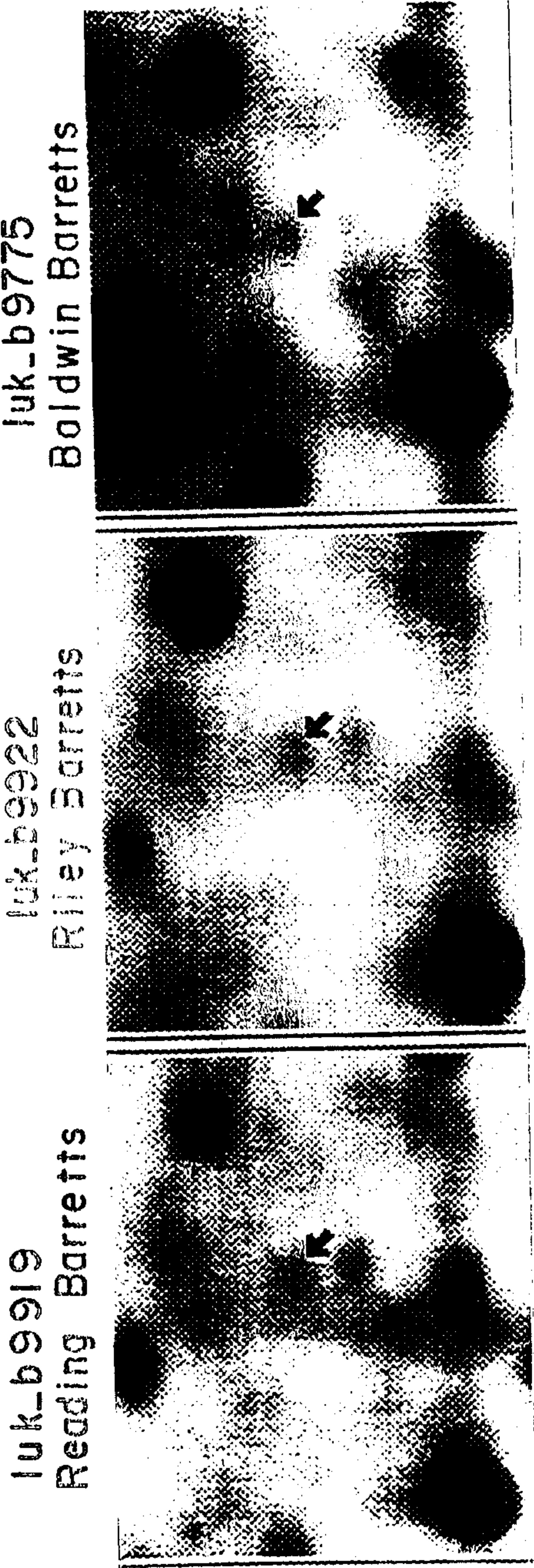


Fig. 17G Fig. 17H Fig. 17I

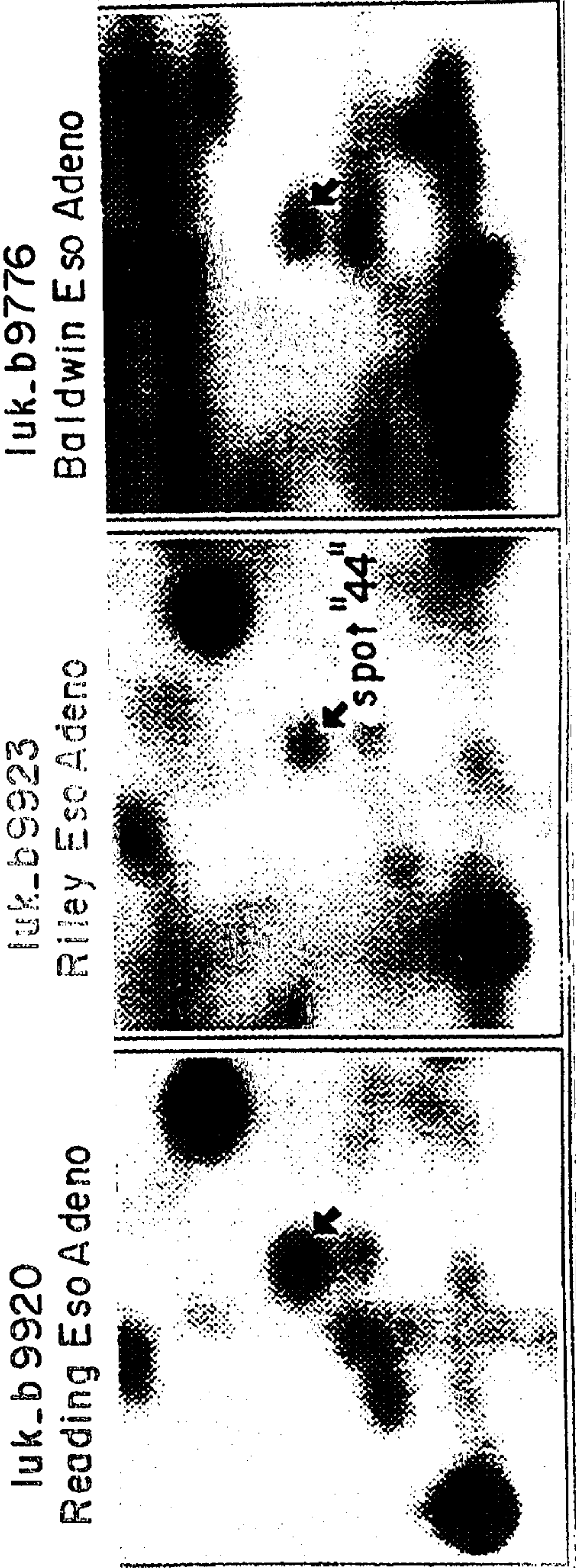


Fig. 17J Fig. 17K Fig. 17L

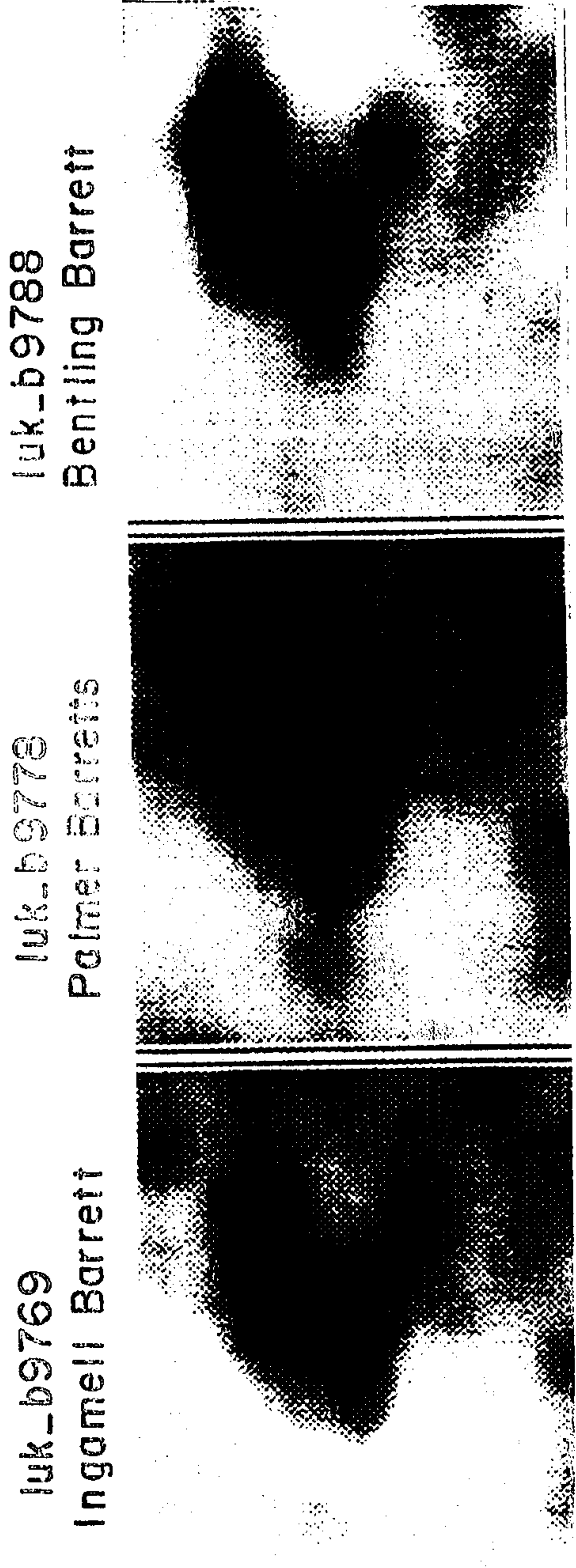


Fig. 18A

Fig. 18B

Fig. 18C

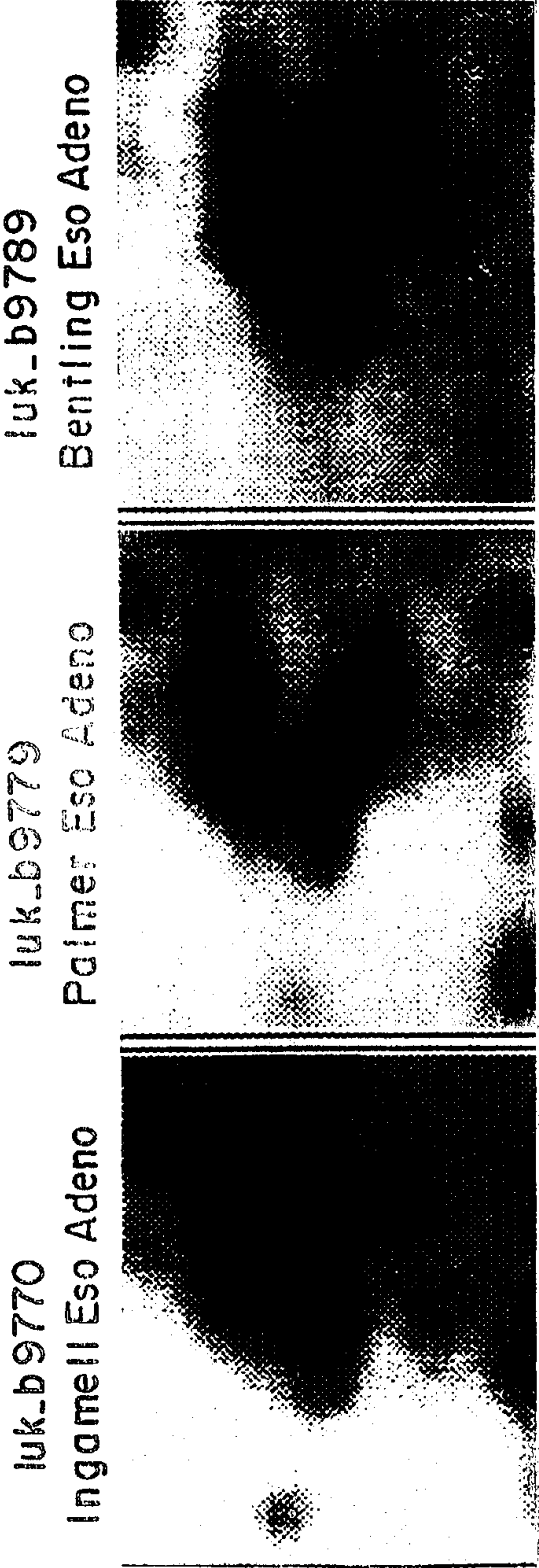


Fig. 18D

Fig. 18E

Fig. 18F



Fig. 18G Fig. 18H Fig. 18I

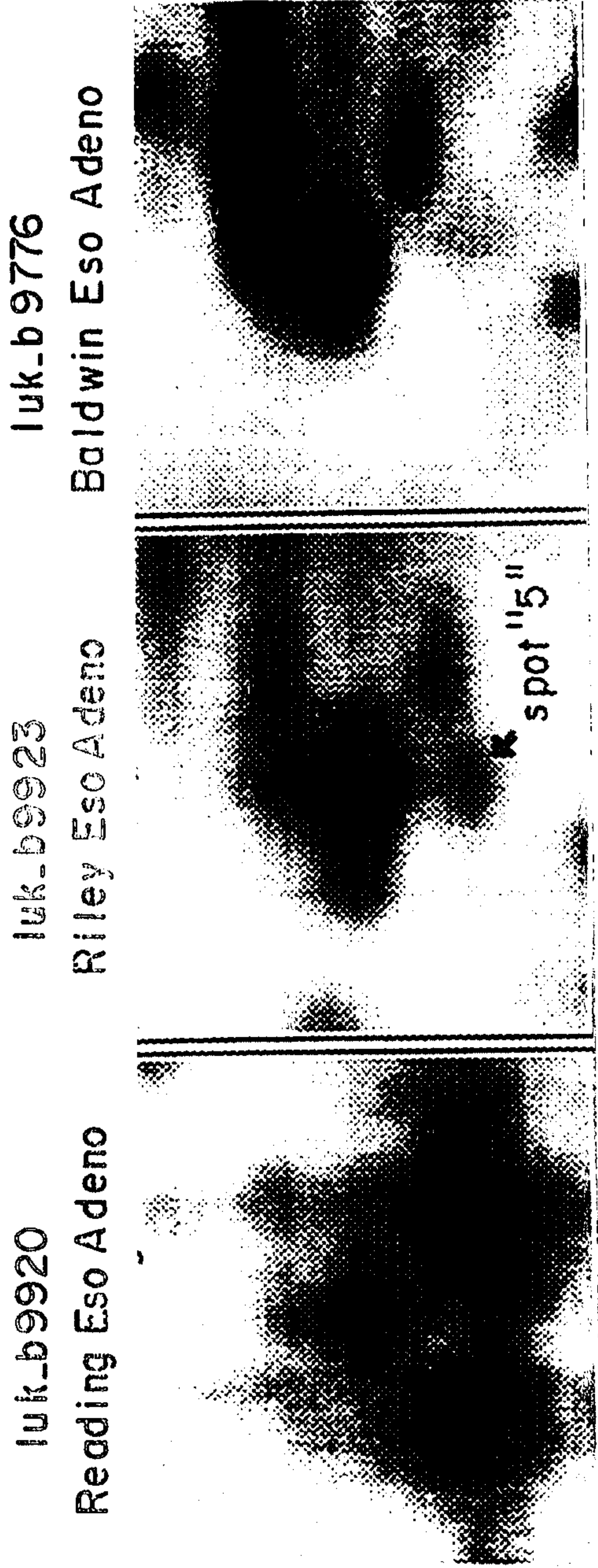


Fig. 18J Fig. 18K Fig. 18L

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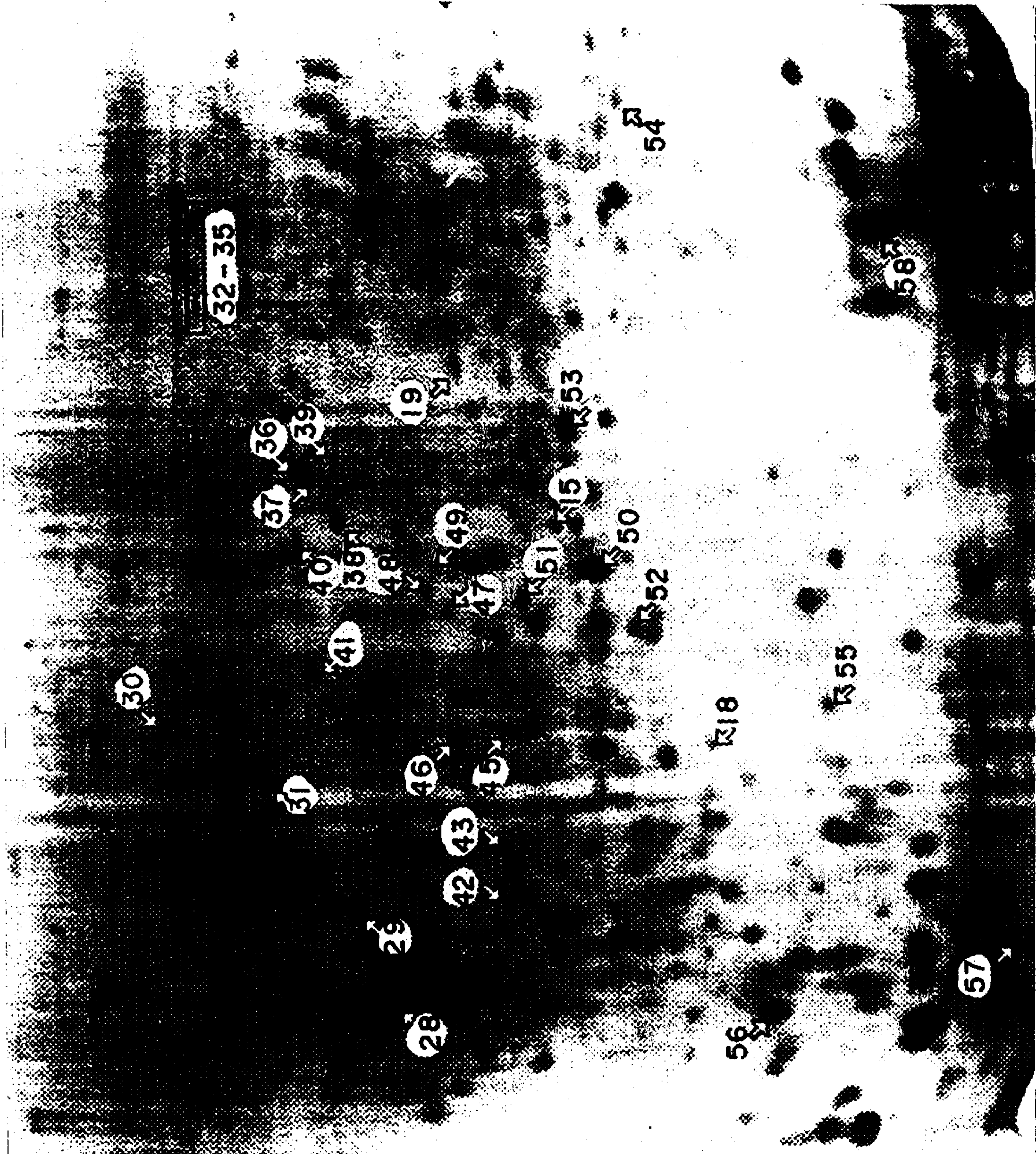


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Fig. 19

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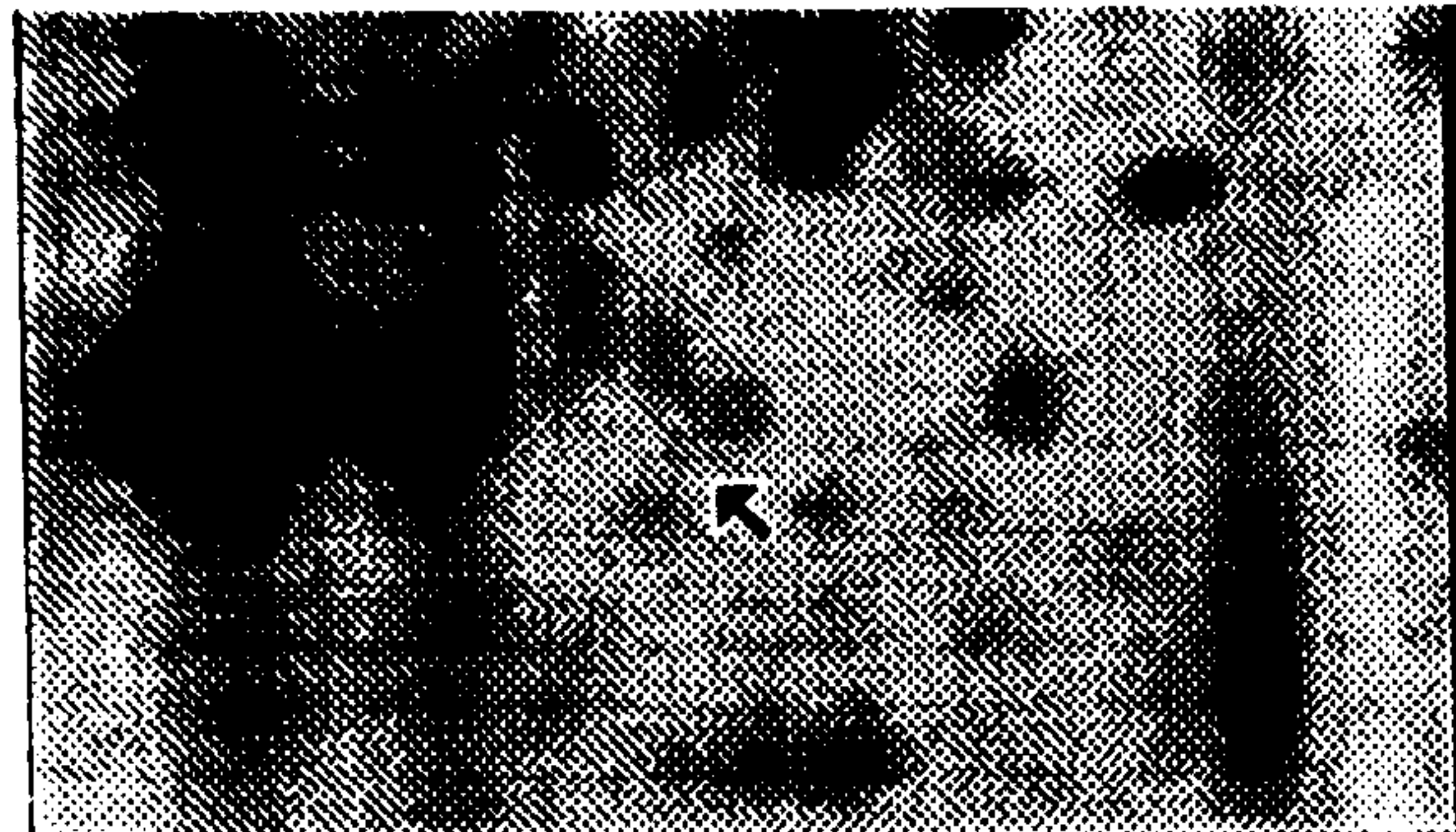


Fig. 21A

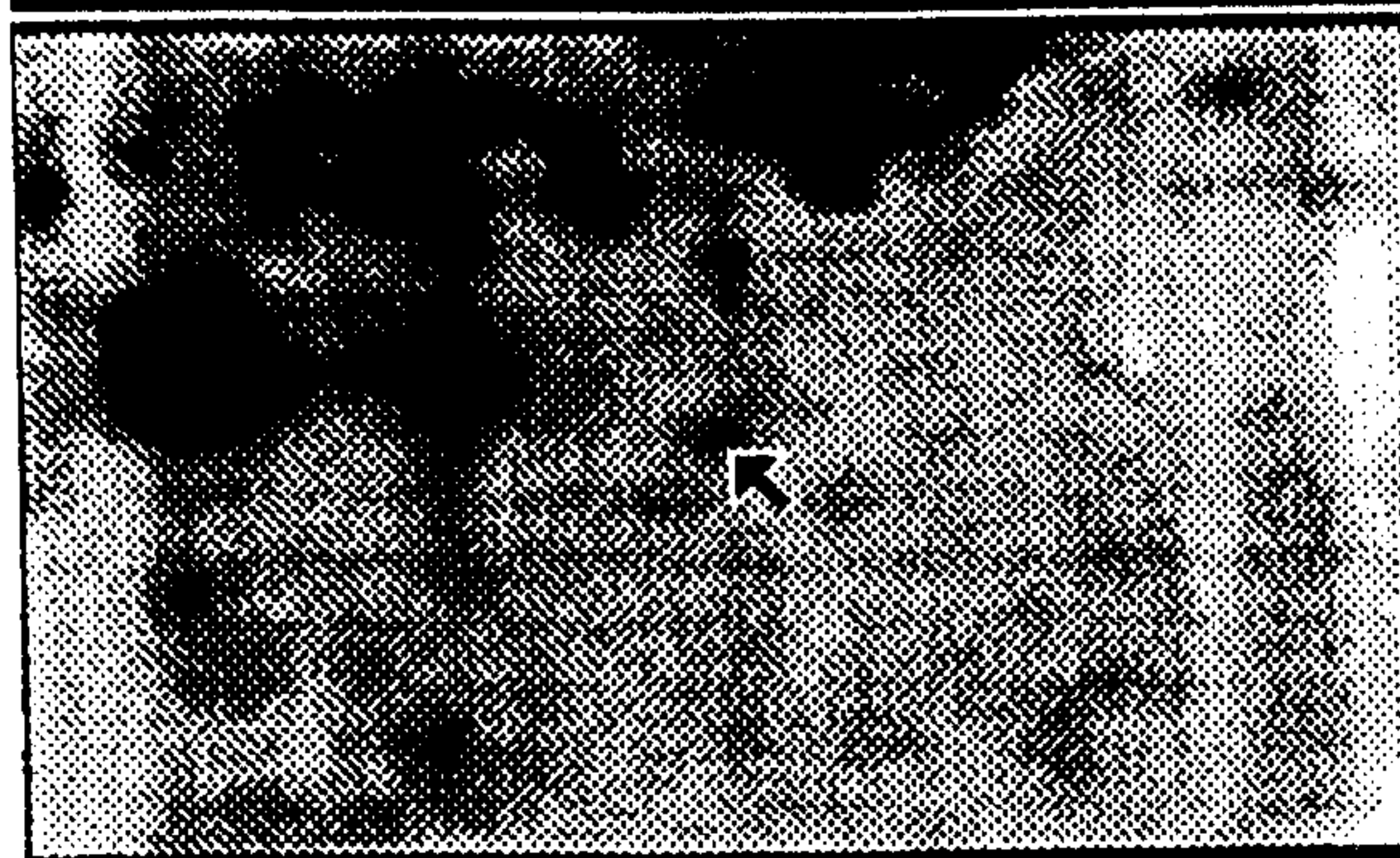


Fig. 21B

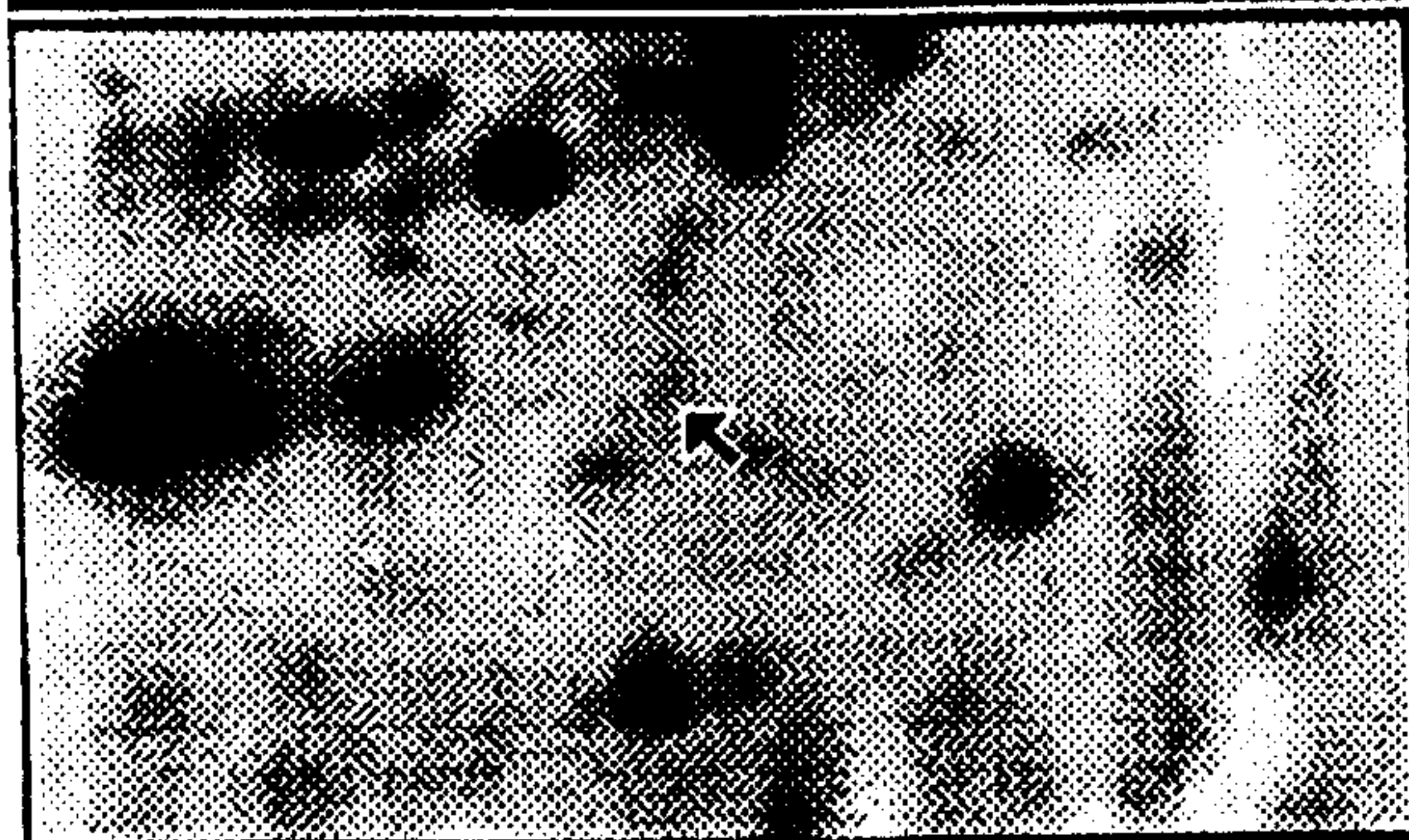


Fig. 21C

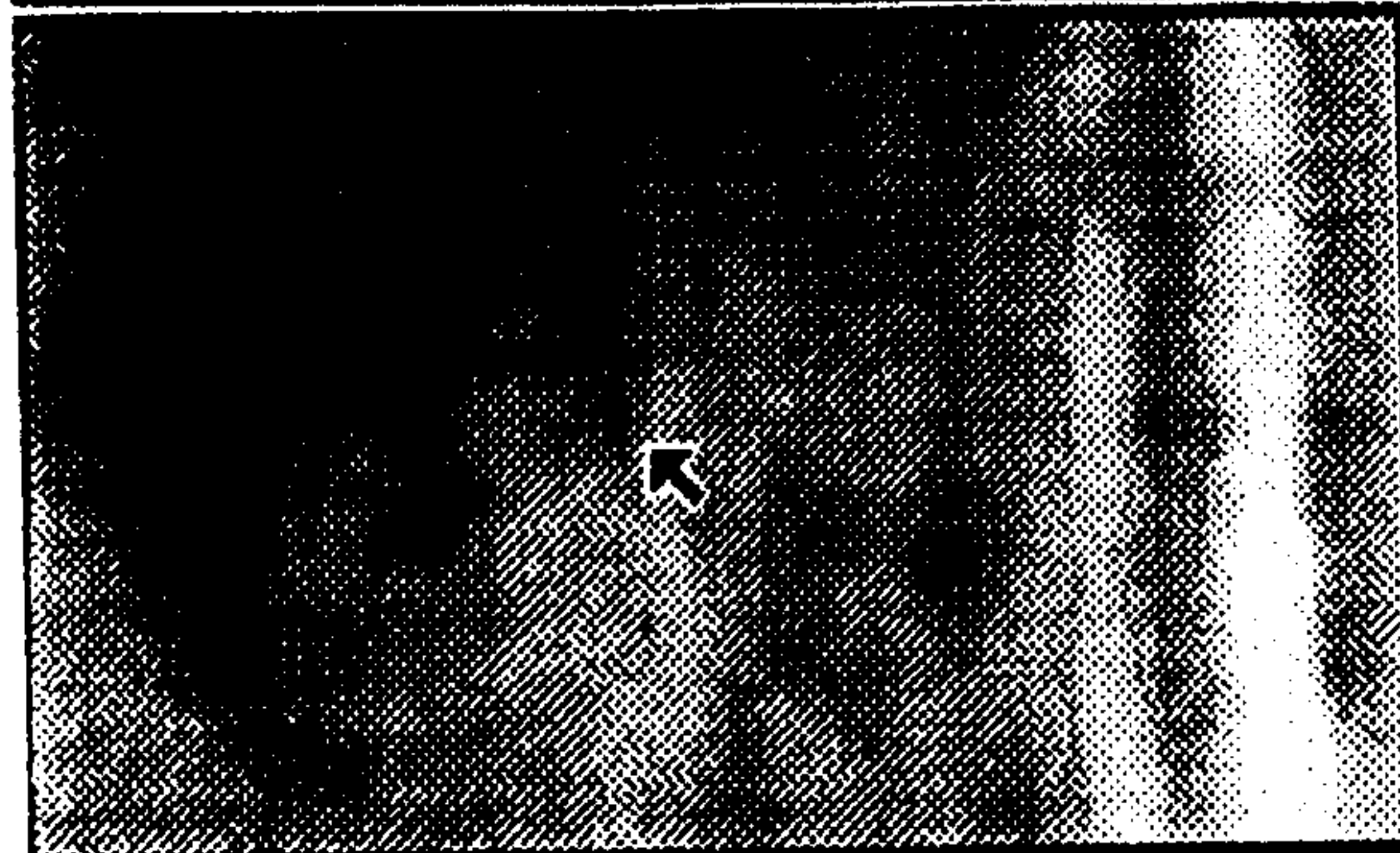


Fig. 21D

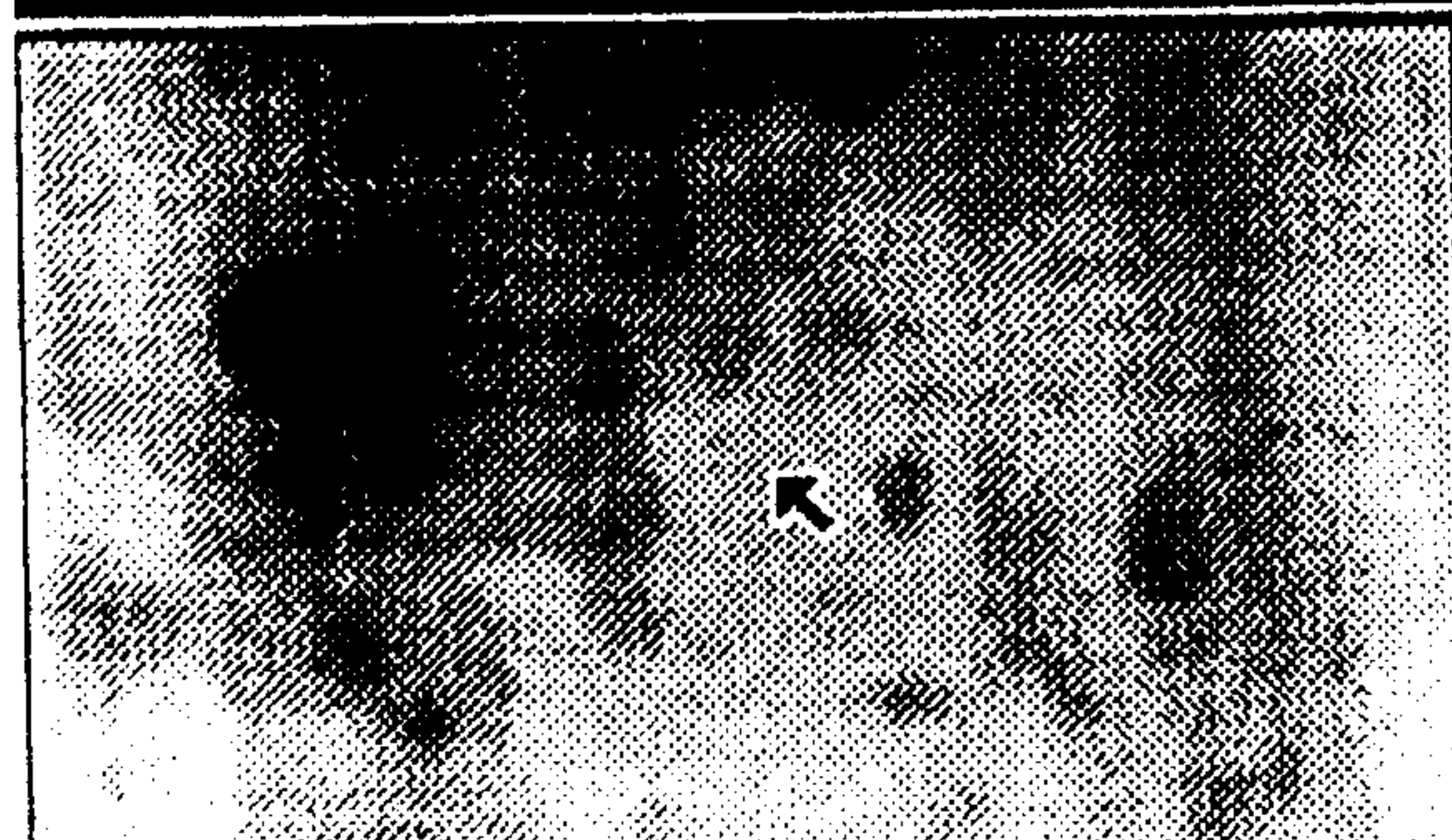


Fig. 21E

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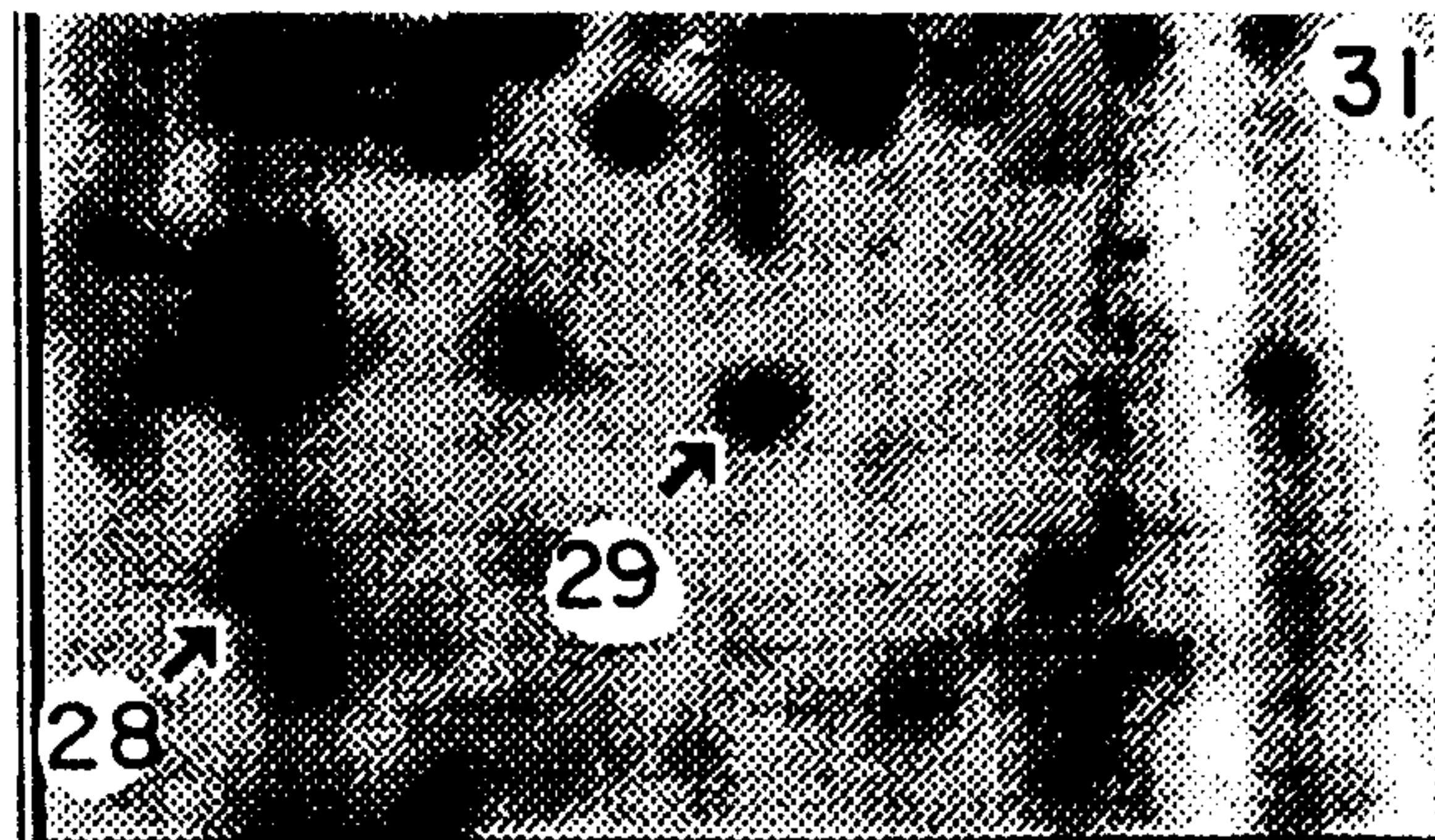


Fig. 21F

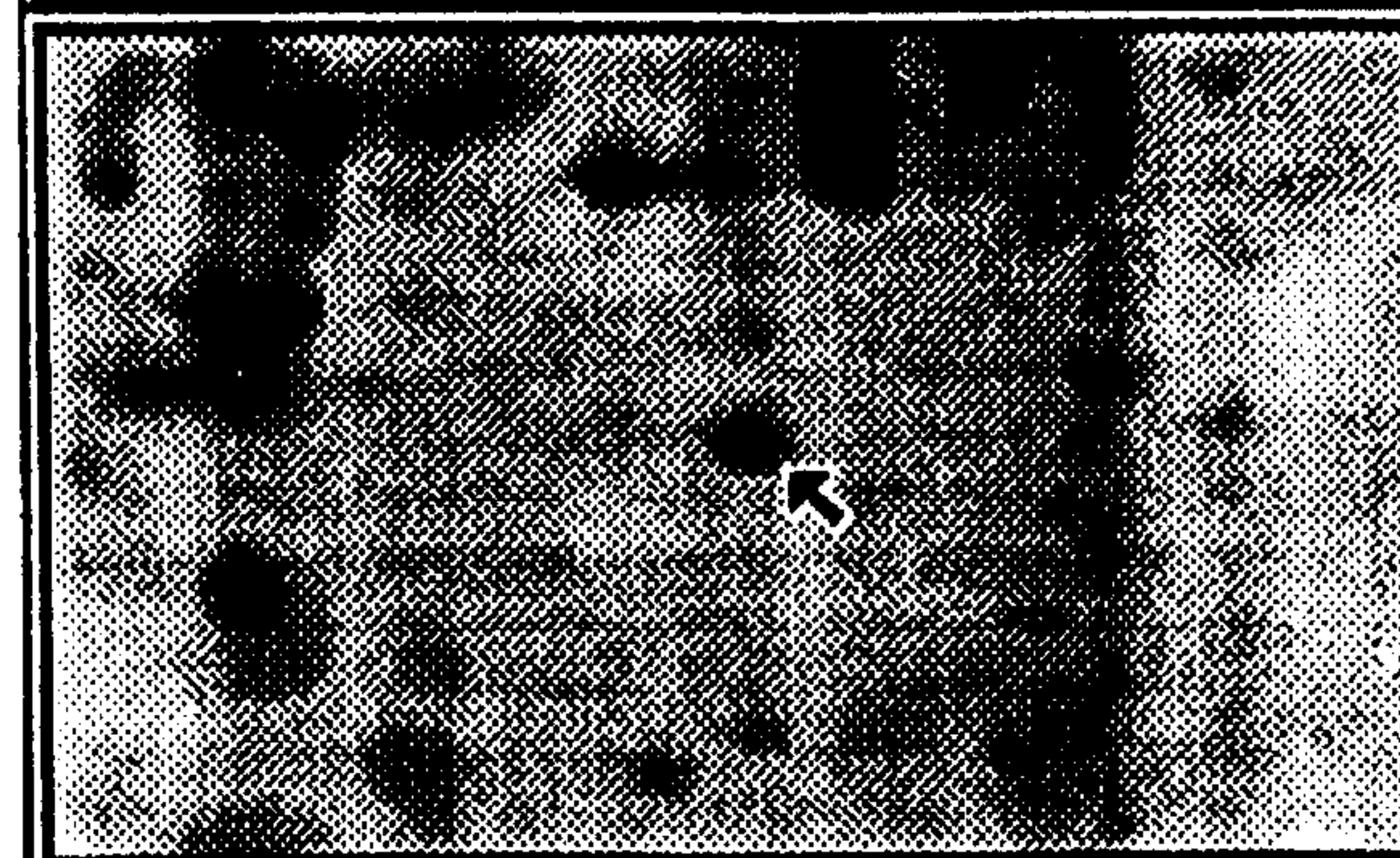


Fig. 21G



Fig. 21H

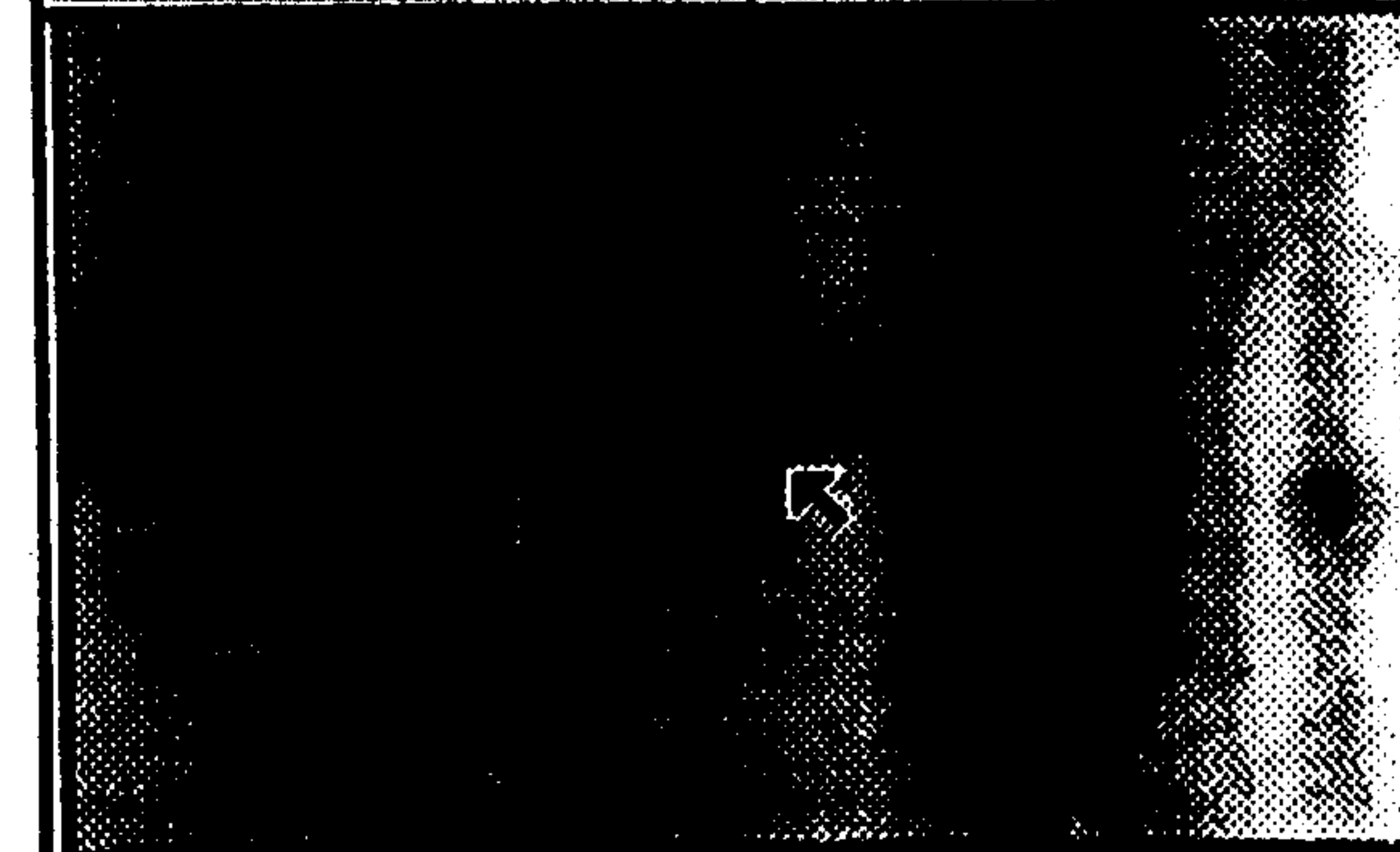


Fig. 21I

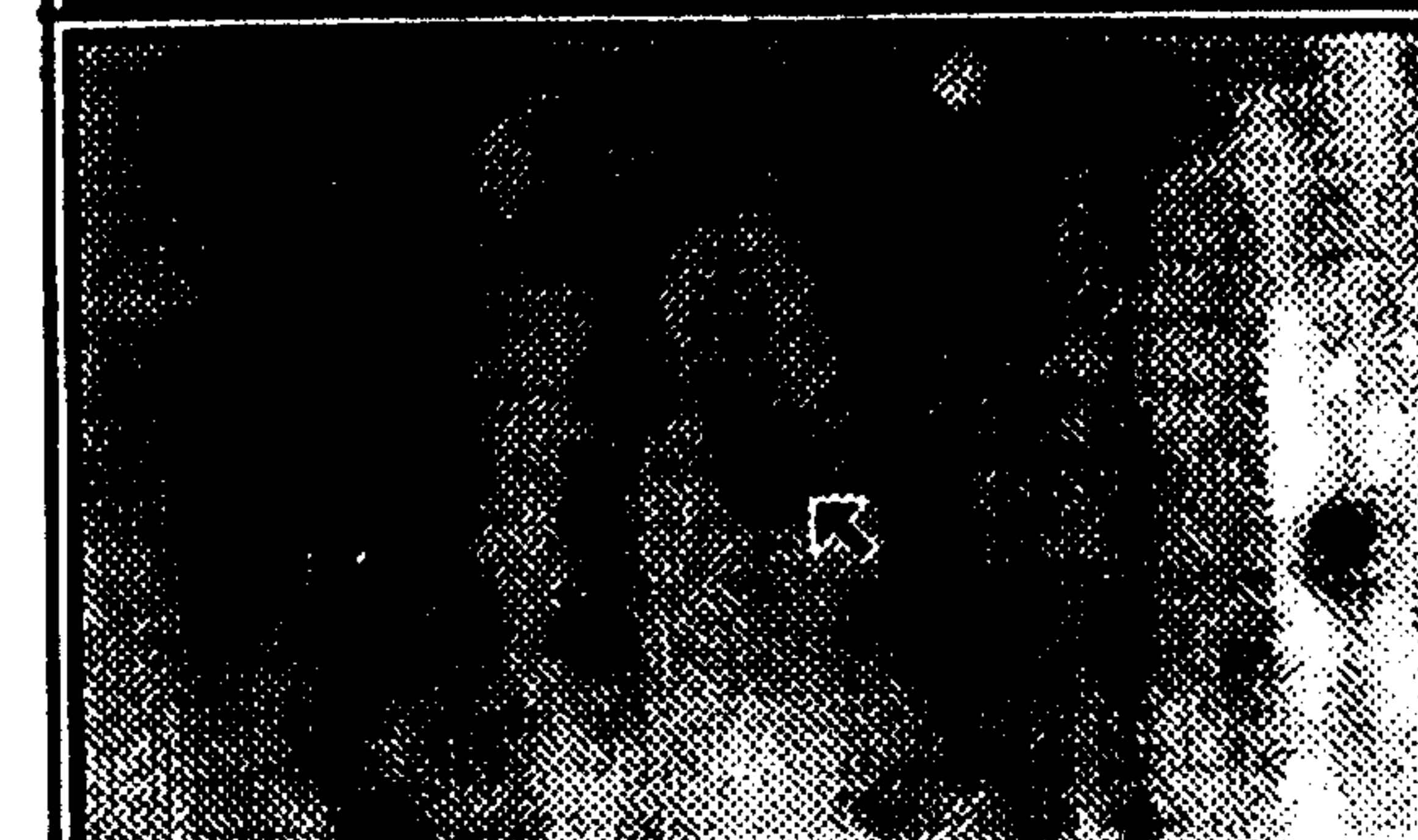


Fig. 21J

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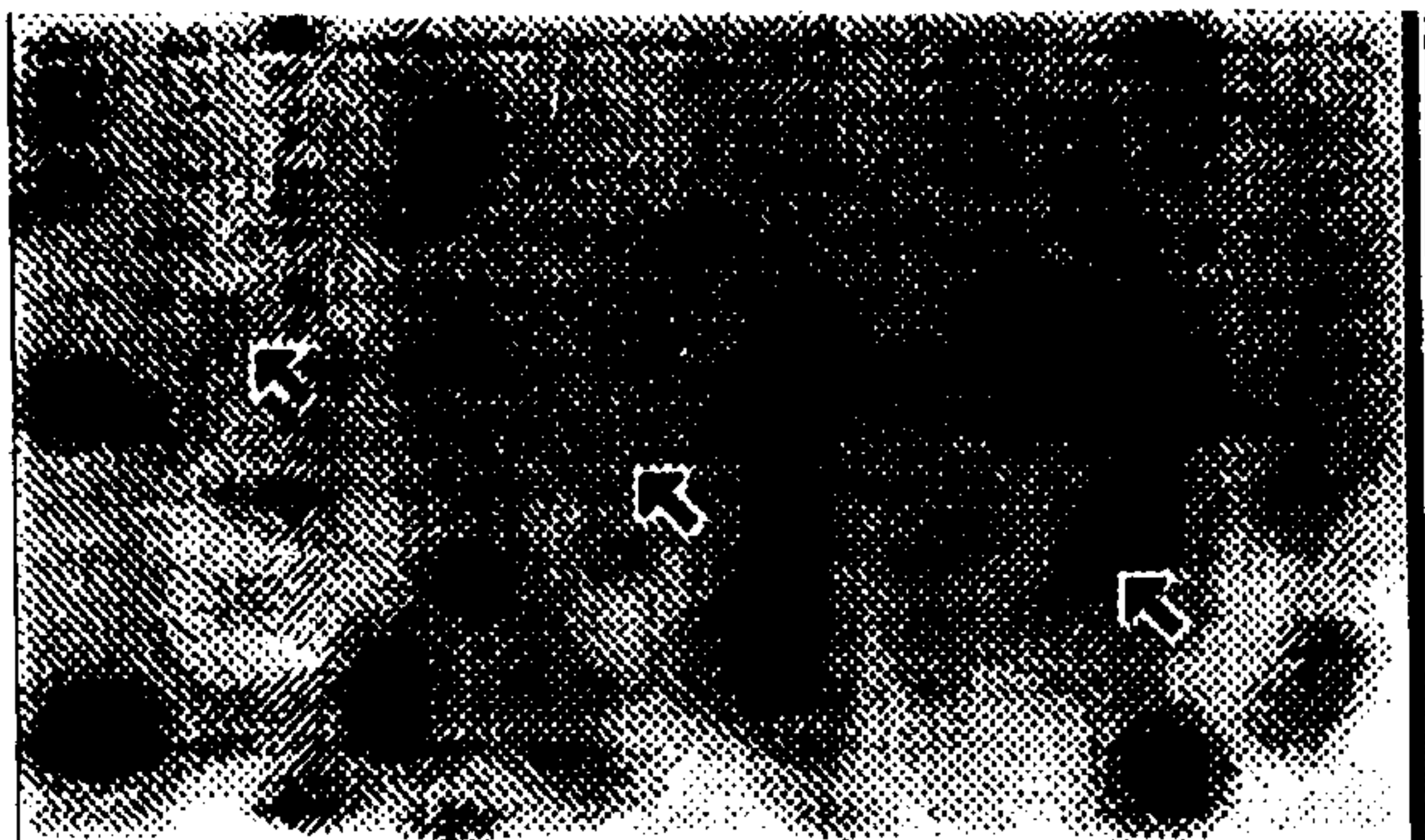


Fig. 22A

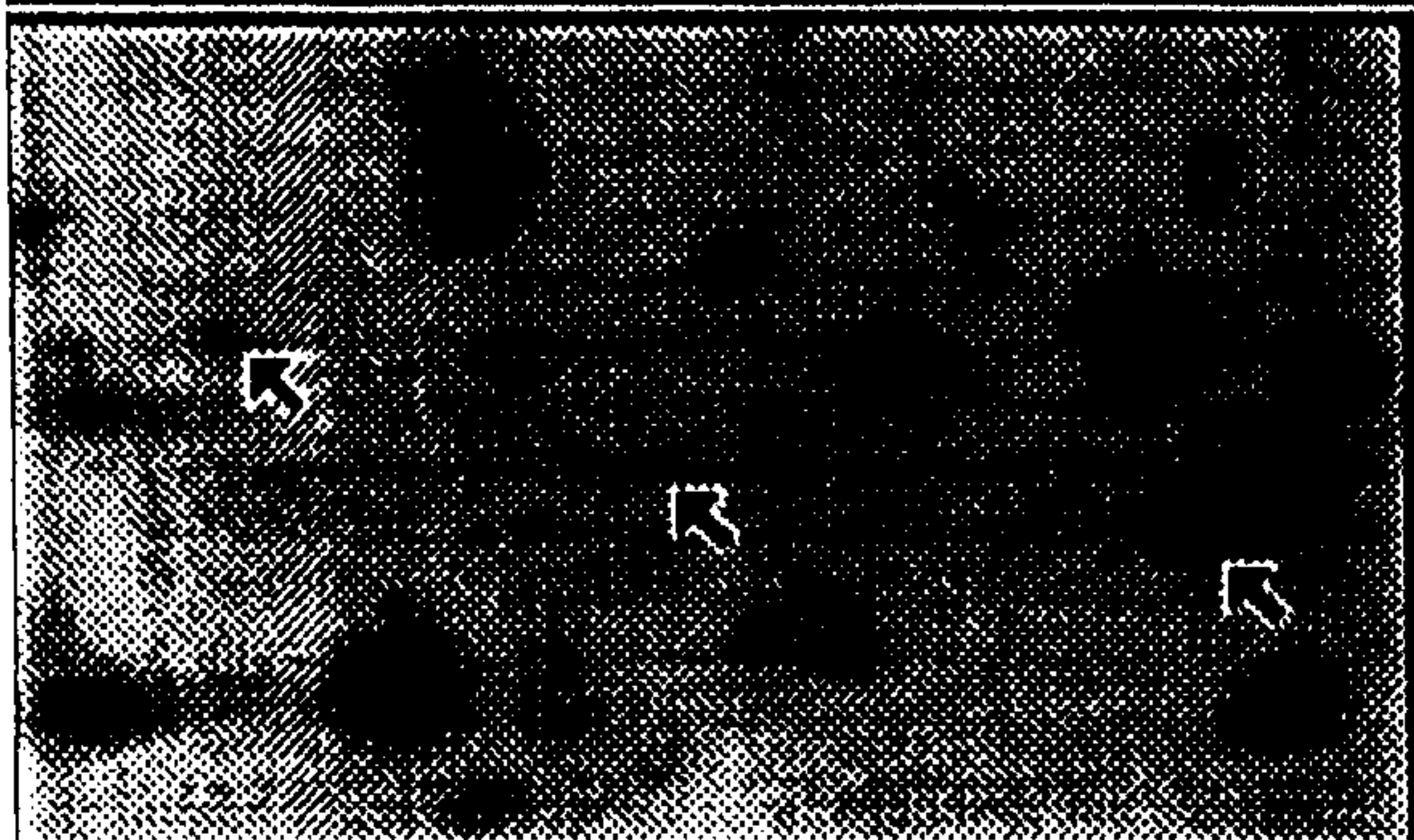


Fig. 22B

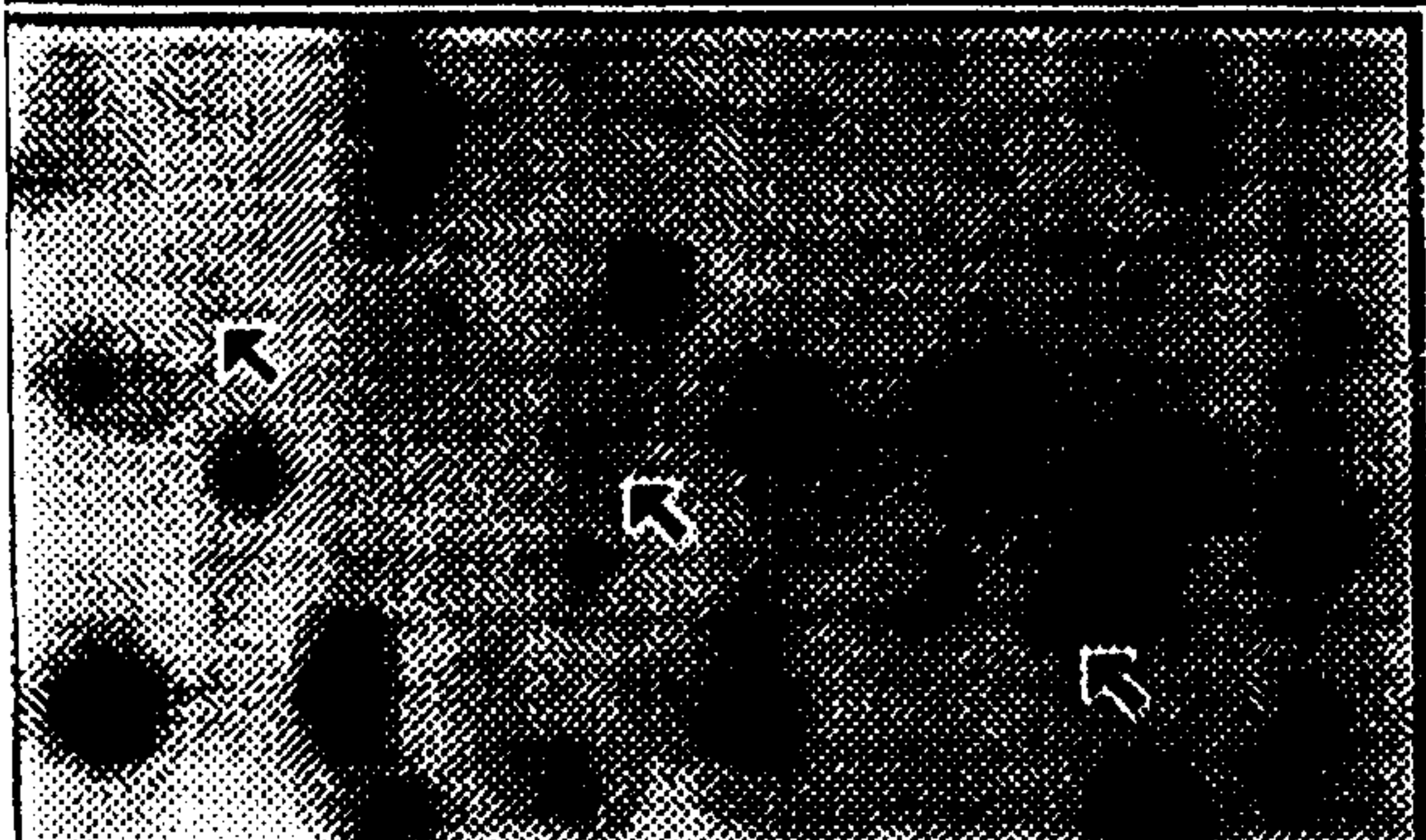


Fig. 22C

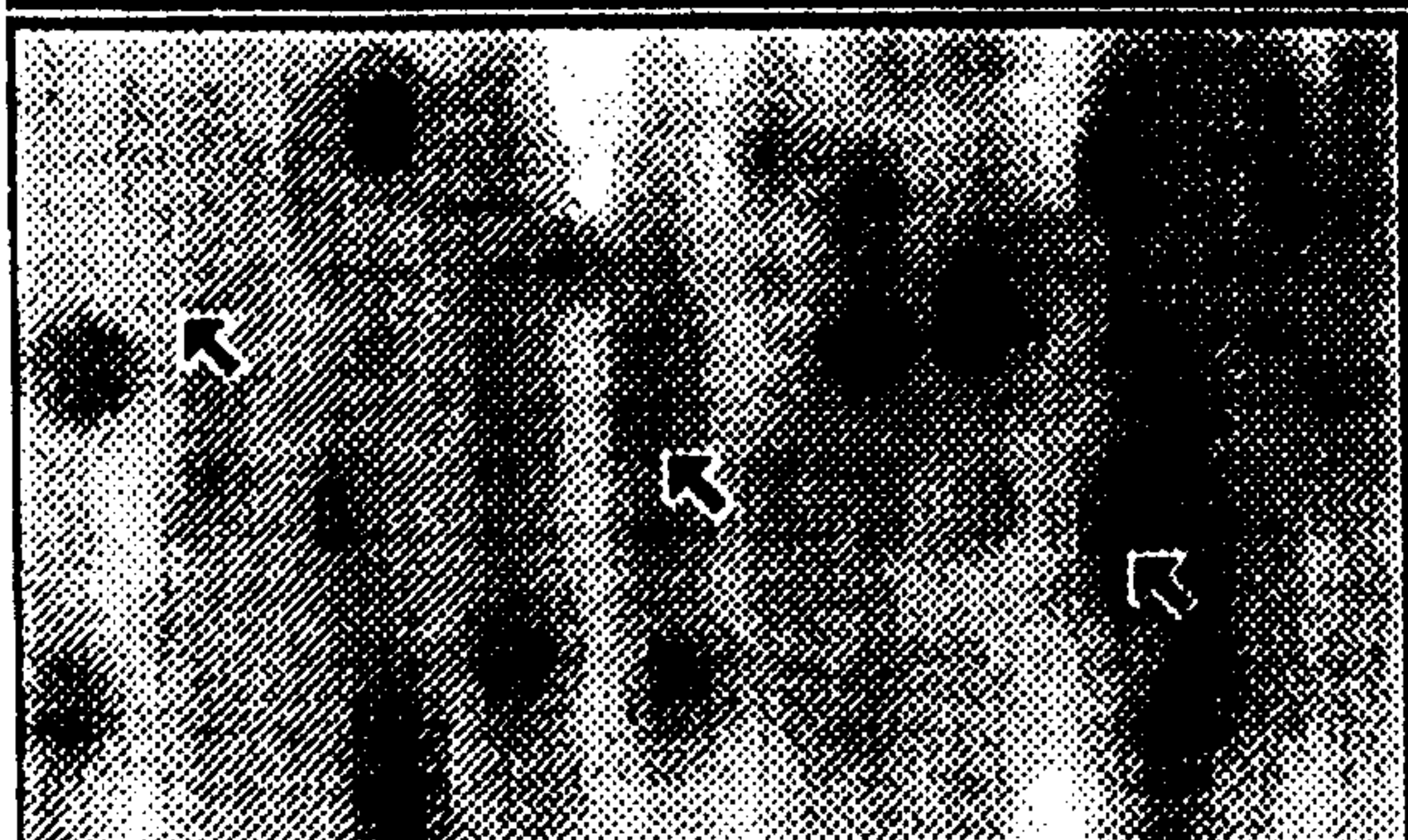


Fig. 22D

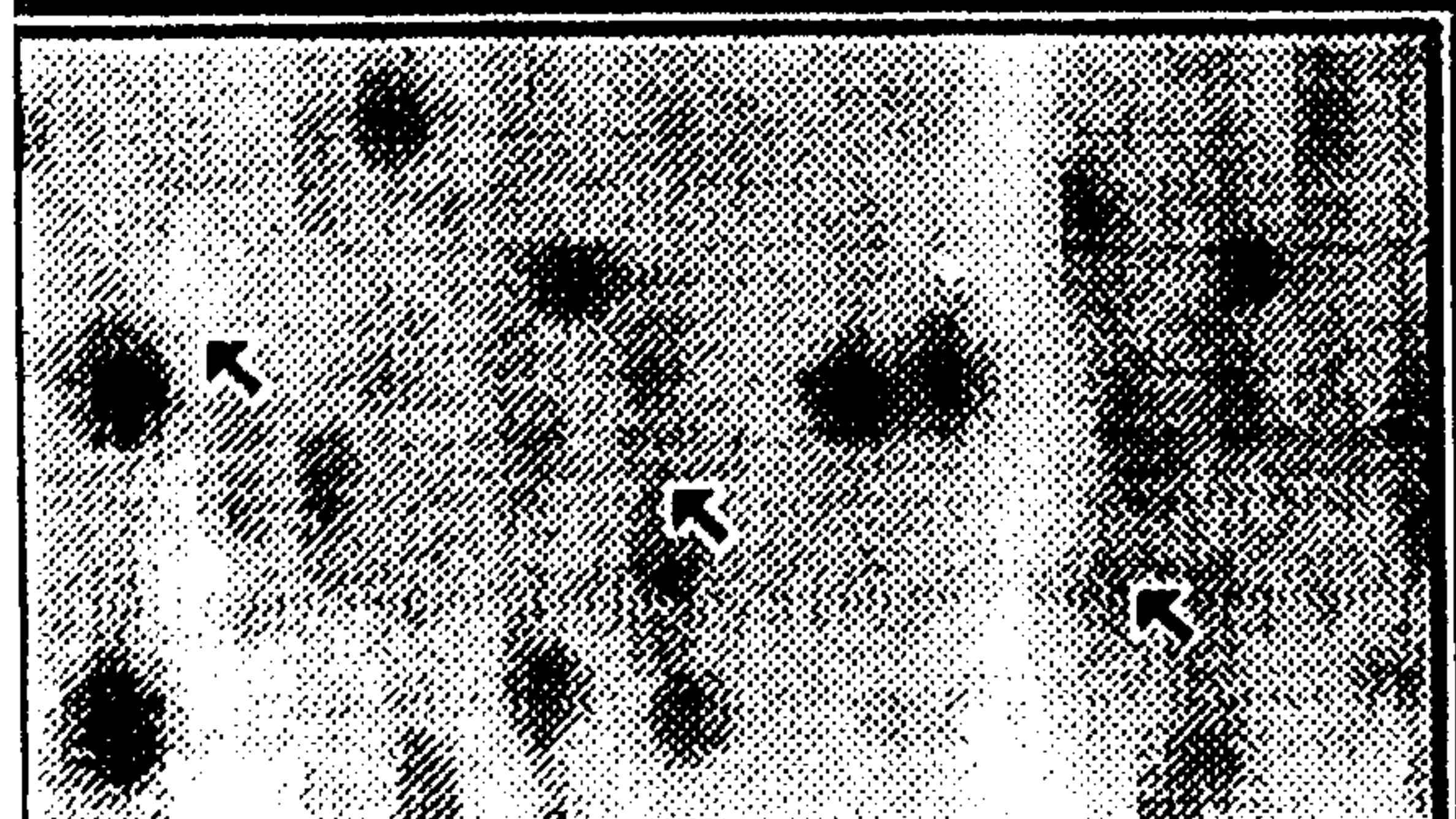


Fig. 22E

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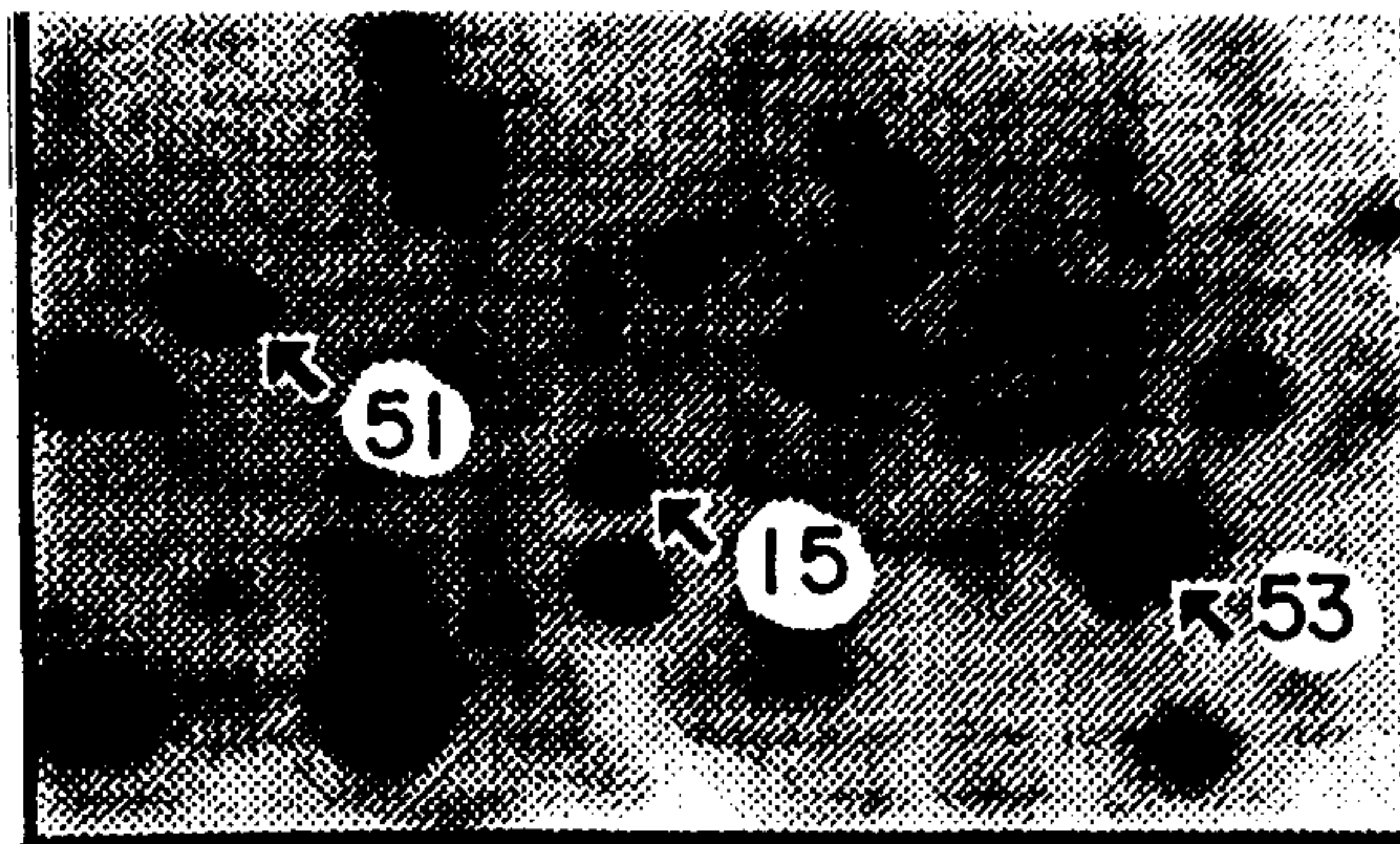


Fig. 22F



Fig. 22G

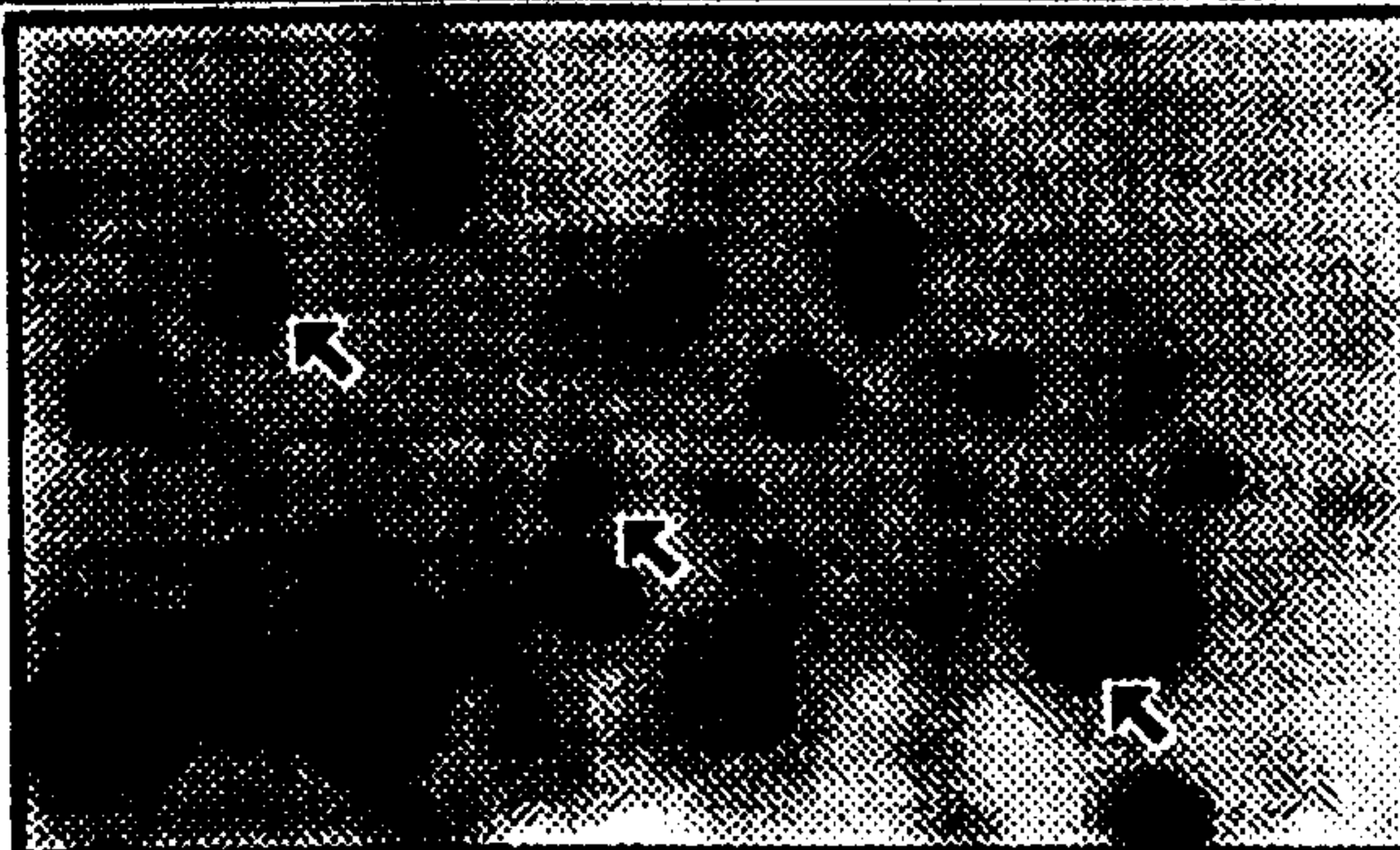


Fig. 22H

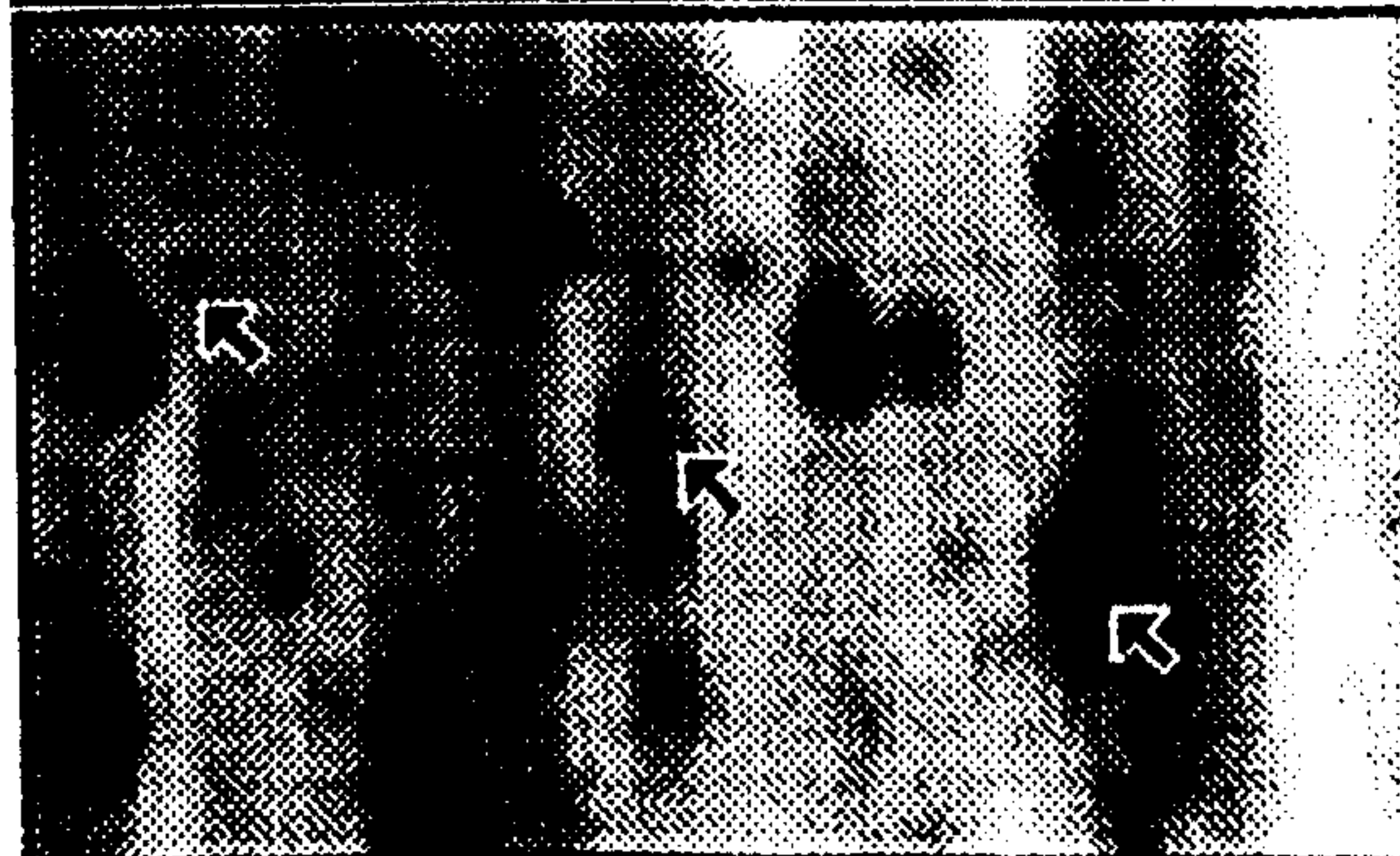


Fig. 22I

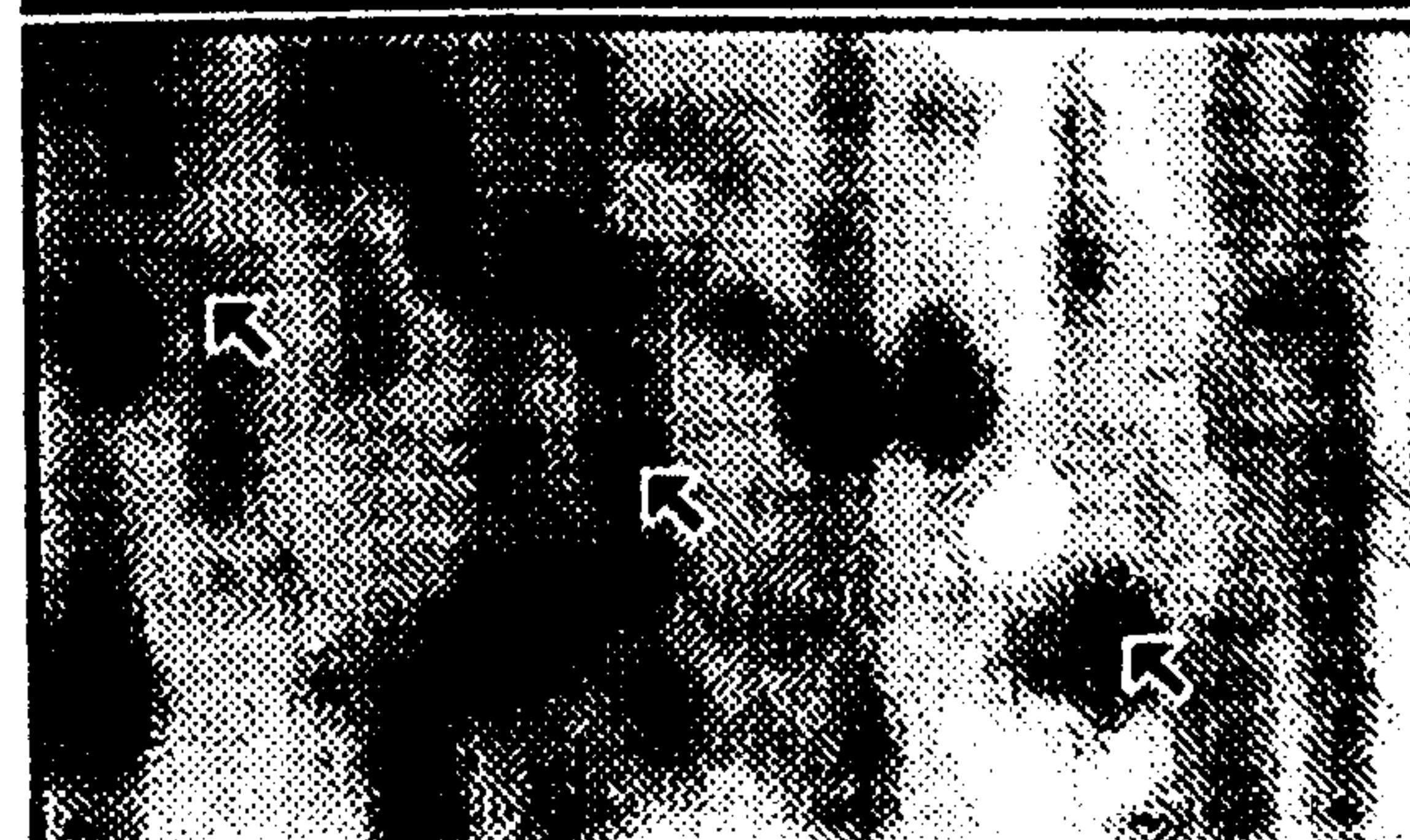


Fig. 22J

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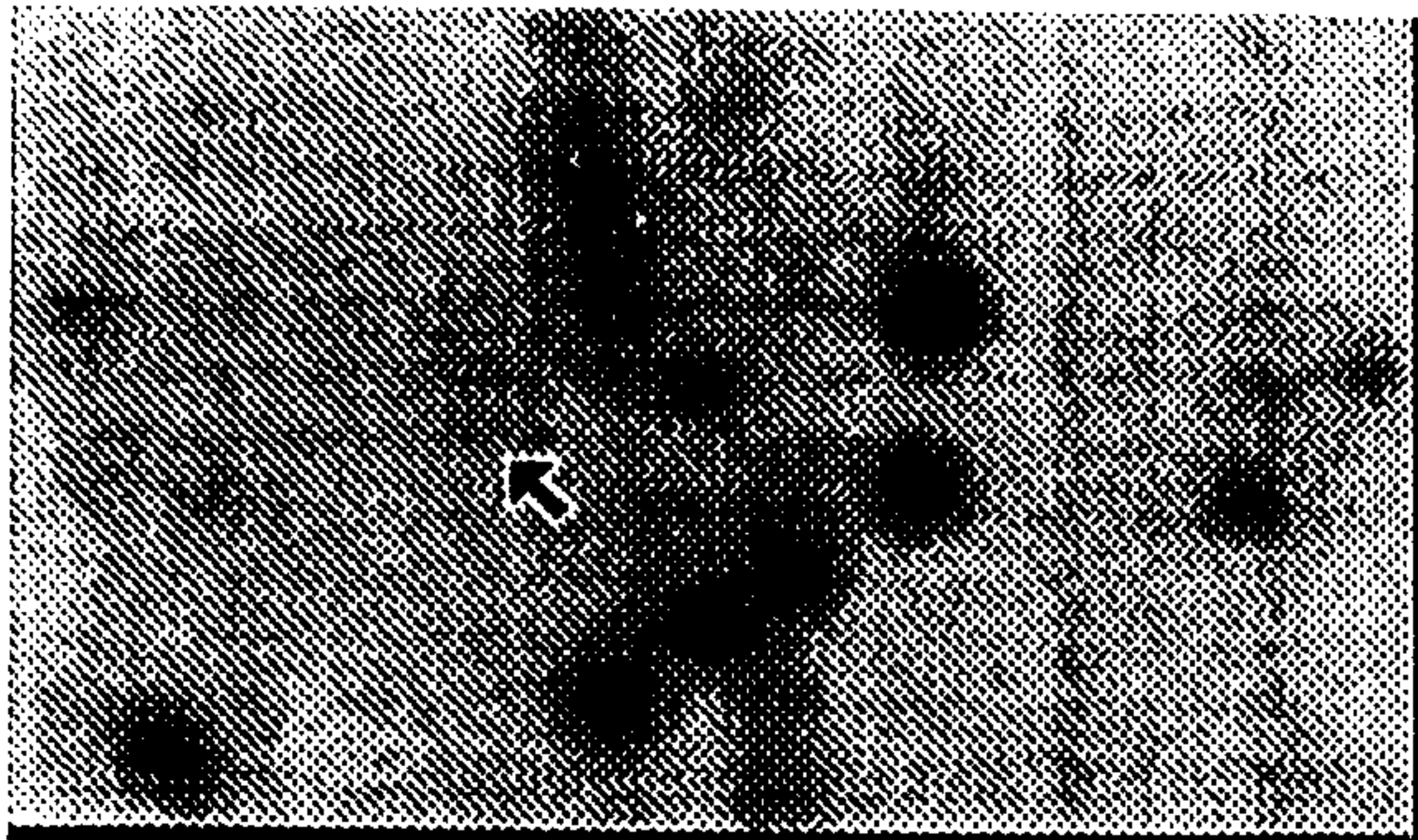


Fig. 23 A

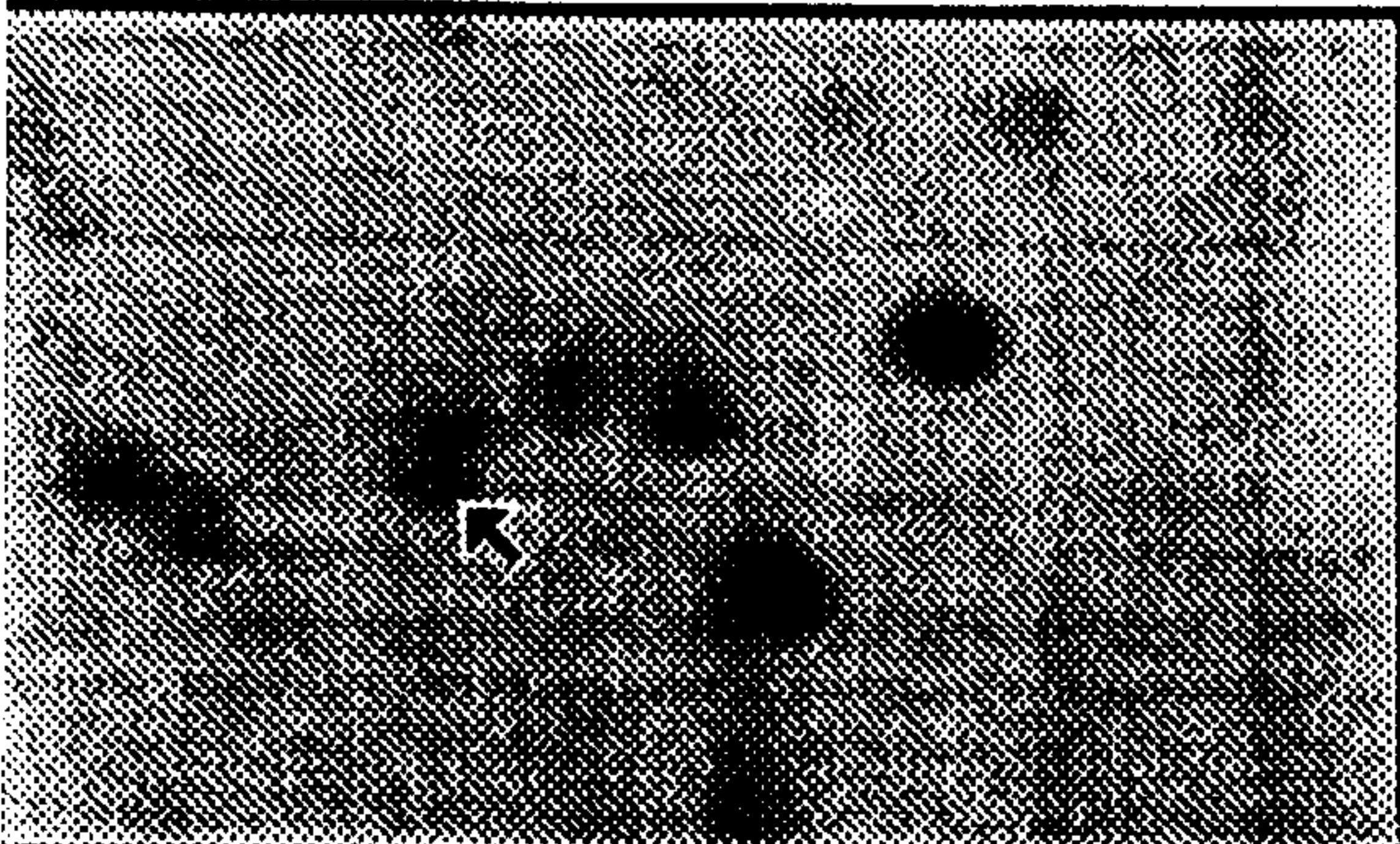


Fig. 23 B

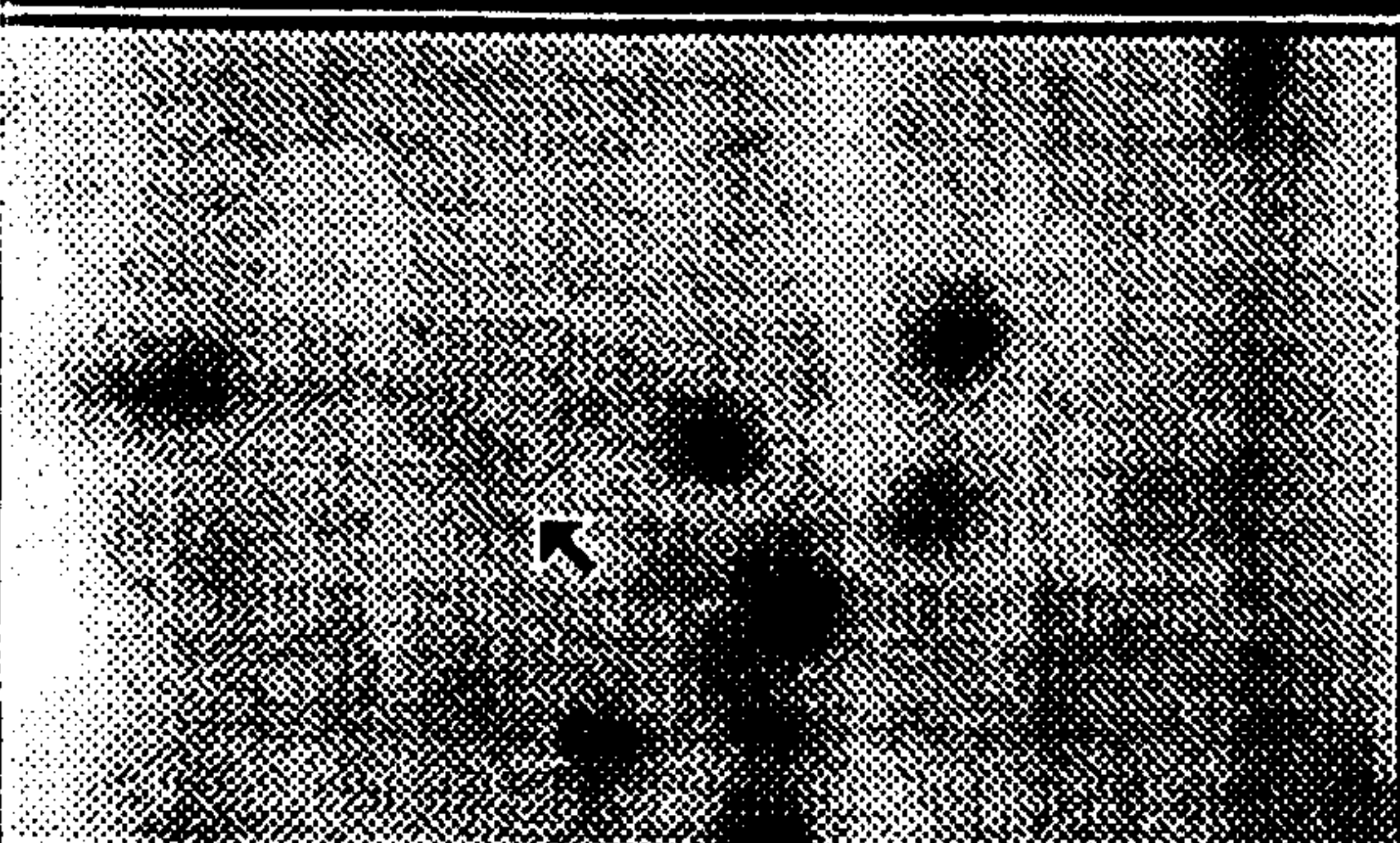


Fig. 23 C

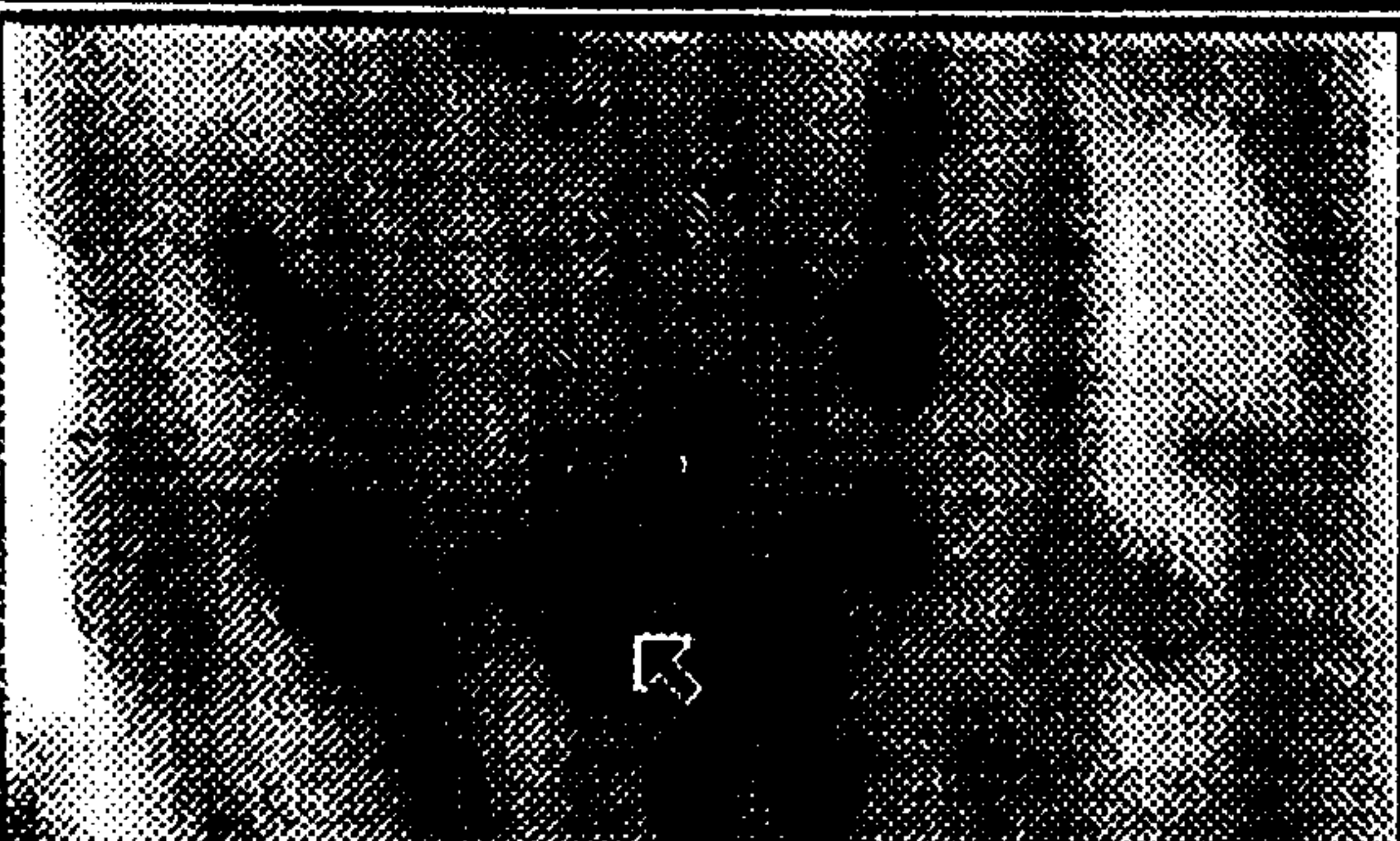


Fig. 23 D

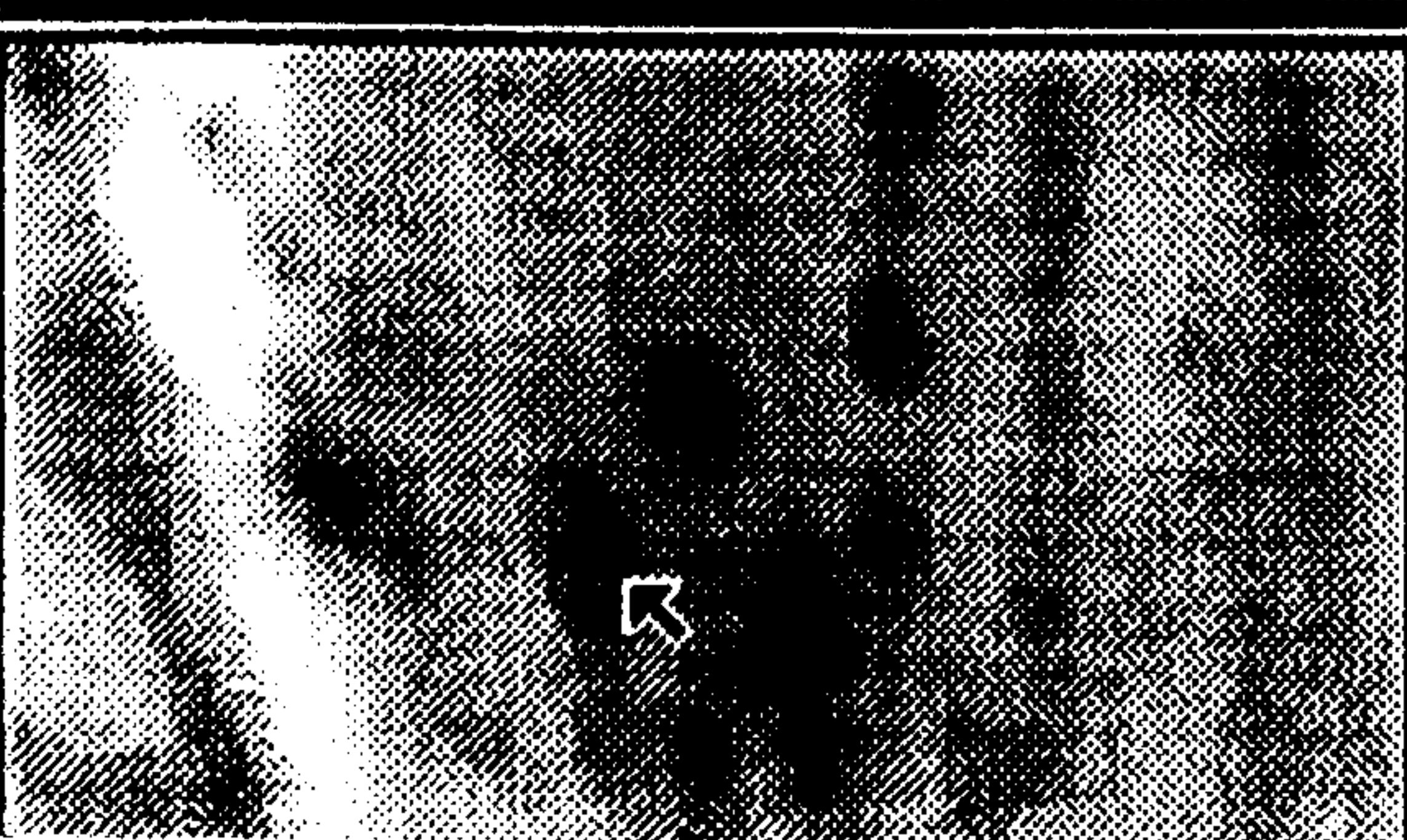


Fig. 23 E

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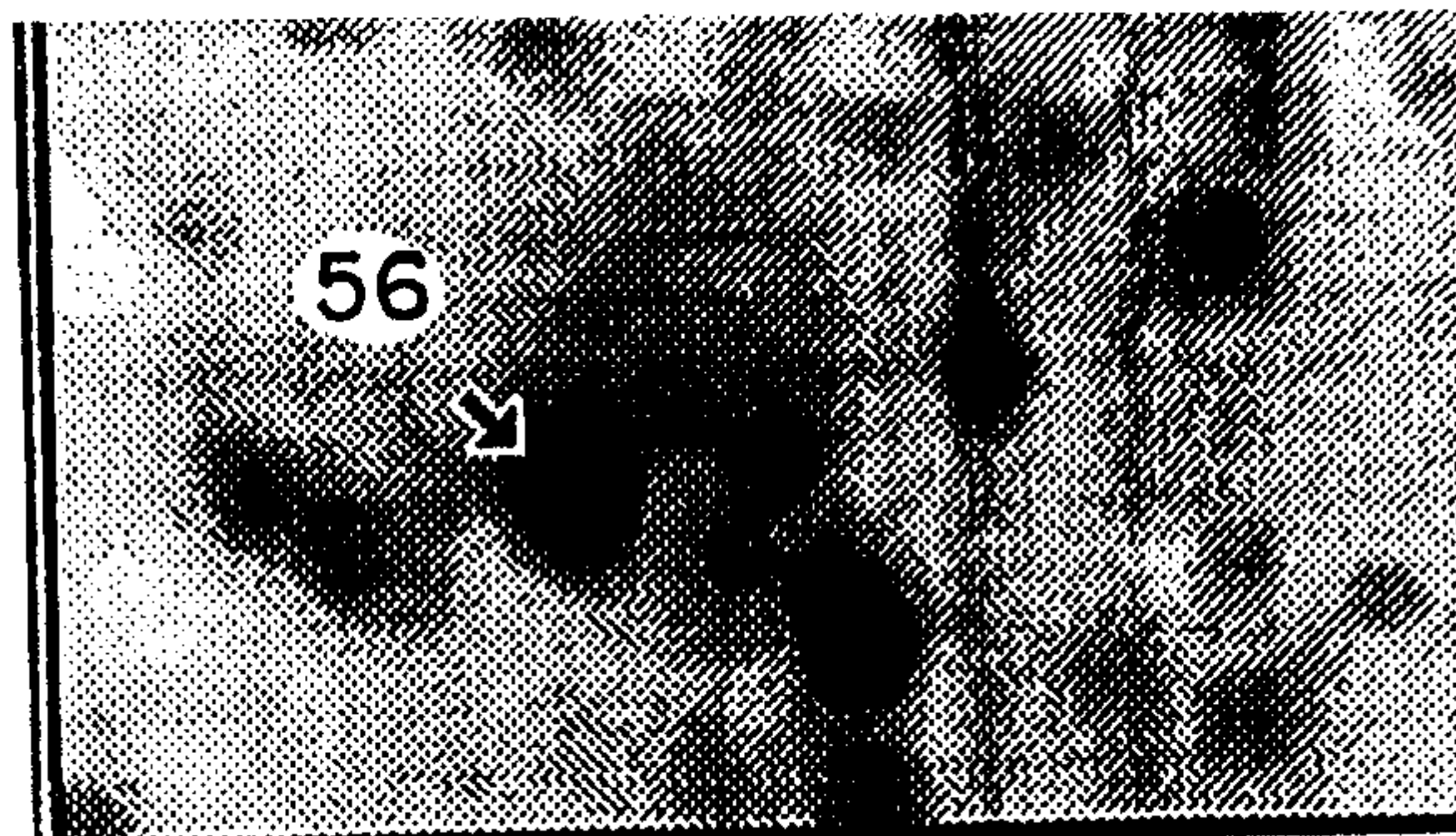


Fig. 23 F

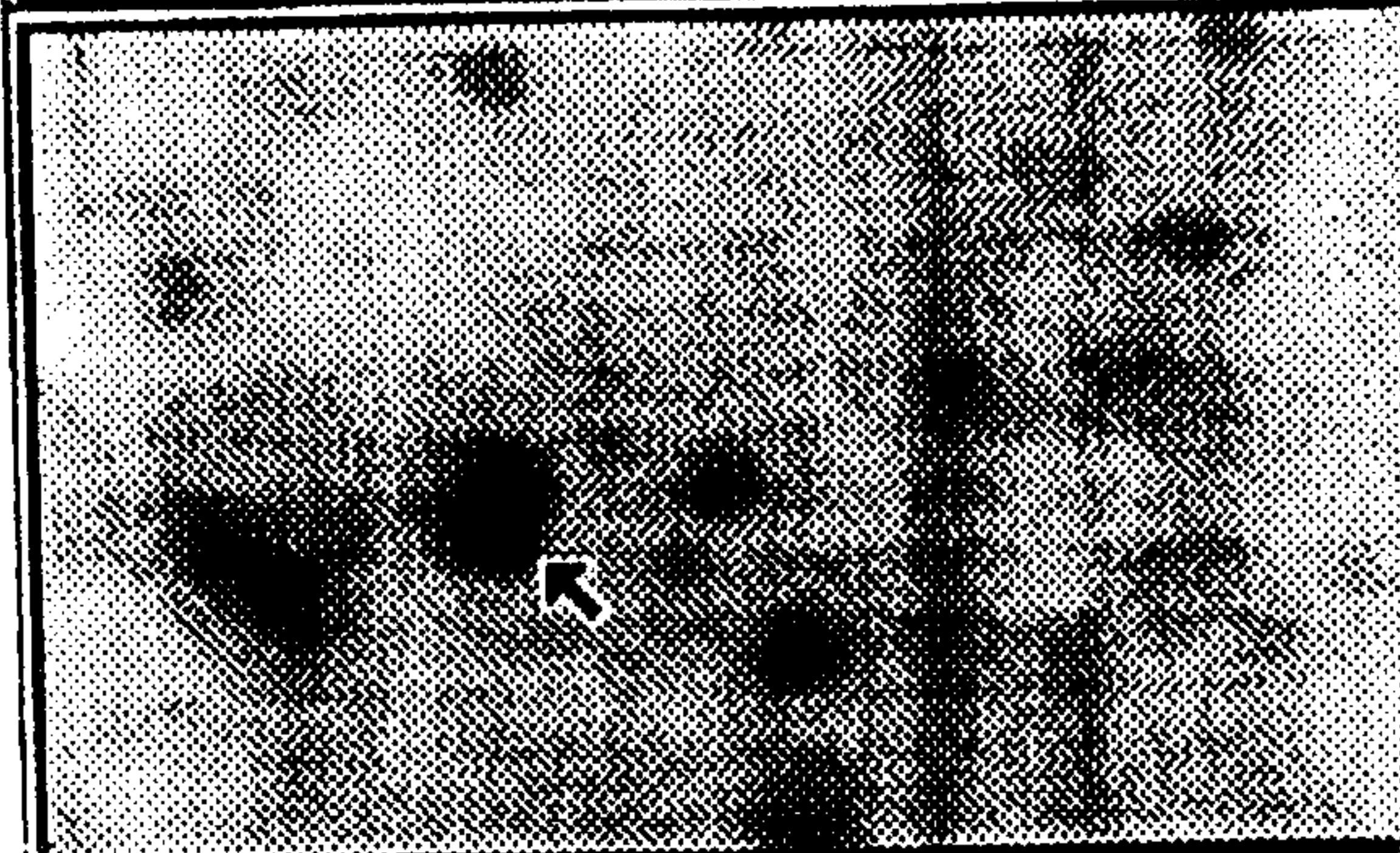


Fig. 23 G

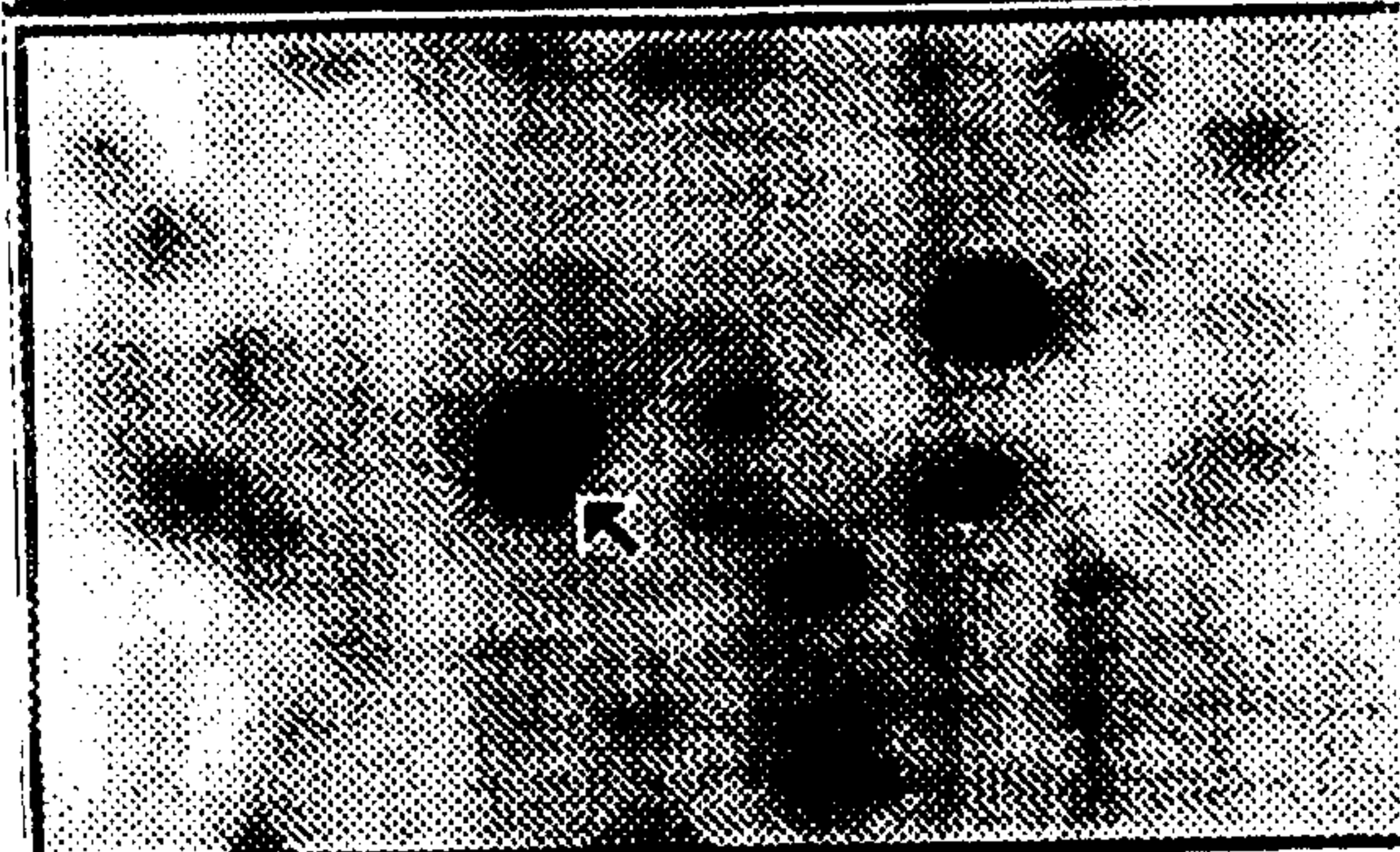


Fig. 23 H

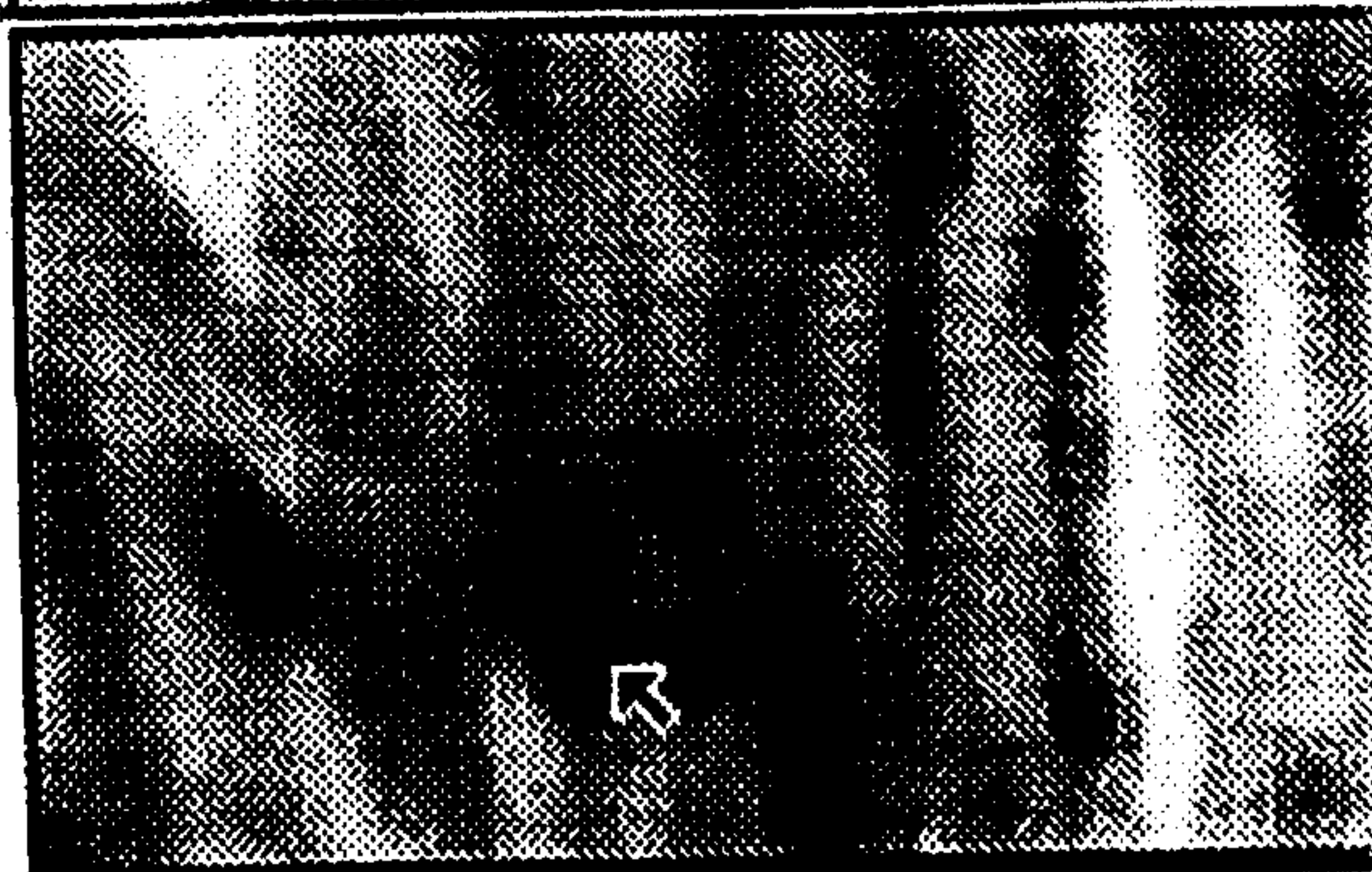


Fig. 23 I



Fig. 23 J

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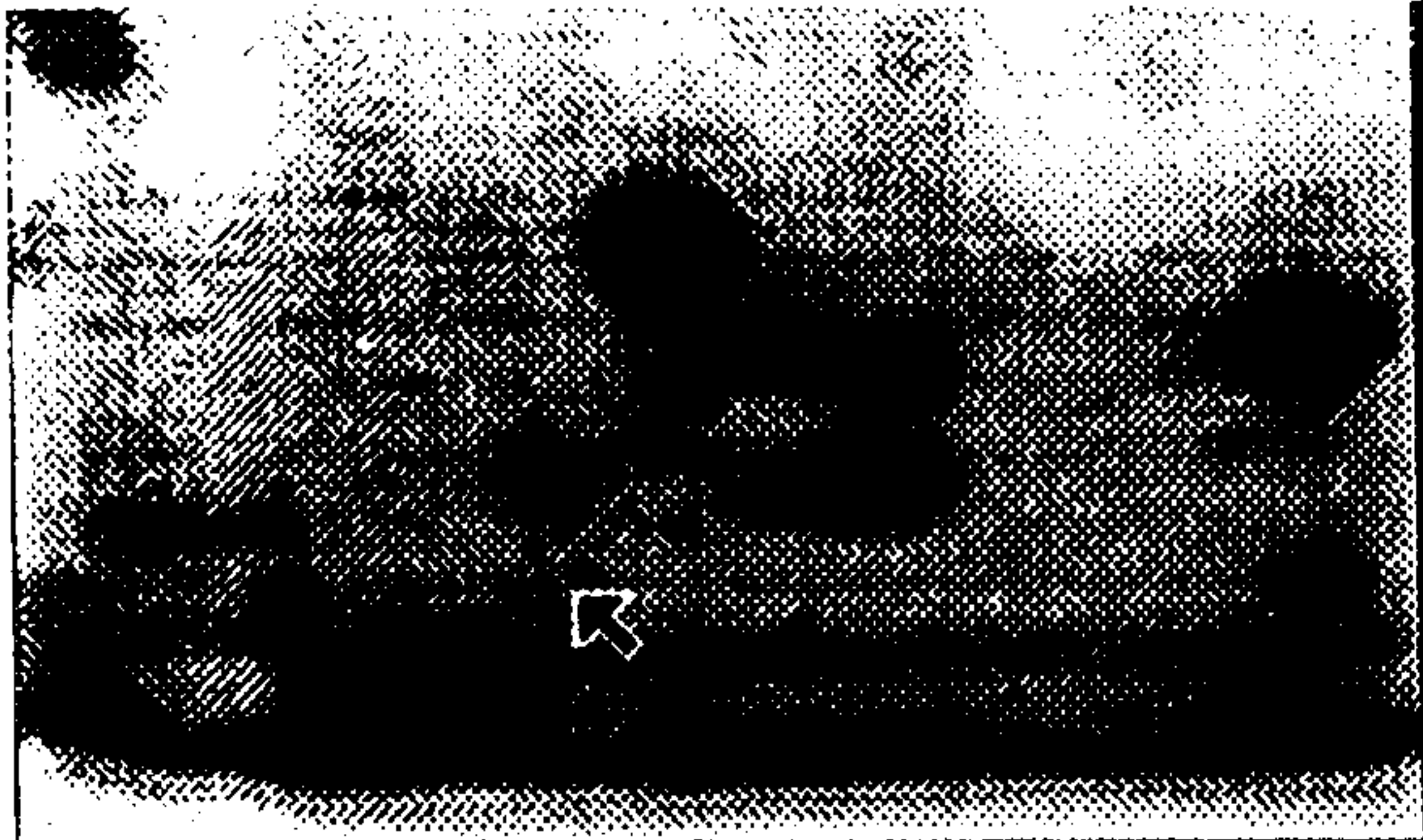


Fig. 24A

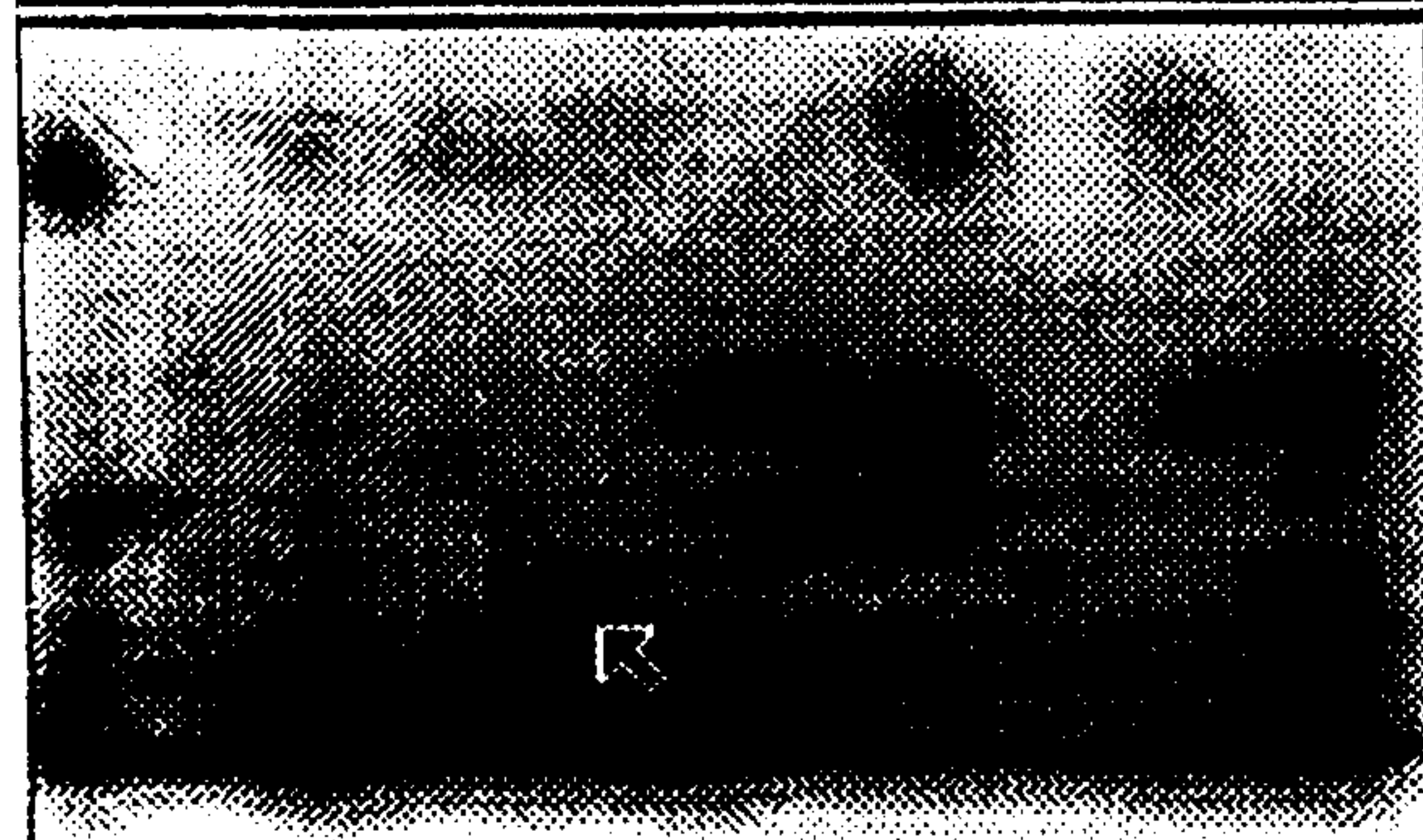


Fig. 24B



Fig. 24C

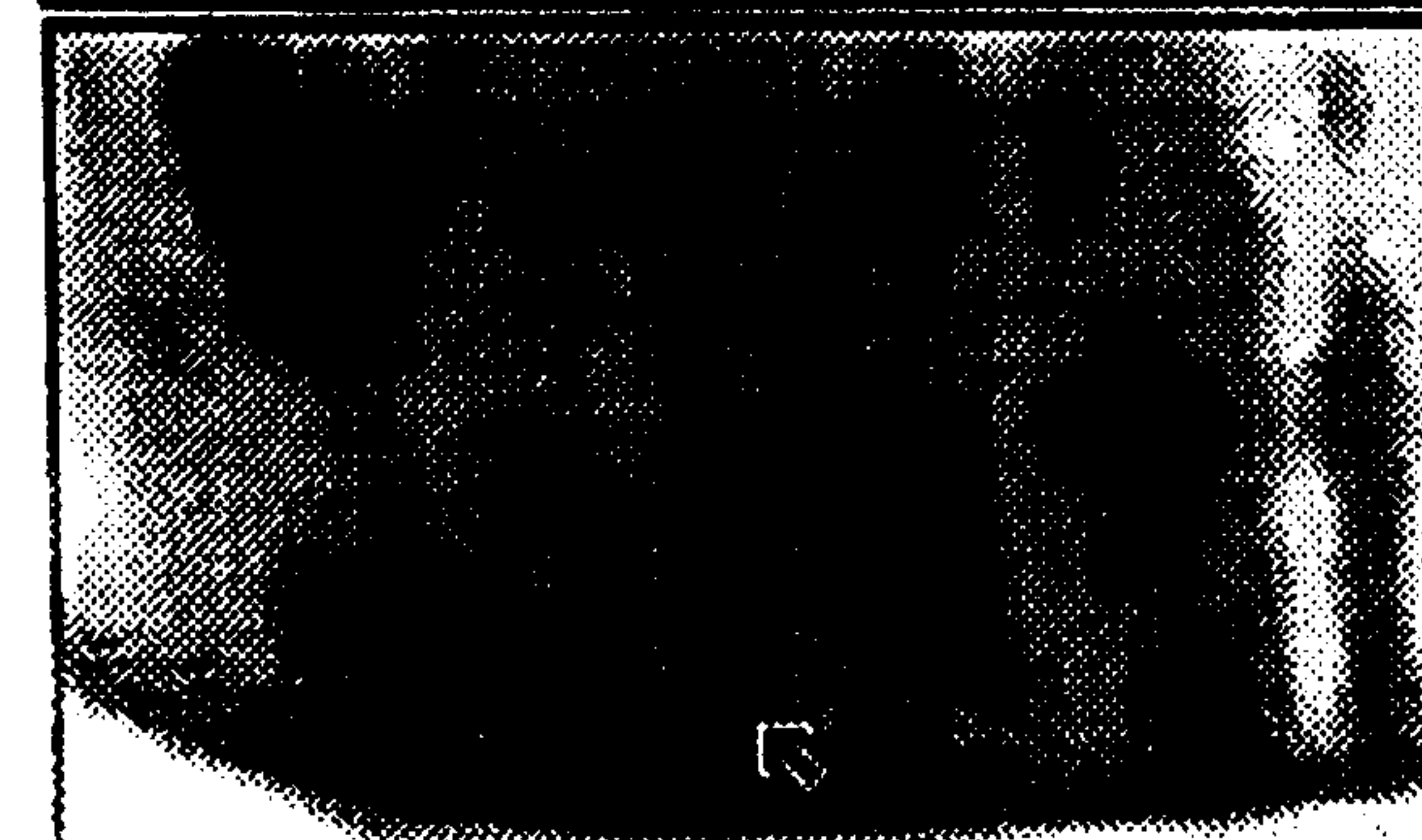


Fig. 24D



Fig. 24E

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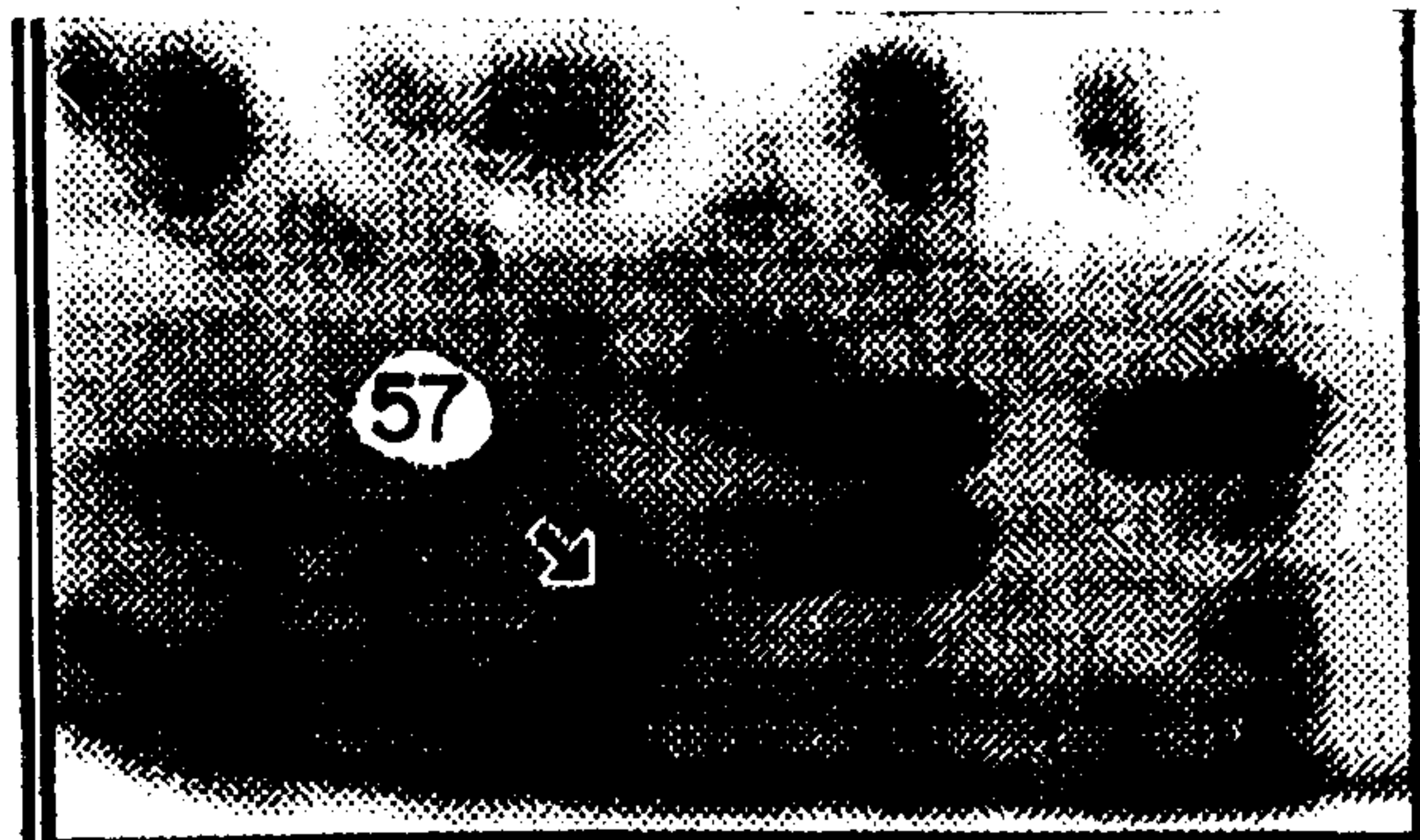


Fig. 24F



Fig. 24G



Fig. 24H

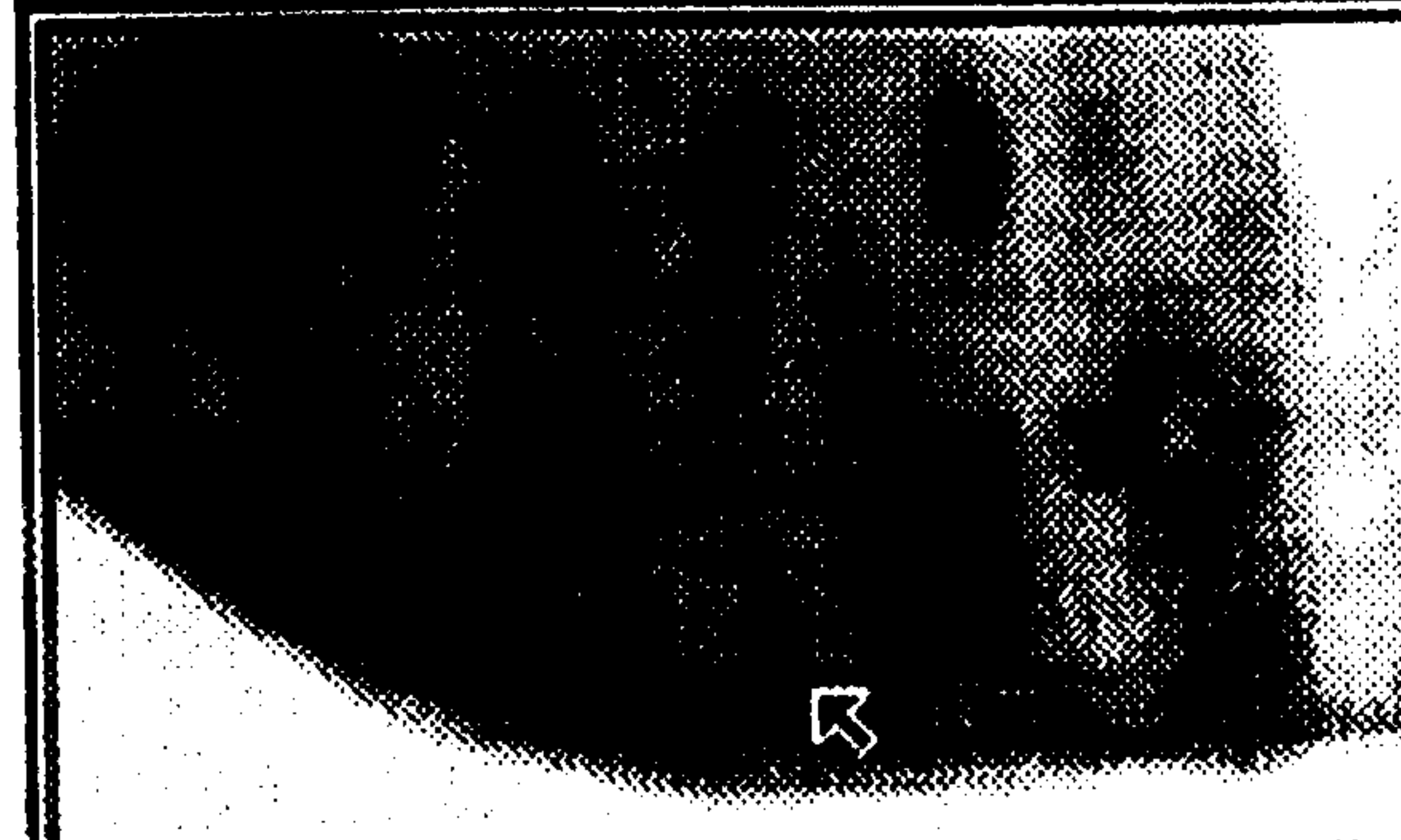


Fig. 24I

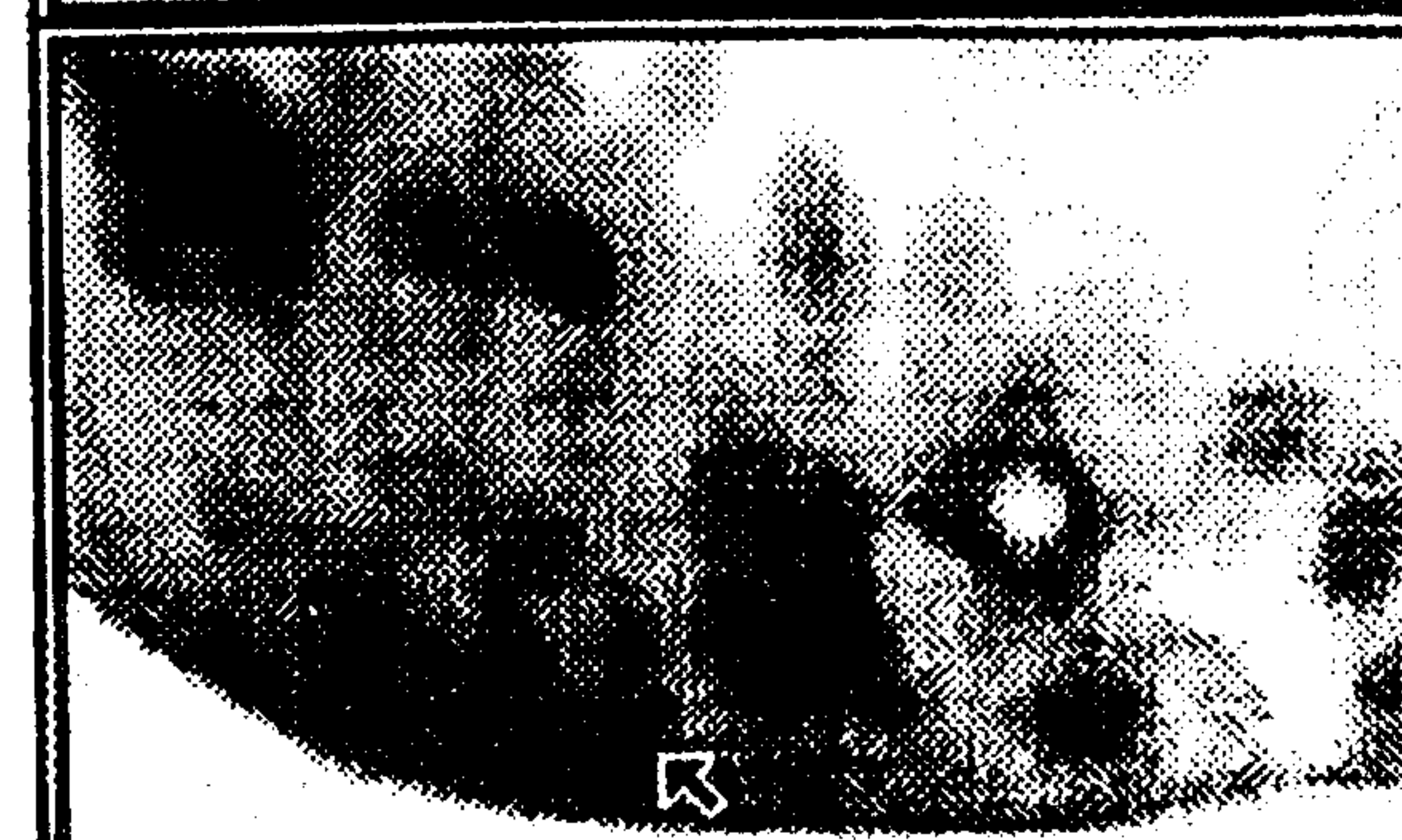
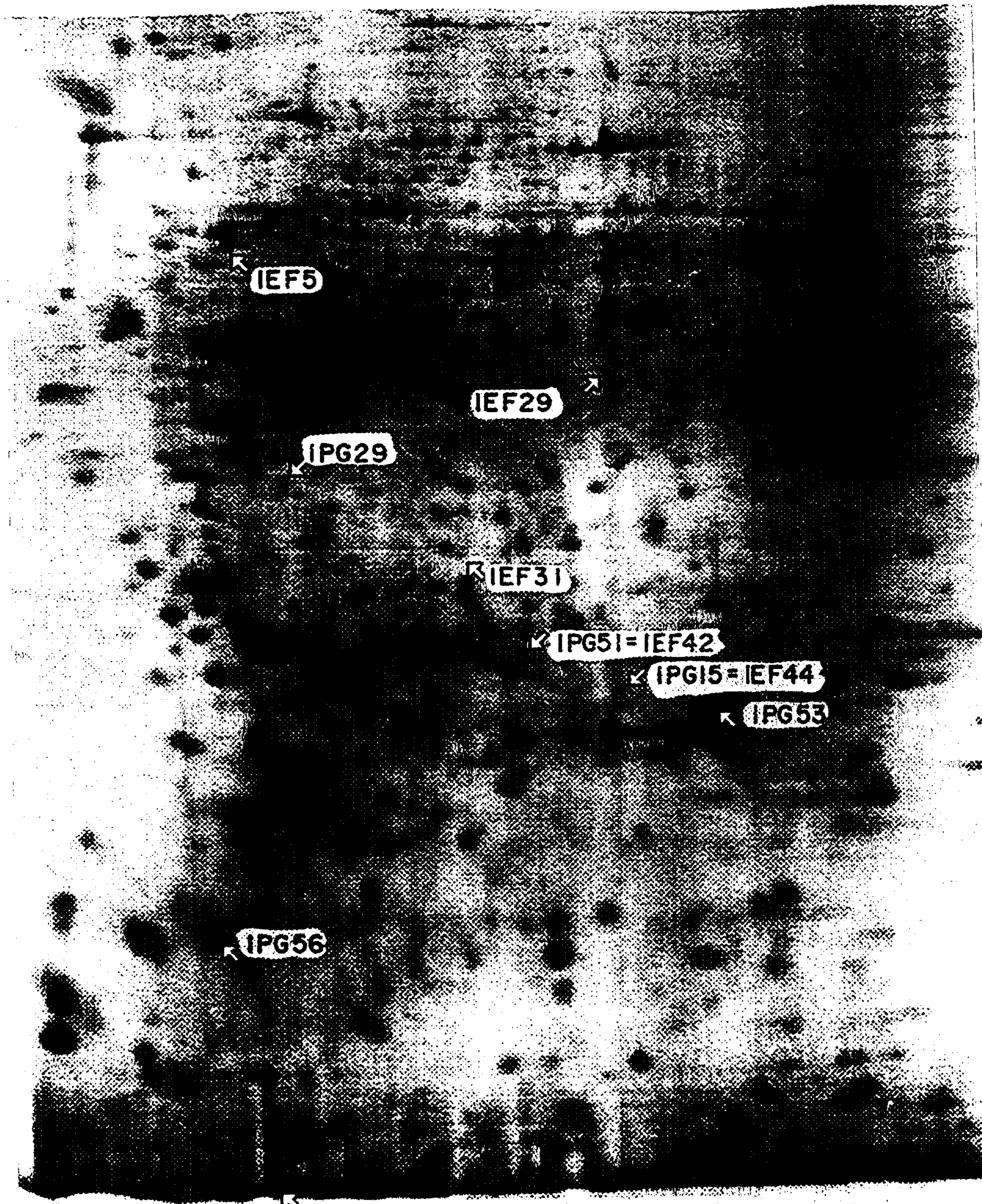


Fig. 24J

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b9923 "Ril" Eso. Adeno.



IPG 57, THIS GEL DOES NOT SHOW IT WELL.

Fig. 25

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