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(72) Inventeurs/Inventors:  
UTERMOHLEN, JOSEPH G., US;  
CONNAUGHTON, JOHN F., US

(73) Propriétaire/Owner:  
VENTANA MEDICAL SYSTEMS, INC., US

(74) Agent: MBM INTELLECTUAL PROPERTY LAW LLP

(54) Titre : FORMULE DE SEQUENCE OLIGONUCLEOTIDIQUE POUR LE MARQUAGE DE SONDÉS  
OLIGONUCLEOTIDIQUES ET DE PROTEINES, DESTINÉ À UNE ANALYSE IN-SITU

(54) Title: OLIGONUCLEOTIDE SEQUENCE FORMULA FOR LABELING OLIGONUCLEOTIDE PROBES AND  
PROTEINS FOR IN SITU ANALYSIS

(57) **Abrégé/Abstract:**

The present invention provides oligonucleotide probes and oligonucleotide probe collections and protein labeling for detecting or localizing a plurality nucleic acid target genes or antigens within a cell or tissue sample. Specifically, the invention provides collections of oligonucleotide probes for use in in situ hybridization analyses in which each probe has a label-domain with the sequence formulas of (CTATTTT)<sub>n</sub> CT, (AAAATAG)<sub>n</sub> or (TTTTATC)<sub>n</sub> or (GATAAAA)<sub>n</sub> in which all cases "n" would equal 1 or greater. The present invention provides collections or "cocktails" of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lambda gene (SEQ ID NOS: 17 through 29 inclusive); the CMV (cytomegalovirus) gene (SEQ ID NOS: 30 through 50 inclusive); EBER (Epstein-Barr early RNA) gene (SEQ ID NOS: 51-54 inclusive); Alu (SEQ ID NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO: 58).

### ABSTRACT OF THE DISCLOSURE

The present invention provides oligonucleotide probes and oligonucleotide probe collections and protein labeling for detecting or localizing a plurality nucleic acid target genes or antigens within a cell or tissue sample. Specifically, the invention provides collections of oligonucleotide probes for use in *in situ* hybridization analyses in which each probe has a label-domain with the sequence formulas of (CTATTTT)<sub>n</sub>CT, (AAAATAG)<sub>n</sub> or (TTTTATC)<sub>n</sub> or (GATAAAA)<sub>n</sub> in which all cases “n” would equal 1 or greater. The present invention provides collections or “cocktails” of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lambda gene (SEQ ID NOS: 17 through 29 inclusive); the CMV (cytomegalovirus) gene (SEQ ID NOS: 30 through 50 inclusive); EBER (Epstein-Barr early RNA) gene (SEQ ID NOS: 51-54 inclusive); Alu (SEQ ID NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO: 58).

# OLIGONUCLEOTIDE SEQUENCE FORMULA FOR LABELING OLIGONUCLEOTIDE PROBES AND PROTEINS FOR IN SITU ANALYSIS

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

This invention relates to oligonucleotide probes and collections of oligonucleotide probes for detecting or localizing nucleic acid genes targets within a cell or tissue sample. In particular, the invention relates to collections of oligoprobes.

### 2. Background of the Invention

*In situ* analysis includes *in situ* hybridization and immunohistochemistry. *In situ* hybridization (ISH) employs labeled DNA or RNA probe molecules that are anti-sense to a target gene sequence or transcript to detect or localize targeted nucleic acid target genes within a cell or tissue sample. ISH has proven to be a useful tool in a number of biomedical fields, including developmental biology, cell biology, and molecular biology. ISH has been used, for example, to diagnose genetic disorders, map genes, study gene expression, and localize sites of target gene expression.

Typically, ISH is performed by exposing a cell or tissue sample immobilized on a glass slide to a labeled nucleic acid probe which is capable of specifically hybridizing to a given target gene in the cell or tissue sample (*In Situ Hybridization: Medical Applications* (G. R. Coulton and J. de Belleruche, eds., Kluwer Academic Publishers, 1992); *In Situ Hybridization: In Neurobiology; Advances in Methodology* (J. H. Eberwine, K. L. Valentino, and J. D. Barchas, eds., Oxford University Press, 1994); *In Situ Hybridization: A Practical Approach* (D. G. Wilkinson, ed., Oxford University Press, 1992)). The hybridization of labeled probe molecules to nucleic acids in the cell or tissue sample can then be detected using, for example, radioactive-based direct detection methods, fluorescence-based direct detection methods, or indirect detection methods based on the binding of a fluorescence-labeled protein binding to a hapten such as BrdU, digoxigenin-labeled or biotin-labeled nucleotides incorporated into probes. Hapten-based methods have been further extended to include those molecules to be bonded by binding protein-enzyme conjugates such as antibody-enzyme-conjugates and colorimetric based detection chemistry. In addition, several target genes can be simultaneously analyzed by exposing a cell or tissue sample to a plurality of nucleic acid probes that have been labeled with a plurality of different nucleic

acid tags. For example, a plurality of nucleic acid probes can be labeled with a plurality of fluorescent compounds having different emission wavelengths, thereby permitting simultaneous multicolored analysis to be performed in a single step on a single target cell or tissue sample.

5 A significant problem associated with incorporation of labeled nucleotides into oligonucleotide probes is that the conjugation moieties that are attached to the nucleotide usually interfere with the formation of Watson-Crick base pairing, thus negatively affecting the hybridization of the probe to its target. This has been seen with use of label attached via N4-substituted cytosine nucleotides, because of steric hinderance and the expected shift to the  
10 less reactive state of a secondary amine (as seen with N4 labeled cytosine), as compared to the natural G-C bond formed with an unsubstituted cytosine (a primary amine). Any small change or interference with G-C bonding in a small oligonucleotide (25 to 50 bases) can reduce the ability of these oligos to hybridize with the intended targeted sequence.

There remains a need in the art to develop suitable probes designs for incorporating  
15 labeled nucleotides in oligonucleotide probes. We demonstrate that a few artificial sequences are viable alternatives for probe labeling and also work both singly and in complex oligonucleotide probe mixtures for detecting or localizing nucleic acid target genes within a cell or tissue sample. The development of such generic sequences and labeling strategy for probe collections has wide application in the medical, genetic, and molecular biological arts.

20 This interference due to labeling chemistry and hybridization stringency and kinetics is solved herein by designing the oligo to have at least two distinct functional domains, one domain or sequence to be gene specific and involved in the base pair formation, and the second domain to be an artificial, non-specific sequence (in reference to the sample's genome) comprised of spacing nucleotides and the labeled nucleotide. These elements are  
25 positioned so that these label-nucleotides are more accessible as haptens for binding proteins (immunoglobulin or avidin(s)) and thus do not interfere with Watson-Crick base pairing in the gene-specific domain.

## SUMMARY OF THE INVENTION

30 The present invention provides a novel strategy to incorporate label into oligonucleotide probes and labeled oligonucleotide probe collections for detecting or

localizing nucleic acid target genes within a cell or tissue sample. In particular, the invention relates to non-gene-specific sequences using sequence formulas for making repetitive polymers of such sequences which can be incorporated into collections of oligonucleotide probes for use in *in situ* hybridization analyses. In addition, using labeled synthetic

5 oligonucleotide polymers, based on sequence formulas, when conjugated to binding proteins, i.e. immunoglobulins, is a very effective and controlled process for labeling such proteins used in immunohistochemical analysis. The present invention provides collections or "cocktails" of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the

10 Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lambda gene (SEQ ID NOS: 17-29); the CMV (cytomegalovirus) gene (SEQ ID NOS: 30-50 inclusive); EBER (Epstein-Barr early RNA) gene (SEQ ID NOS: 51-54 inclusive); Alu (SEQ ID NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO: 58).

The invention is directed to an oligonucleotide label-domain comprising the sequence

15 (CTATTTT)<sub>n</sub> and its complement (AAAATAG)<sub>n</sub> wherein "n" is at least 1.

The invention is also directed to a probeset for detecting Kappa immunoglobulin light

20 chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 1 through 16, inclusive.

The invention is also directed to a probeset for detecting Lambda immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 17-29.

25 The invention is also directed to a probeset for detecting cytomegalovirus (CMV) immediate early RNA and /or corresponding mRNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 30 through 50.

The invention is also directed to a probeset for detecting Epstein Barr virus (EBV) early RNA, RNA 1 and RNA 2, (EBER) wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 51 through 54.

The invention is also directed to a probeset for detecting Human Alu repetitive satellite  
5 genomic DNA sequences wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 55 and 56.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

## 10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a generic probe structure of the two-domain probe design. This is the oligonucleotide design used for the probes in the gene specific cocktails described in the following examples. Each probe is composed of two domains: a 5' labeling domain and a 3' target gene target gene-specific domain. The labeling domain consists of this specific  
15 sequence (CTATTTT)<sub>n</sub>, wherein each cytosine may be labeled with a fluorophore or a cytosine-hapten conjugate, the hapten being fluorescein in this embodiment. This illustration specifically shows nucleic acid sequences for the 301 (SEQ ID NO: 55) and 302 (SEQ ID NO: 56) probes, each of which possesses target gene gene-specific domains corresponding to human repetitive Alu sequences and labeling domains having a fluorescein hapten.

20 Figure 2 illustrates the results obtained for *in situ* hybridization (ISH) analysis of human skin tissue using a probe comprising the labeling domain (330 probe; SEQ ID NO: 58). The absence of a detectable signal indicates that the sequence formula, (CTATTTT)<sub>n</sub>CT, of the labeling domain common to the oligonucleotides used in these ISH examples is non-specific, and non-reactive in its ability to form Watson-Crick base pairing with human  
25 nucleic acid sequences because it does not hybridize.

Figure 3 illustrates the results obtained for ISH analysis of human skin tissue using a probe comprising the labeling domain and a poly d(T) target gene-specific domain (320 probe; SEQ ID NO: 57). The presence of a detectable signal localized to the cytoplasm

indicates that this probe is capable of specifically hybridizing to polyadenylated region of messenger RNA.

Figures 4A-4B illustrate the results obtained for ISH analysis of human skin tissue using the 320 probe, wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to polyadenylated region common to messenger RNA.

Figures 5A-5B illustrate the results obtained for ISH analysis of human spleen tissue using the 320 probe, wherein the hybridization and stringency wash were performed at room temperature (A), or at 37°C (B). This result illustrates that the intensity of color is related to the stringency of hybridization conditions, with the more intense color indicating less stringent conditions.

Figure 6 illustrates the results obtained for ISH analysis of the human Raji cell line using the 320 probe. This shows that this probe design also is functional with embedded cell lines as well as embedded tissue.

Figure 7 illustrates the results obtained for ISH analysis of the human Raji cell line using a probe collection consisting of the 301 and 302 probes.

Figure 8 illustrates the results obtained for ISH analysis of the human HT cell line using a probe collection consisting of the 301 and 302 probes.

Figure 9 illustrates the results obtained for ISH analysis of a rat cell line using a probe collection consisting of the 301 and 302 probes. The absence of a detectable signal indicates that this probe collection is specific for human nucleic acid sequences.

Figure 10 illustrates the results obtained for ISH analysis of an Epstein-Barr virus (EBV)-negative human HT cell line using a probe possessing a target gene-specific domain corresponding to EBV EBER nuclear RNA (SEQ ID NOS: 51 through 54 inclusive).

Figure 11 illustrates the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA (SEQ ID NOS: 51 through 54 inclusive).

Figure 12 illustrates the results obtained for ISH analysis of human tonsil tissue using a probe collection consisting of probes possessing target gene-specific domains

corresponding to EBV EBER 1 and 2 nuclear RNA. (SEQ ID NO: 51 through SEQ ID NO: 54).

Figures 13A-13B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA. (SEQ ID NO: 51 through SEQ ID NO: 54), wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to human EBER 1 and EBER 2 nuclear RNA.

Figure 14 illustrates the results obtained for ISH analysis of kappa light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain mRNA (SEQ ID NO:15).

Figure 15 illustrates the results obtained for ISH analysis of lymphoma tissues using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin kappa light chain mRNA (SEQ ID NOS: 2-4, SEQ ID NOS: 7-12, SEQ ID NOS: 14, 15). The lymphoma tissue in (A) over expresses the kappa light chain and the tissue in (B) over expresses the lambda light chain. The absence of a detectable signal in (B) indicates that the kappa light chain probe collection is specific to kappa light chain mRNA.

Figure 16 illustrates the results obtained for ISH analysis of lambda light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain variable region mRNA (SEQ ID NOS:19 through 29).

Figure 17 illustrates the results obtained for ISH analysis of a lambda light chain-positive human RPMI 8226 cell line using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA (SEQ ID NOS:19 through 29).

Figures 18A-18B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA (SEQ ID NOS: 19 through 29). The tissue in (A) over expresses the lambda light chain and the tissue in (B)

over expresses the kappa light chain. The absence of a detectable signal in (B) indicates that the lambda light chain probe collection is specific to human lambda light chain mRNA.

Figure 19 illustrates the results obtained for ISH analysis of cytomegalovirus (CMV)-positive human lung tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA (SEQ ID NOS: 30-32, SEQ ID NOS: 34-35, SEQ ID NO:38, SEQ ID NO: 50). (CMV infected cell)

Figure 20 illustrates the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has not been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early mRNA (SEQ ID NOS: 30-32, SEQ ID NOS: 34-35, SEQ ID NO:38, SEQ ID NO: 50).

Figures 21A-21B illustrate the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA (SEQ ID NOS: 30-32, SEQ ID NOS: 34-35, SEQ ID NO:38, SEQ ID NO: 50). The tissue in (A) is shown at a magnification of 40X and the tissue in (B) is shown at a magnification of 20X.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides oligonucleotide probes and oligonucleotide probe collections for detecting or localizing nucleic acid target genes within a cell or tissue sample. In particular, the invention relates to collections of oligonucleotide probes for use in *in situ* hybridization analyses.

More specifically, this invention relates to the use of specific sequence formulas for nucleotide polymers or label-domains to attach a detectable moiety ( a label) to oligonucleotide probes or proteins. The specific utility of these sequences or derivatives thereof, is the inert or non-reactive characteristic that does not hybridize to human DNA or RNA at a detectable level under standard stringency of hybridization conditions. These label-domains or polymers were demonstrated to be useful generic sequences for incorporation into oligonucleotide probes for detecting gene-specific sequences within cells or tissue samples in *in situ* hybridization analyses. Additionally, this inert set of sequences

are useful for attaching a label to immunoglobulins or other proteins for detecting haptens and antigens in immunohistochemical analyses.

As used herein, the terms “probe” or “oligonucleotide probe” refers to a nucleic acid molecule used to detect a complementary nucleic acid target gene.

5 As used herein, the term “hybridization” refers to the process whereby complementary nucleic acid sequences join to form a double-stranded nucleic acid molecule. By labeling the target nucleic acid molecule with, for example, a radioactive or fluorescent tag, interactions between probe and target genes can be detected.

10 The oligonucleotide probes and oligonucleotide probes of the collections of the present invention are synthesized using conventional methods. *See e.g., Methods in Molecular Biology, Vol 20: Protocols for Oligonucleotides and Analogs* 165-89 (S. Agrawal, ed., 1993); *Oligonucleotides and Analogues: A Practical Approach* 87-108 (F. Eckstein, ed., 1991).

15 In a preferred embodiment of the present invention, oligonucleotide probes possess two distinct domains: a 5' (or labeling) domain and a 3' (or gene-specific target) domain (See Figure 1A). In more preferred embodiments of the present invention, the oligonucleotide probe possesses a labeling domain which consists of the sequences (CTATTTT)<sub>n</sub> and/or (CTATTTT)<sub>n</sub>CT. Other embodiments are also demonstrated herein, including a triple-domain embodiment having two terminal labeling domains, and a central gene-specific target domain. Specifically, SEQ ID NOS: 125-126 depict this labeling scheme. Yet 20 a further preferred embodiment of a labeling domain is TC(TTTTATC)<sub>n</sub> or its complement. This sequence is predicted to be as unique as the (CTATTTT)<sub>n</sub>CT label-domain. The oligonucleotide probes of the present invention are labeled so that hybridization between said probes and target nucleic acids in a particular cell or tissue can be detected. Labels that are 25 acceptable for use in *in situ* hybridization (ISH) analysis are known to those with skill in the art. Such labels permit interactions between probe and target genes to be detected using, for example, radioactive-based direct detection methods, fluorescence-based direct detection methods, digoxigenin-labeled or biotin-labeled probes coupled with fluorescence-based detection methods, or digoxigenin-labeled or biotin-labeled probes coupled with antibody- 30 enzyme-based detection methods. In preferred embodiments of the present invention, oligonucleotide probes are labeled with fluorescein. In more preferred embodiments of the

present invention, the oligonucleotide probe possesses a labeling domain which consists of the sequence (CTATTTT)<sub>n</sub>CT, wherein the cytosine nucleotides may be labeled with a flurophore for direct detection, or a hapten for indirect detection. In either, the fluorescein-cytosine nucleotide conjugate and the fluorescein molecule is linked at the N4 position of cytosine through an OBEA linkage (See Mishra *et al.*, U.S. Patent No. 5,684,142 ).

In a preferred embodiment, the density of fluorophore attached to the label-domain is at least 7 mole percent, preferably at least 10 mole percent, and most preferably at least 16 mole percent, when measured against the label-domain solely. For example, if probe 401 is considered (a 2-domain probe) it comprises a label-domain of 30 bases including a 3' terminal CT wherein the C is also labeled, the mole percent is  $5/30 = 16.7$  mole percent label. In the overall probe, the mole percent is 8.3.

In some embodiments of the present invention, several target genes are simulanteously analyzed by exposing a cell or tissue sample to a plurality of nucleic acid probes that have been labeled with a plurality of different nucleic acid tags. For example, a plurality of nucleic acid probes can be labeled with a plurality of fluorescent compounds having different emission wavelengths, thereby permitting simultaneous multicolored analysis to be performed in a single step on a single target cell or tissue sample.

The oligonucleotide probes and oligonucleotide probe collections of the present invention may be used in ISH analysis to detect or localize nucleic acid target genes within a cell or tissue sample. ISH may be performed as described, for example, in *In Situ Hybridization: Medical Applications* (G. R. Coulton and J. de Belleruche, eds., Kluwer Academic Publishers, 1992); *In Situ Hybridization: In Neurobiology; Advances in Methodology* (J. H. Eberwine, K. L. Valentino, and J. D. Barchas, eds., Oxford University Press, 1994); or *In Situ Hybridization: A Practical Approach* (D. G. Wilkinson, ed., Oxford University Press, 1992)).

The preferred embodiment of the probes and probe collections of the present invention are best understood by referring to Figures 1-21 and Examples 1-2. The Examples, which follow, are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

**EXAMPLE 1****Probe Collection Preparation**

Probe collections consisting of a plurality of oligonucleotide probes of 55 to 60 bases in length were designed as follows. In this Example, each oligonucleotide probe possessed two distinct domains: a 5' (or labeling) domain and a 3' (or target gene-specific) domain (*See* Figure 1).

In this embodiment, the labeling domain consists of the sequence (CTATTTT)<sub>n</sub>CT, wherein the cytosine nucleotide represents a fluorescein-cytosine nucleotide conjugate and the fluorescein molecule is linked at the N4 position of cytosine through an OBEA linkage.

The target gene-specific domain consists of a 25-30 base sequence that is complementary to a specific nucleic acid target gene. Oligonucleotide probes were designed to possess target gene-specific domains corresponding to the human immunoglobulin kappa light chain variable region (*See* Table 1; oligonucleotide probes 401-416), the human immunoglobulin lambda light chain variable region (oligonucleotide probes 501-515), human cytomegalovirus (CMV) sequences (oligonucleotide probes 221-241), human Epstein-Barr virus (EBV) EBER (Epstein-Barr early RNA) sequences (oligonucleotide probes 100A2, 100C2, 100A1, and 100B1), human repetitive Alu sequences (oligonucleotide probes 301 and 302), and poly d(T) (oligonucleotide probe 320).

**EXAMPLE 2****Label-domain design: Alu repetitive sequence probe**

Four probes all against the Alu human repetitive sequence were used to evaluate label-domain design. The probes numbered 301 (SEQ ID NO: 55), 301A (SEQ ID NO:116), 301A2/2 (SEQ ID NO: 121), and 301A3/2 (SEQ ID NO: 122) are shown in Table 1.

The four probes were evaluated at the concentrations of 100, 75, 50, and 25 ng/ml per mL of probe in the reaction, respectively. This hybridization analysis was done manually, using standard protocols. The target, paraffin-embedded cell line MBA MD 468 (Oncor INFORM™ Her-2/neu Control Slides, Cat. No. S8100, Level 1, available from Ventana Medical Systems, Inc., Tucson, AZ) was the target sample and was processed by removing paraffin by standard xylene methods. The tissue was subjected to Ventana's Protease 1 for 12 minutes at 50 degrees C as a 1:2 dilution with Ventana's APK buffer. The hybridization reaction was accomplished with the addition of probe diluent as 100 ul probe (25%

formamide, 5% dextran sulfate, 2X SSC, 1% Triton) to a residual 100 ul volume of 2XSSC/Triton X-100. The slide was heated to 85 degrees C for 5 minutes and then incubated for 1 hr at 37 degrees C. Standard SSC washes followed for removing excess probe. The hybrids were detected with an antibody against FITC. The mouse antibody was detected  
5 colormetrically using Ventana Enhanced Alkaline Phosphatase Blue Detection (cat# 760-061). Unless otherwise indicated, all reagents were obtained from Ventana Medical Systems, Inc., Tucson, AZ. The results were observed by colormetric detection using brightfield microscopy.

The results of these experiments were that signal intensity was a function of the total  
10 number of fluorescein hapten conjugated to the probe and signal was of the specific label-domain design. The greater the number of fluoresceins per probe molecule, the greater the signal observed. Comparison of design and placement of haptens on the probe showed that this was not a factor in signal intensity. The two probes that contained five fluoresceins, (301A3/2 (SEQ ID NO: 122) and 301 (SEQ ID NO:55) both yielded equivalent signal. These  
15 two probes yielded greater signal than seen for 301A2/2, a probe with a split label-domain design with four fluoresceins. The probe 301A2/2 yielded a signal greater than probe 301A a probe with a single label-domain design at the 5' end and with three fluoresceins.

### EXAMPLE 3

#### 20 Label Domain Design: EBER probes

This experiment compared two label-domain designs and sequences to determine whether greater spacing between the fluorescein haptens improves the production of signal during probe detection steps during *in situ* hybridization analysis.

The tissue used was an EBV-infected human spleen tissue fixed in neutral buffered  
25 formalin paraffin embedded section of 4-micron thickness placed on silane plus glass microscope slides. The tissue sections were deparaffinized on a Ventana DISCOVERY™ machine, followed by a 6-min digestion with Ventana's Protease 1, at a temperature of 37 C. The probe was dissolved in hybridization buffer diluent at a concentration of 50 ng/mL as a 100 ul applied to an equal volume of 2 X SSC/ Triton X-100 residual volume left on the slide  
30 after prepared by the Ventana Medical Systems, Inc. automated ISH staining system, Discovery. The probe diluent-mixed with the residual volume on slide for 6 min at 37 C,

then the solution was heated to 85C and held there for a total of 10 min. The slide was then taken to a 37C temperature and held at that temperature for 1 hour. All of these aqueous reactions on the slide were all done under a film of LIQUID COVERSLIP™, to prevent evaporative loss of water during processing. Each slide after hybridization was washed 3 times with 2X SSC/Triton solution, with a 6 min incubation between each wash, the slide volume being approximately 300 ul (+/- 10% vol). The hybrids were detected with an antibody against FITC. The mouse antibody was detected colormetrically using Ventana Enhanced Alkaline Phosphatase Blue Detection (cat# 760-061).

The two oligonucleotide probes used for this study probe 100A1 (SEQ ID NO: 53) and 1002A32 (SEQ ID NO: 120). The two differences between these probes were the label-domain sequence and structure. The probe 100A1 label domain was 5' to gene target domain, contained 5 fluoresceins attached to cytosine residues via the OBEA linker, with the sequence formula of (CTATTTT)<sub>4</sub>CT (SEQ ID NO: 58). The label domain of the oligo probe 1002A32, was similiar, (SEQ ID NO:125). Besides the different sequence the primary difference was that the fluoresceine labeled cytosines were spaced 10 bases apart compared to the oligo 100A1 the cytosine spacing was closer at 7 bases apart. The result of this comparison as deduced by H score analysis were that these oligonucleotide were equivalent as to the amount of signal generated on the slide. The data was that for 100A2, for the 368 cells analysed in a total of 3 fields the H score was 106, and for probe 1002A32 for the 345 cell analysed in three field the H score was 109. The H score is a spectrographic analysis done with microscope that factors into the score background to signal ratio on the tissue section to yield a relative comparison of total target specific signal on the slide. (See reference Giroud, F. Perrin C, and Simony Lafontaine, J.; Quantitative Immunocytochemistry and Immunohistochemistry. Third Conference of the European Society for Analytical Cellular Pathology, 1994; and AutoCyte Quic Immuno User's Manual, 1998, document number PA-029, Co AutoCyte Inc. Burlington NC 2721). The histograms and the score sheet indicated that each oligo were equally efficient in yielding a colormetric signal. This indicates that the position of the label domain can be either 3 prime or 5 prime to the gene target sequence or the gene target sequence can be positioned between two label domains.

#### EXAMPLE 4

##### In Situ Hybridization

The probe collections prepared in Example 1 were first diluted in a solution consisting of 20% dextran sulfate (wt/vol), 50% formamide (vol/vol), 2X SSC, 10 mM Tris-HCl, 5 mM EDTA, and 0.05% Brij-35, at a final pH of 7.3. Probe collections were then mixed with an equal volume of a solution consisting of 2X SSC and 0.05% Triton<sup>™</sup> X-100.

5 Samples for ISH analysis were prepared by cutting formalin-fixed and paraffin-embedded cells or tissue samples into 4  $\mu$ m sections and placing the sections onto a glass slide. Subsequent processing and ISH of samples was carried out in an automated device, such as the DISCOVERY<sup>™</sup> Automated ISH/IHC Stainer (Ventana Medical Systems, Inc., Tuscon, AZ) described in co-owned PCT Publication Nos. 99/44030 and WO 00/14507.

10 To remove paraffin from the samples, the slides were immersed in an aqueous solution, heated for approximately 20 minutes, and then rinsed. The automated deparaffinization procedure is more fully described in PCT Publication No. WO 00/14507. The

15 samples were then treated with protease and the slides were heated to 85°C (for hybridization to RNA target genes) or 90-95°C (for hybridization to DNA target genes) for 4 to 10 minutes.

Hybridization reactions were typically performed in a hybridization buffer consisting of 10% dextran sulfate (wt/vol), 25% formamide (vol/vol), 2X SSC, 5 mM Tris, 2.5 mM EDTA, 0.025% Brij-35, 0.25% Triton<sup>™</sup> X-100, and between 25 to 125 ng/mL of each individual probe molecule. ISH reactions were performed at between 37°C to 54°C. For ISH  
20 using the probe collections described in Example 1, hybridization reactions were optimally carried out for 1 hr at 47°C (except for the poly d(T) probe, wherein the hybridization reaction was optimally carried out at 37°C for 1 hr).

The hybridization of fluorescein-labeled probe molecules to a particular target gene in the sample was detected by using a sequential series of binding proteins, i.e., secondary  
25 antibody detection. However, it is equally possible to use detect detection when visualizing the bound probes. In secondary detection, first, an anti-fluorescein mouse monoclonal antibody directed against the fluorescein-labeled probe molecule was added to the sample. Next, a biotin-labeled polyclonal goat antibody directed against the mouse antibody was added to the sample. Finally, hybridization reactions were colormetrically detected using a 5-  
30 bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate. This technique, termed "secondary antibody detection," is routine for one of skill in the art.

Primary and secondary antibodies are available from numerous suppliers, including Ventana Medical Systems, Tucson, AZ, which are optimized for use on the Ventana autostaining systems (ES<sup>®</sup>, NexES<sup>®</sup>, DISCOVERY<sup>™</sup>, and BENCHMARK<sup>™</sup>).

Figures 2-21 illustrate the results obtained for *in situ* hybridization analysis of various cell lines or tissue samples using the probes disclosed and claimed herein having the structural motif illustrated in Figure 1 or probe collections consisting of such probes.

Figure 1 illustrates a generic probe structure of the two-domain probe design. This is the oligonucleotide design used for the probes in the gene specific cocktails described in the following examples. Each probe is composed of two domains: a 5' labeling domain and a 3' target gene target gene-specific domain. The labeling domain consists of this specific sequence (CTATTTT)<sub>n</sub>CT, wherein the cytosine nucleotide is a cytosine-hapten conjugate, the hapten being fluorescein in this embodiment. This illustration specifically shows nucleic acid sequences for the 301 (SEQ ID NO: 55) and 302 (SEQ ID NO: 56) probes, each of which possesses target gene gene-specific domains corresponding to human repetitive Alu sequences and labeling domains having a fluorescein hapten.

Figure 2 illustrates the results obtained for *in situ* hybridization (ISH) analysis of human skin tissue using a probe comprising the labeling domain (330 probe; SEQ ID NO: 58). The absence of a detectable signal indicates that the sequence formula, (CTATTTT)<sub>n</sub>CT, of the labeling domain common to the oligonucleotides used in these ISH examples is non-specific, and non-reactive in its ability to form Watson-Crick base pairing with human nucleic acid sequences because it does not hybridize.

Figure 3 illustrates the results obtained for ISH analysis of human skin tissue using a probe comprising the labeling domain and a poly d(T) target gene-specific domain (320 probe; SEQ ID NO: 57). The presence of a detectable signal localized to the cytoplasm indicates that this probe is capable of specifically hybridizing to polyadenylated region of messenger RNA.

Figures 4A-4B illustrate the results obtained for ISH analysis of human skin tissue using the 320 probe, wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to polyadenylated region common to messenger RNA.

Figures 5A-5B illustrate the results obtained for ISH analysis of human spleen tissue using the 320 probe, wherein the hybridization and stringency wash were performed at room temperature (A), or at 37°C (B). This result illustrates that the intensity of color is related to the stringency of hybridization conditions, with the more intense color indicating less  
 5 stringent conditons.

Figure 6 illustrates the results obtained for ISH analysis of the human Raji cell line using the 320 probe. This shows that this probe design also is functional with embedded cell lines as well as embedded tissue.

Figure 7 illustrates the results obtained for ISH analysis of the human Raji cell line  
 10 using a probe collection consisting of the 301 and 302 probes.

Figure 8 illustrates the results obtained for ISH analysis of the human HT cell line using a probe collection consisting of the 301 and 302 probes.

Figure 9 illustrates the results obtained for ISH analysis of a rat cell line using a probe collection consisting of the 301 and 302 probes. The absence of a detectable signal indicates  
 15 that this probe collection is specific for human nucleic acid sequences.

Figure 10 illustrates the results obtained for ISH analysis of an Epstein-Barr virus (EBV)-negative human HT cell line using a probe possessing a target gene-specific domain corresponding to EBV EBER nuclear RNA (SEQ ID NO: 51 through SEQ ID NO: 54).

Figure 11 illustrates the results obtained for ISH analysis of human spleen tissue using  
 20 a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA (SEQ ID NO: 51 through SEQ ID NO: 54).

Figure 12 illustrates the results obtained for ISH analysis of human tonsil tissue using a probe collection consisting of probes possessing target gene-specific domains  
 25 corresponding to EBV EBER 1 and 2 nuclear RNA (SEQ ID NO: 51 through SEQ ID NO: 54).

Figures 13A-13B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA (SEQ ID NO: 51 through SEQ ID  
 30 NO: 54), wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The

decrease in detectable signal in (B) indicates that this probe specifically hybridizes to human EBER 1 and EBER 2 nuclear RNA .

Figure 14 illustrates the results obtained for ISH analysis of kappa light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain mRNA (SEQ ID NO : 15).

Figure 15 illustrates the results obtained for ISH analysis of lymphoma tissues using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin kappa light chain mRNA (SEQ ID NOS: 2-4, SEQ ID NOS: 7-12, SEQ ID NOS: 14, 15). The lymphoma tissue in (A) over expresses the kappa light chain and the tissue in (B) over expresses the lambda light chain. The absence of a detectable signal in (B) indicates that the kappa light chain probe collection is specific to kappa light chain mRNA.

Figure 16 illustrates the results obtained for ISH analysis of lambda light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain variable region mRNA (SEQ ID NOS: 19 through 29).

Figure 17 illustrates the results obtained for ISH analysis of a lambda light chain-positive human RPMI 8226 cell line using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA (SEQ ID NOS: 19 through 29).

Figures 18A-18B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA (SEQ ID NOS: 19 through 29). The tissue in (A) over expresses the lambda light chain and the tissue in (B) over expresses the kappa light chain. The absence of a detectable signal in (B) indicates that the lambda light chain probe collection is specific to human lambda light chain mRNA.

Figure 19 illustrates the results obtained for ISH analysis of cytomegalovirus (CMV)-positive human lung tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA (SEQ ID NOS: 30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50). Arrow indicates CMV infected cell.

Figure 20 illustrates the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has not been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA (SEQ ID NOS: 30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50).

Figures 21A-21B illustrate the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA (SEQ ID NOS: 30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50) expression of the CMV immediate early RNA with cyclohexamide. The tissue in (A) is shown at a magnification of 40X and the tissue in (B) is shown at a magnification of 20X.

Table 1

| Probe ID | Sequence                                                                      | SEQ ID |
|----------|-------------------------------------------------------------------------------|--------|
| 401      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT<br><u>CCAGAGTAGCAGGAGCCCCAGGAGCTGAGC-3'</u> | 1      |
| 402      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GGATGGAGACTGGGTCAACTGGATGTCACA-3'</u>    | 2      |
| 403      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GCAAGCGATGGTGACTCTGTCTCCTACAGC-3'</u>    | 3      |
| 404      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>TCTGTCCCAGATCCACTGCCACTGAACCTT-3'</u>    | 4      |
| 405      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GCAGCCACAGTTCGCTTCATCTGCACCTTG-3'</u>    | 5      |
| 406      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>TTTCAACTGCTCATCAGATGGCGGGAAGAT-3'</u>    | 6      |
| 407      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>AAGTTATTCAGCAGGCACACAACAGAGGCA-3'</u>    | 7      |
| 408      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GGCGTTATCCACCTTCCACTGTACTTTGGC-3'</u>    | 8      |
| 409      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>TAGGTGCTGTCCTTGCTGTCCTGCTCTGTG-3'</u>    | 9      |
| 410      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GTAGTCTGCTTTGCTCAGCGTCAGGGTGCT-3'</u>    | 10     |
| 411      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GATGGGTGACTTCGCAGGCGTAGACTTTGT-3'</u>    | 11     |
| 412      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>CTCTCCCCTGTTGAAGCTCTTTGTGACGGG-3'</u>    | 12     |
| 413      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>TGGAAGTGAAGAGCAGGTGGGGGCACTTCT-3'</u>    | 13     |
| 414      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GAAAAAGGGTCAGAGGCCAAAGGATGGGAG-3'</u>    | 14     |
| 415      | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>AGATGAGCTGGAGGACCGCAATAGGGGTAG-3'</u>    | 15     |

|     |                                     |                                            |    |
|-----|-------------------------------------|--------------------------------------------|----|
| 416 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>GCATAATTAAAGCCAAGGAGGAGGAGGGG</u> - 3'  | 16 |
| 501 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>CCTGAGTGAGGAGGGTGAGGAGCAGCAGAG</u> - 3' | 17 |
| 502 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>AGACCCAGACACGGAGGCAGGCTGAGTCAG</u> - 3' | 18 |
| 503 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>TGTTGGTTCCAGTGCAGGAGATGGTGATCG</u> - 3' | 19 |
| 504 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>TAAATCATGATTTTGGGGGCTTGCCTGGG</u> - 3'  | 20 |
| 505 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>TGTTGCCAGACTTGGAGCCAGAGAAGCGAT</u> - 3' | 21 |
| 506 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>AATAATCAGCCTCGTCCTCAGCCTGGAGCC</u> - 3' | 22 |
| 507 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>GGTCCCTCCGCCGAAAACCACAGTGTAAC</u> - 3'  | 23 |
| 508 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>TTATGAGACACACCAGTGTGGCCTTGTGG</u> - 3'  | 24 |
| 509 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>CTGCTCAGGCGTCAGGCTCAGATAGCTGCT</u> - 3' | 25 |
| 511 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>ATGCGTGACCTGGCAGCTGTAGCTTCTGTG</u> - 3' | 26 |
| 512 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>ATTCTGTAGGGGCCACTGTCTTCTCCACGG</u> - 3' | 27 |
| 513 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>CCTCCCCTGGGATCCTGCAGCTCTAGTCTC</u> - 3' | 28 |
| 515 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>TGAGGGTTTATTGAGTGCAGGAGAGAGGGC</u> - 3' | 29 |
| 221 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>GGAGGTCAAAACAGCGTGGATGGCG</u> - 3'      | 30 |
| 222 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>GAGGCTGGATCGGTCCCGGTGTCTT</u> - 3'      | 31 |
| 223 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>AATCCGCGTTCCAATGCACCGTTCC</u> - 3'      | 32 |
| 224 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>TAAAACTGCGGGCACTGGGGACGG</u> - 3'       | 33 |
| 225 | 5' - CTATTTTCTATTTTCTATTTTCTATTTTCT | <u>ACCCGAGATTGCGGTGGAGATCCCA</u> - 3'      | 34 |

|       |                                        |                                        |    |
|-------|----------------------------------------|----------------------------------------|----|
| 226   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>GAGCAAGGAGCTGCCGAGCGACCAT-3'</u>    | 35 |
| 227   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>ACACTGGTGGTGGTGGGCATCGTGC-3'</u>    | 36 |
| 228   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>TTCAAATGCGTCAGCGGTGCAAGC-3'</u>     | 37 |
| 229   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>AGCTGCCTGCATCTTCTTCTGCCGC-3'</u>    | 38 |
| 230   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>CCCTCCACCGTTAACAGCACCGCAA-3'</u>    | 39 |
| 231   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>TTGGTCACGGGTGTCTCGGGCCTAA-3'</u>    | 40 |
| 232   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>TCGGCCAACCTCTGGAAACAGCGGGT-3'</u>   | 41 |
| 233   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>TCGGGGTTCTCGTTGCAATCCTCGG-3'</u>    | 42 |
| 234   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>ATCTCGATGCCCCGCTCACATGCAA-3'</u>    | 43 |
| 235   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>TGCCGCACCATGTCCACTCGAACCT-3'</u>    | 44 |
| 236   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>GTTAGCGGCGCCCTTGCTCACATCA-3'</u>    | 45 |
| 237   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>TGCAGATCTCCTCAATGCGGCGCTT-3'</u>    | 46 |
| 238   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>TCTCAGAGGATCGGCCCCCAGAATG-3'</u>    | 47 |
| 239   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>CCTCATCTGACTCCTCGGCGATGGC-3'</u>    | 48 |
| 240   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>CGGGTACAGGGGACTCTGGGGGTGA-3'</u>    | 49 |
| 241   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>GGGTGGGTGCTCTTGCCCTCCAGAGG-3'</u>   | 50 |
| 100A2 | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>GACCTCGGGTCGGTAGCACCGCACT-3'</u>    | 51 |
| 100C2 | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>GGAAGCCTCTCTTCTCCTCCCCCGG-3'</u>    | 52 |
| 100A1 | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>CCACAGACACCGTCCTCACCACCCG-3'</u>    | 53 |
| 100B1 | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>GGCTACAGCCACACACGTCTCCTCC-3'</u>    | 54 |
| 301   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>CGAGGCGGGCGGATCACCTGAGGTC-3'</u>    | 55 |
| 302   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>CGGGAGGCGGAGGTTGCAGTGAGCC-3'</u>    | 56 |
| 320   | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT      | <u>TTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'</u> | 57 |
|       | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT-3'   |                                        | 58 |
|       | 5'-CCAGAGTAGC AGGAGCCCCA GGAGCTGAGC-3' |                                        | 59 |
|       | 5'-GGATGGAGAC TGGGTCAACT GGATGTCACA-3' |                                        | 60 |
|       | 5'-GCAAGCGATG GTGACTCTGT CTCCTACAGC-3' |                                        | 61 |
|       | 5'-TCTGTCCCAG ATCCACTGCC ACTGAACCTT-3' |                                        | 62 |
|       | 5'-GCAGCCACAG TTCGCTTCAT CTGCACCTTG-3' |                                        | 63 |
|       | 5'-TTTCAACTGC TCATCAGATG GCGGGAAGAT-3' |                                        | 64 |
|       | 5'-AAGTTATTCA GCAGGCACAC AACAGAGGCA-3' |                                        | 65 |

|  |                                                 |    |
|--|-------------------------------------------------|----|
|  | <u>5' -GGCGTTATCC ACCTTCCACT GTACTTTGGC-3'</u>  | 66 |
|  | <u>5' -TAGGTGCTGT CCTTGCTGTC CTGCTCTGTG-3'</u>  | 67 |
|  | <u>5' -GTAGTCTGCT TTGCTCAGCG TCAGGGTGCT-3'</u>  | 68 |
|  | <u>5' -GATGGGTGAC TTCGCAGGCG TAGACTTTGT-3'</u>  | 69 |
|  | <u>5' -CTCTCCCCTG TTGAAGCTCT TTGTGACGGG-3'</u>  | 70 |
|  | <u>5' -TGGAAGTGA GAGCAGGTGG GGGCACTTCT-3'</u>   | 71 |
|  | <u>5' -GAAAAAGGGT CAGAGGCCAA AGGATGGGAG-3'</u>  | 72 |
|  | <u>5' -AGATGAGCTG GAGGACCGCA ATAGGGGTAG-3'</u>  | 73 |
|  | <u>5' -GCATAATTAA AGCCAAGGAG GAGGAGGGGG-3'</u>  | 74 |
|  | <u>5' -CCTGAGTGAG GAGGGTGAGG AGCAGCAGAG-3'</u>  | 75 |
|  | <u>5' -AGACCCAGAC ACGGAGGCAG GCTGAGTCAG-3'</u>  | 76 |
|  | <u>5' -TGTTGGTTCC AGTGCAGGAG ATGGTGATCG-3'</u>  | 77 |
|  | <u>5' -TAAATCATGA TTTTGGGGGC TTGCTTGGG-3'</u>   | 78 |
|  | <u>5' -TGTTGCCAGA CTTGGAGCCA GAGAAGCGAT-3'</u>  | 79 |
|  | <u>5' -AATAATCAGC CTCGTCCTCA GCCTGGAGCC-3'</u>  | 80 |
|  | <u>5' -GGTCCCTCCG CCGAAAACCA CAGTGTAAGT-3'</u>  | 81 |
|  | <u>5' -TTATGAGACA CACCAGTGTG GCCTTGTGTTG-3'</u> | 82 |
|  | <u>5' -CTGCTCAGGC GTCAGGCTCA GATAGCTGCT-3'</u>  | 83 |
|  | <u>5' -ATGCGTGACC TGGCAGCTGT AGCTTCTGTG-3'</u>  | 84 |
|  | <u>5' -ATTCTGTAGG GGCCACTGTC TTCTCCACGG-3'</u>  | 85 |
|  | <u>5' -CCTCCCCTGG GATCCTGCAG CTCTAGTCTC-3'</u>  | 86 |
|  | <u>5' -TGAGGGTTTA TTGAGTGCAG GGAGAAGGGC-3'</u>  | 87 |
|  | <u>5' -GGAGGTCAAA ACAGCGTGGA TGGCG-3'</u>       | 88 |
|  | <u>5' -GAGGCTGGAT CGGTCCCGGT GTCTT-3'</u>       | 89 |
|  | <u>5' -AATCCGCGTT CCAATGCACC GTTCC-3'</u>       | 90 |
|  | <u>5' -TAAAACTGC GGGCACTGGG GACGG-3'</u>        | 91 |
|  | <u>5' -ACCCGAGATT CGCGTGGAGA TCCCA-3'</u>       | 92 |
|  | <u>5' -GAGCAAGGAG CTGCCGAGCG ACCAT-3'</u>       | 93 |
|  | <u>5' -ACACTGGTGG TGGTGGGCAT CGTGC-3'</u>       | 94 |
|  | <u>5' -TTCCAAATGC GTCAGCGGTG CAAGC-3'</u>       | 95 |
|  | <u>5' -AGCTGCCTGC ATCTTCTTCT GCCGC-3'</u>       | 96 |

|         |                                                                       |     |
|---------|-----------------------------------------------------------------------|-----|
|         | 5'-CCCTCCACCG TTAACAGCAC CGCAA-3'                                     | 97  |
|         | 5'-TTGGTCACGG GTGTCTCGGG CCTAA-3'                                     | 98  |
|         | 5'-TCGGCCAACCT CTGGAAACAG CGGGT-3'                                    | 99  |
|         | 5'-TCGGGGTTCT CGTTGCAATC CTCGG-3'                                     | 100 |
|         | 5'-ATCTCGATGC CCCGCTCACA TGCAA-3'                                     | 101 |
|         | 5'-TGCCGCACCA TGTCCACTCG AACCT-3'                                     | 102 |
|         | 5'-GTTAGCGGCG CCCTTGCTCA CATCA-3'                                     | 103 |
|         | 5'-TGCAGATCTC CTCAATGCGG CGCTT-3'                                     | 104 |
|         | 5'-TCTCAGAGGA TCGGCCCCCA GAATG-3'                                     | 105 |
|         | 5'-CCTCATCTGA CTCCTCGGCG ATGGC-3'                                     | 106 |
|         | 5'-CGGGTACAGG GGA CTCTGGG GGTGA-3'                                    | 107 |
|         | 5'-GGGTGGGTGC TCTGCCTCC AGAGG-3'                                      | 108 |
|         | 5'-GACCTCGGGT CGGTAGCACC GCACT-3'                                     | 109 |
|         | 5'-GGAAGCCTCT CTCTCCTCC CCCGG-3'                                      | 110 |
|         | 5'-CCACAGACAC CGTCCTCACC ACCCG-3'                                     | 111 |
|         | 5'-GGCTACAGCC ACACACGTCT CCTCCC-3'                                    | 112 |
|         | 5'-CGAGGCGGGC GGATCACCTG AGGTC-3'                                     | 113 |
|         | 5'-CGGGAGGCGG AGGTTGCAGT GAGCC-3'                                     | 114 |
|         | 5'-TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT-3'                                | 115 |
| 301A    | 5'-CTATTTTCTATTTTCTTTT CGAGGCGGGCGGATCACCTGAGGTC-3'                   | 116 |
| 302C    | 5'-CTATTTTCTATTTTCTTTT CGGGAGGCGGAGGTTGCAGTGAGCC-3'                   | 117 |
| 302A4   | 5'-CTATTTTATACTTTATATTTTATATTTTATCT CGGGAGGCGGAGGTTGCAGTGAGCC-3'      | 118 |
| 302A3/2 | 5'-CTATTTTATATTTATATTTCT CGGGAGGCGGAGGTTGCAGTGAGCC ACTATTTTATACTT-3   | 119 |
| 1002A32 | 5'-CTATTTTATACTTTATATTTCT GACCTCGGGTCGGTAGCACCAC TACTATTTTATACTT-3'   | 120 |
| 301A2/2 | 5'-CTATTTTCTT CGAGGCGGGCGGATCACCTGAGGTC TTCFTTTTATCTT-3               | 121 |
| 301A3/2 | 5'-CTATTTTATACTTTATATTTCT CGAGGCGGGCGGATCACCTGAGGTC ACTATTTTATACTT-3' | 122 |

Table 2

5

| Probe<br>ID # | Sequence               | SEQ<br>ID # |
|---------------|------------------------|-------------|
|               | 5'-CTATTTTCTATTTTCTTTT | 123         |

|     |                                                |     |
|-----|------------------------------------------------|-----|
|     | 5'-CTATTTTATACTTTATATTTTCATATTTTATCT           | 124 |
| 330 | 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT              | 58  |
|     | 5'-CTATTTTATACTTTATATTTCT.....ACTATTTTATACTT-3 | 125 |
|     | 5'-CTATTTTCTT.....TTCTTTTATCTT-3               | 126 |

## SEQUENCE TABLE

<110> Ventana Medical Systems

<120> Oligonucleotide Sequence Formula for Labeling Oligonucleotide Probes and Proteins for In Situ Analysis

<130> 663-111div

<140> N/A (division of 2,422,440)

<141> 2001-09-06

<150> 60/233,177

<151> 2000-09-15

<160> 126

<170> PatentIn version 3.1

<210> 1

<211> 60

<212> DNA

<213> Artificial Sequence

<220>

<223> oligonucleotide probe

<400> 1

ctatttttcta ttttctatatt tctatttttct ccagagtagc aggagcccca ggagctgagc 60

<210> 2

<211> 60

<212> DNA

<213> Artificial Sequence

<220>

<223> oligonucleotide probe

<400> 2

ctatttttcta ttttctatatt tctatttttct ggatggagac tgggtcaact ggatgtcaca 60

<210> 3

<211> 60

<212> DNA

<213> Artificial Sequence

<220>

<223> oligonucleotide probe

<400> 3

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<210> 4  
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 <213> Artificial Sequence  
  
 <220>  
 <223> oligonucleotide probe  
  
 <400> 4  
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<210> 5  
 <211> 60  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> oligonucleotide probe  
  
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<210> 6  
 <211> 60  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> oligonucleotide probe  
  
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<210> 7  
 <211> 60  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> oligonucleotide probe  
  
 <400> 7  
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<210> 8  
 <211> 60  
 <212> DNA  
 <213> Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide probe

&lt;400&gt; 8

ctatatttcta ttttctatatt tctatatttct ggcgttatcc accttcact gtactttggc 60

&lt;210&gt; 9

&lt;211&gt; 60

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide probe

&lt;400&gt; 9

ctatatttcta ttttctatatt tctatatttct taggtgctgt ccttgctgtc ctgctctgtg 60

&lt;210&gt; 10

&lt;211&gt; 60

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&lt;220&gt;

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide probe

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&lt;211&gt; 60

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide probe

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&lt;220&gt;

&lt;223&gt; oligonucleotide probe

&lt;400&gt; 45

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&lt;210&gt; 46

&lt;211&gt; 55

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide probe

&lt;400&gt; 46

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&lt;211&gt; 55

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide probe

&lt;400&gt; 47

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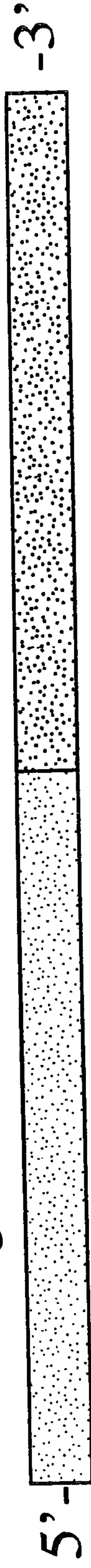
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**THE EMBODIMENTS OF THE INVENTION FOR WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:**

1. A synthetic oligonucleotide probe comprising a label-domain consisting of a non-gene-specific sequence of a polymer of the repetitive sequence (AAAATAG)<sub>n</sub>, and a target domain comprising a gene-specific target sequence, wherein the label-domain is detectably labeled, wherein n is at least 4.
2. The oligonucleotide probe according to claim 1, wherein the label is a fluorophore or a hapten.
3. The oligonucleotide probe according to claim 2, wherein the fluorophore is present at a density of greater than 7 mole percent of the label-domain.
4. The oligonucleotide probe according to claim 1, wherein the label-domain is located at the 5' end of the oligonucleotide probe, and the gene-specific target sequence is 3' to the label-domain.
5. The oligonucleotide probe according to claim 1, wherein the label-domain is located at the 3' end of the oligonucleotide probe, and the gene-specific target sequence is 5' to the label-domain.

# Oligonucleotide Probe Design

Common Signal Detection Region      Unique Target Detection Region



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Alu301 5'-CTATTTCTATTTCTATTTCTATTTCT-CGAGGCGGCGGATCACCTGAGGTC-3'

Alu302 5'-CTATTTCTATTTCTATTTCTATTTCT-CGGGAGGCGGAGGTTGCAGTGAGCC-3'

FIG. 1

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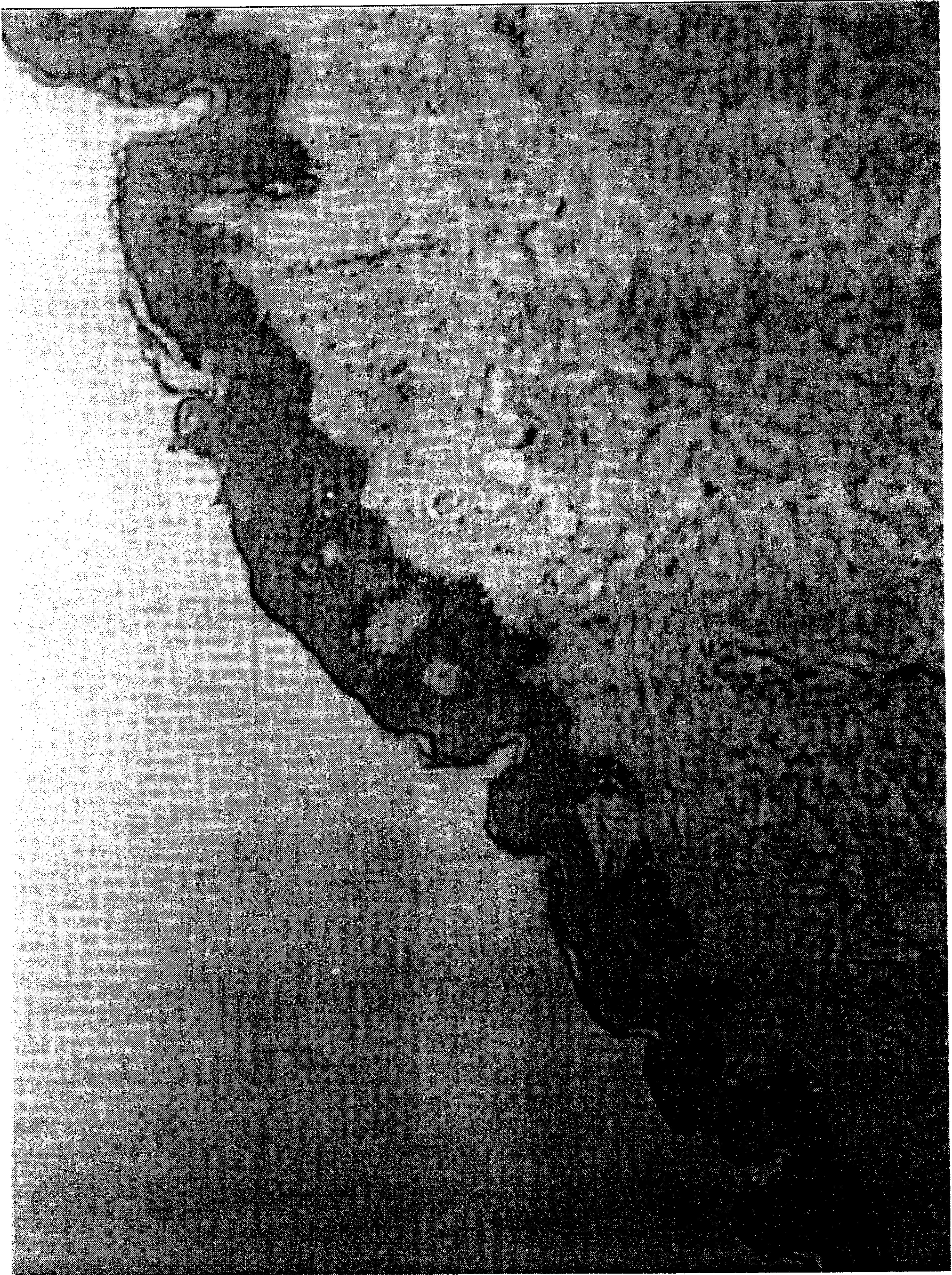


FIG. 2

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FIG. 3

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PLUS RNase



FIG. 4B

MINUS RNase



FIG. 4A

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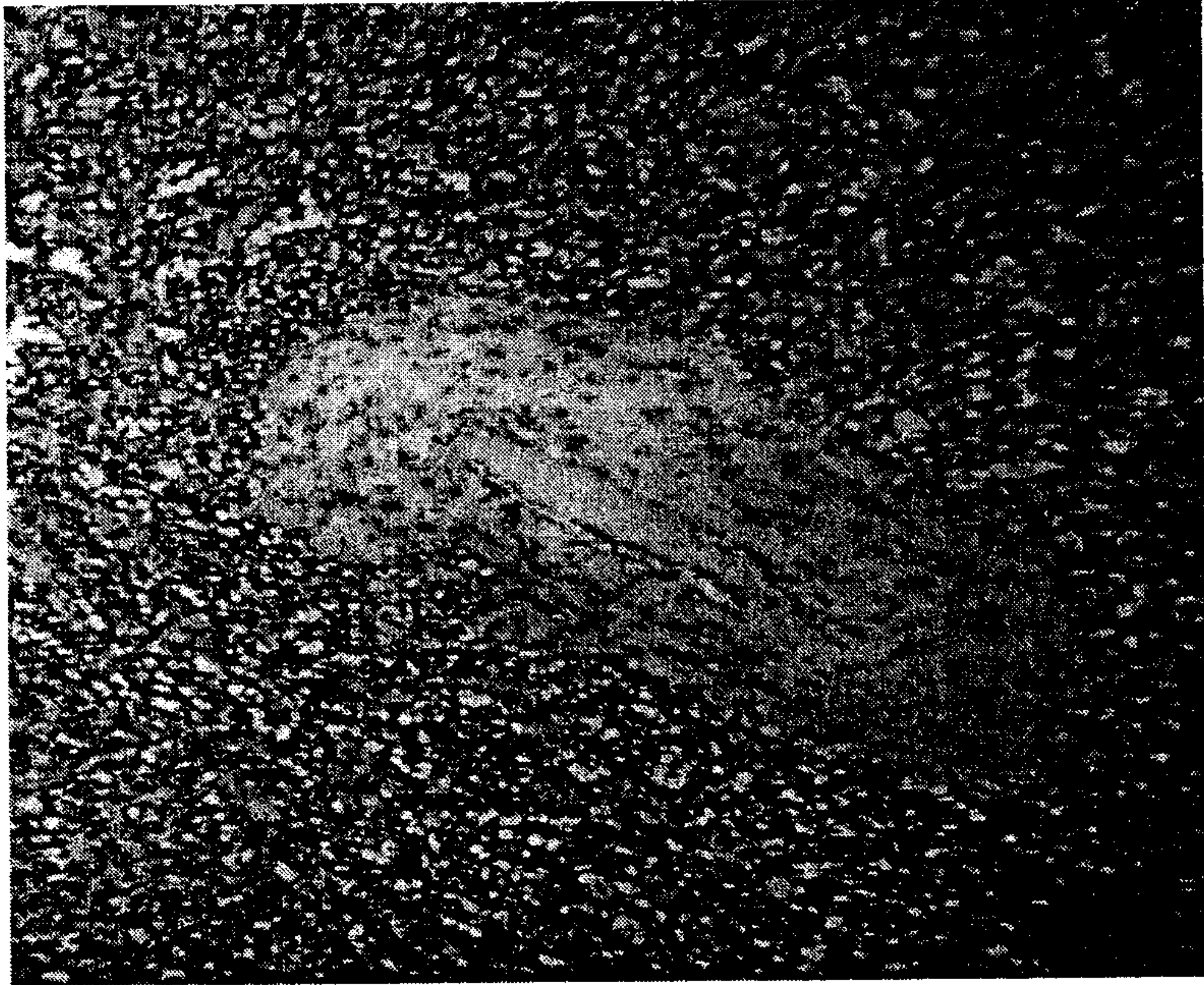


FIG. 5B

RT/RT



FIG. 5A

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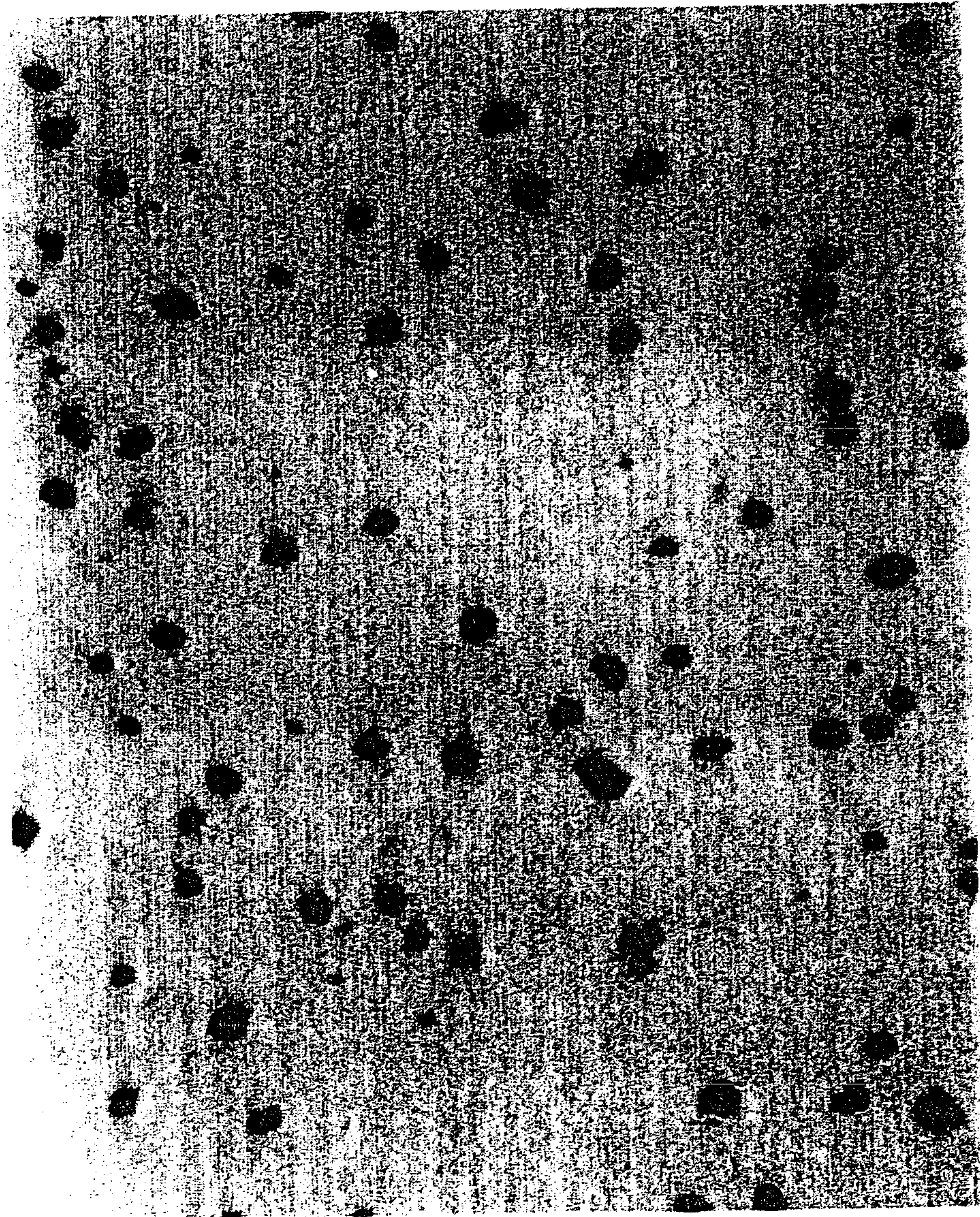


FIG. 6

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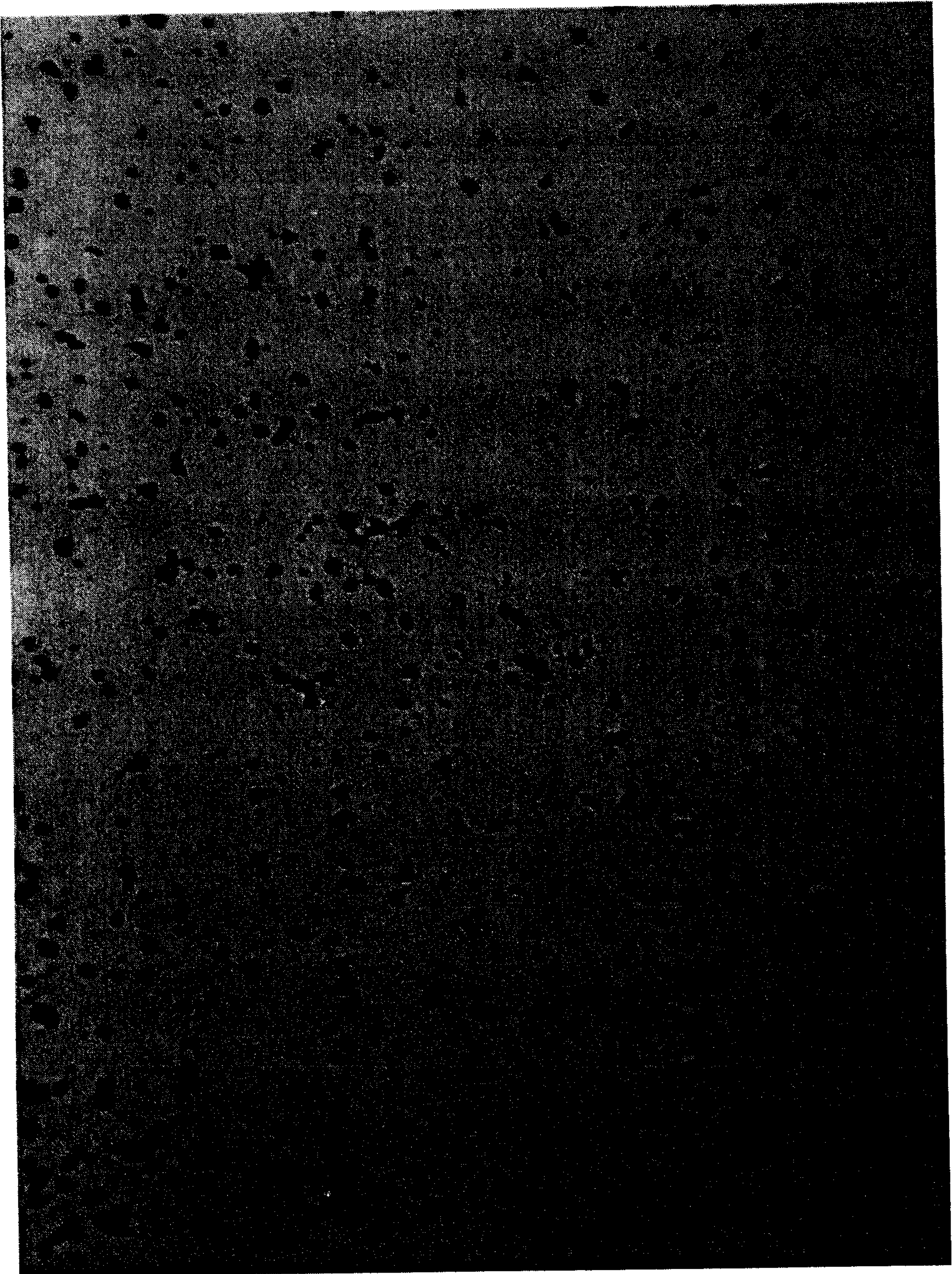


FIG. 7

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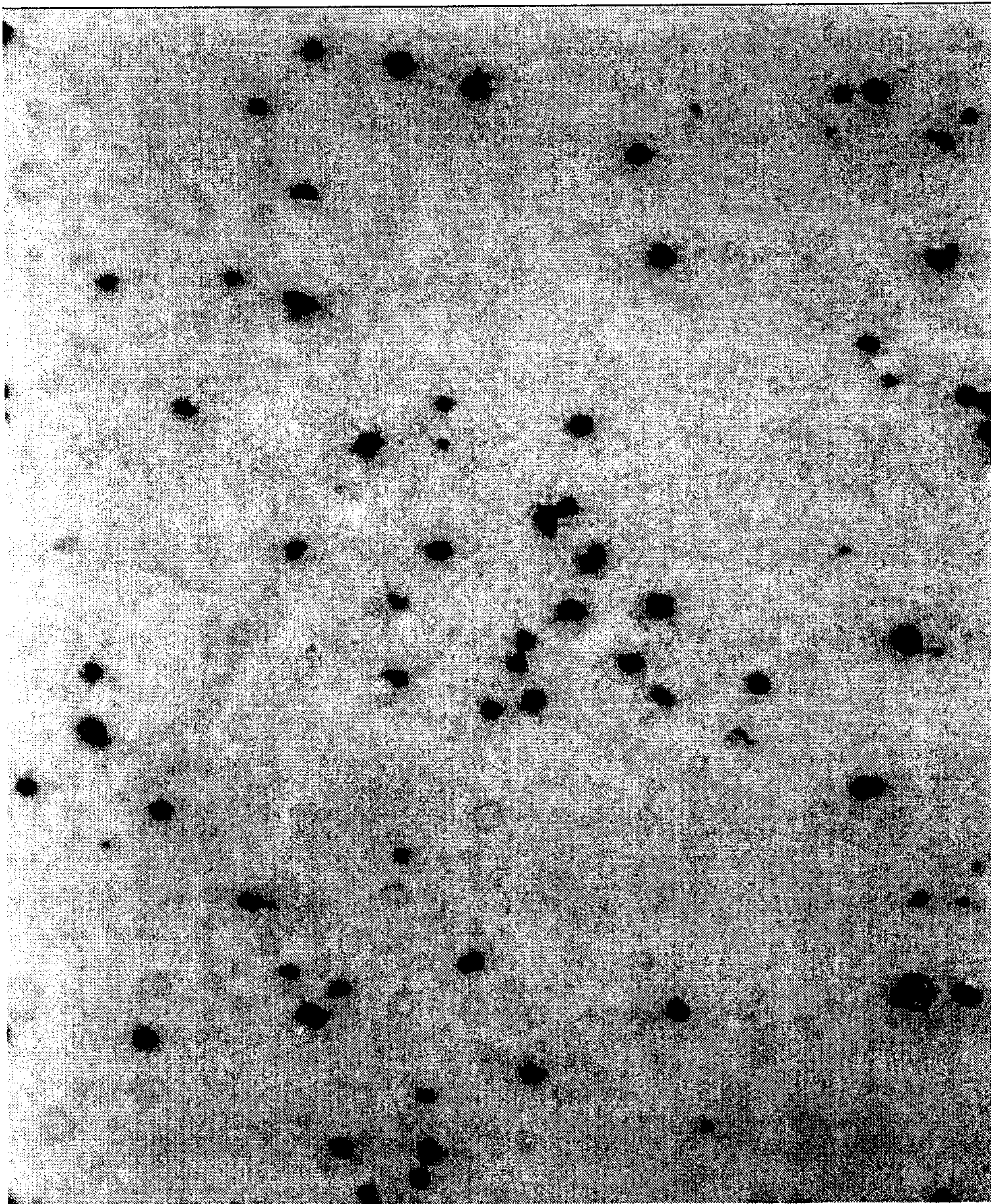


FIG. 8

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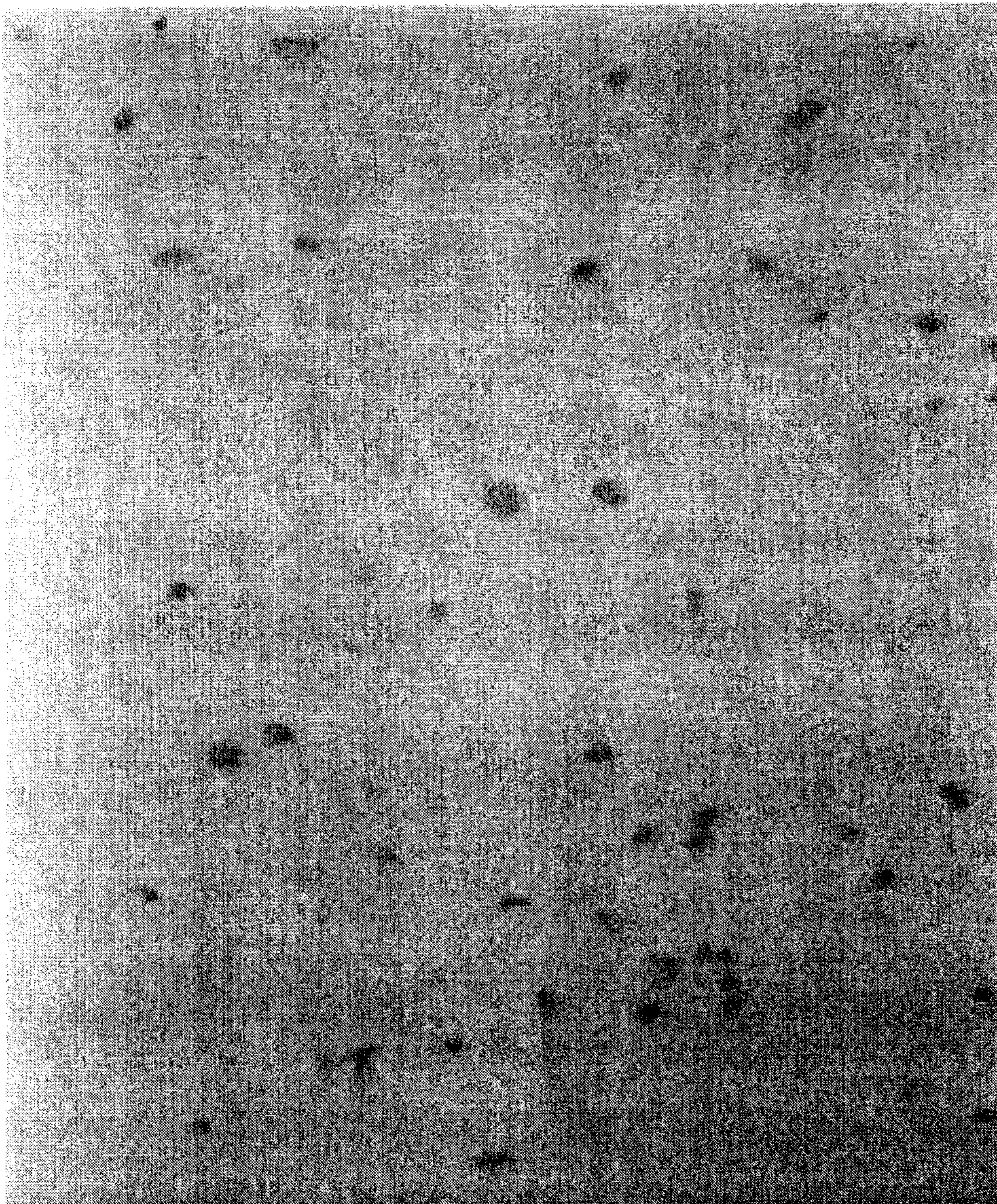


FIG. 9

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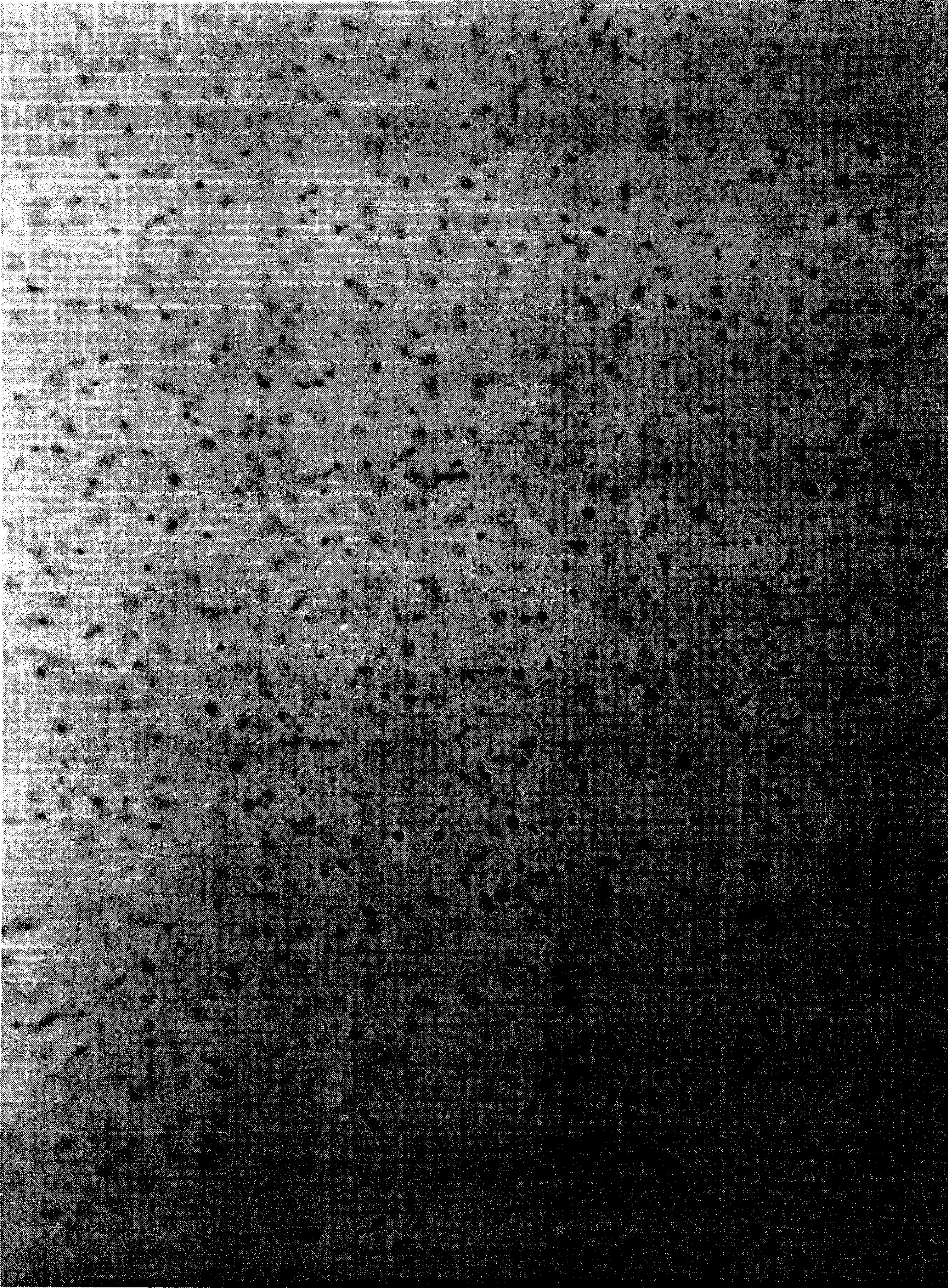


FIG. 10

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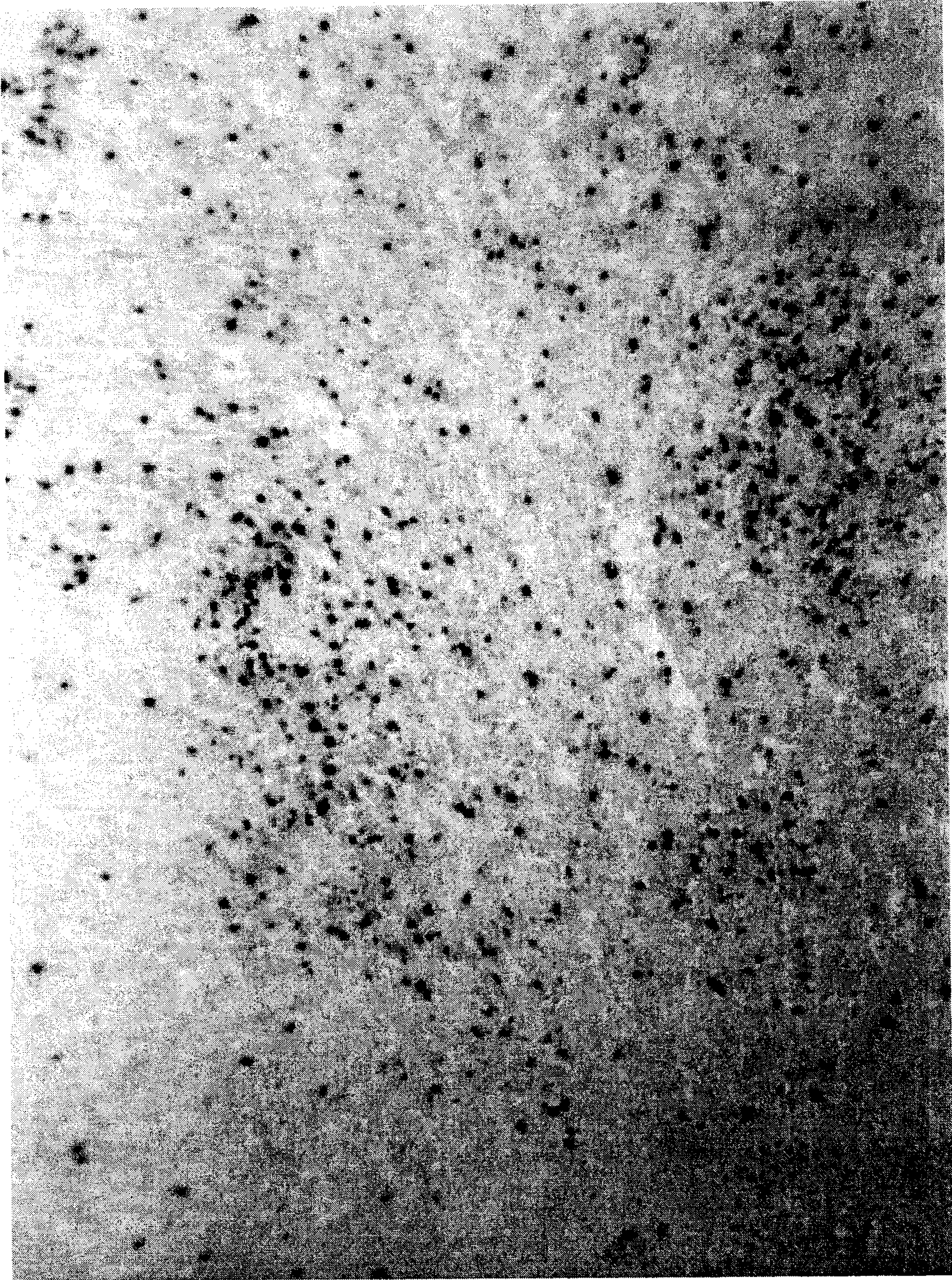


FIG. 11

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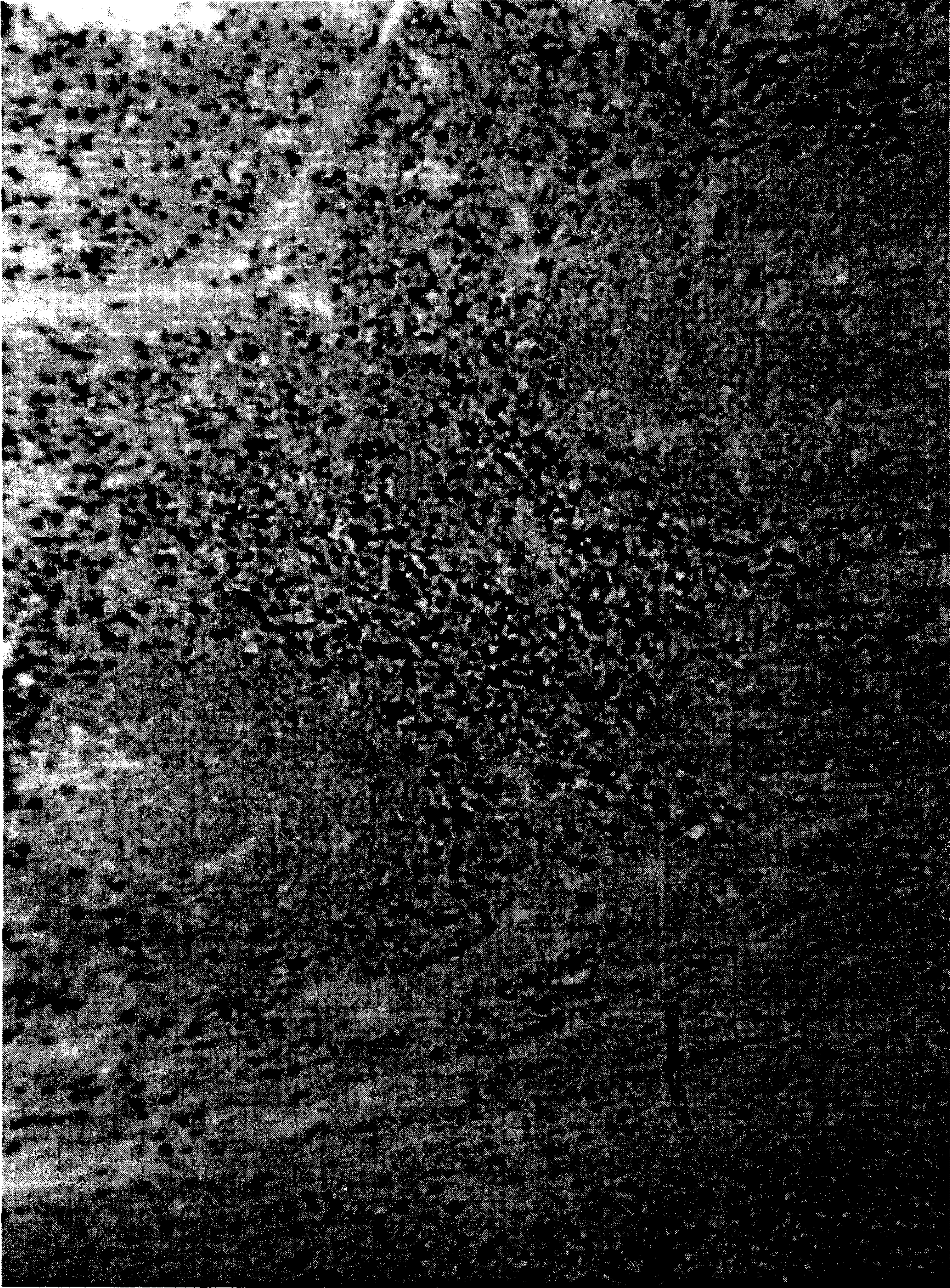


FIG. 12

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PLUS RNase

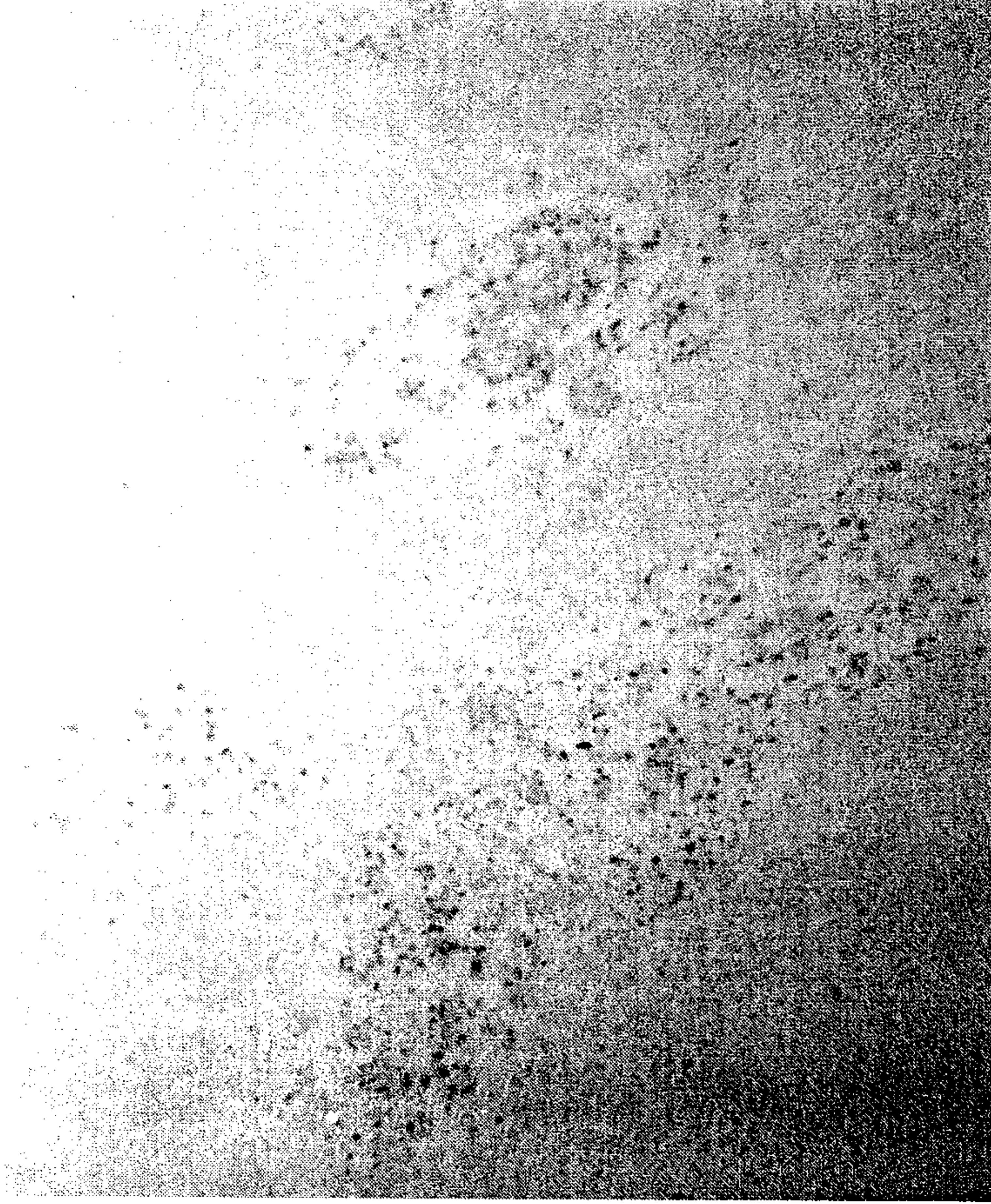


FIG. 13B

MINUS RNase

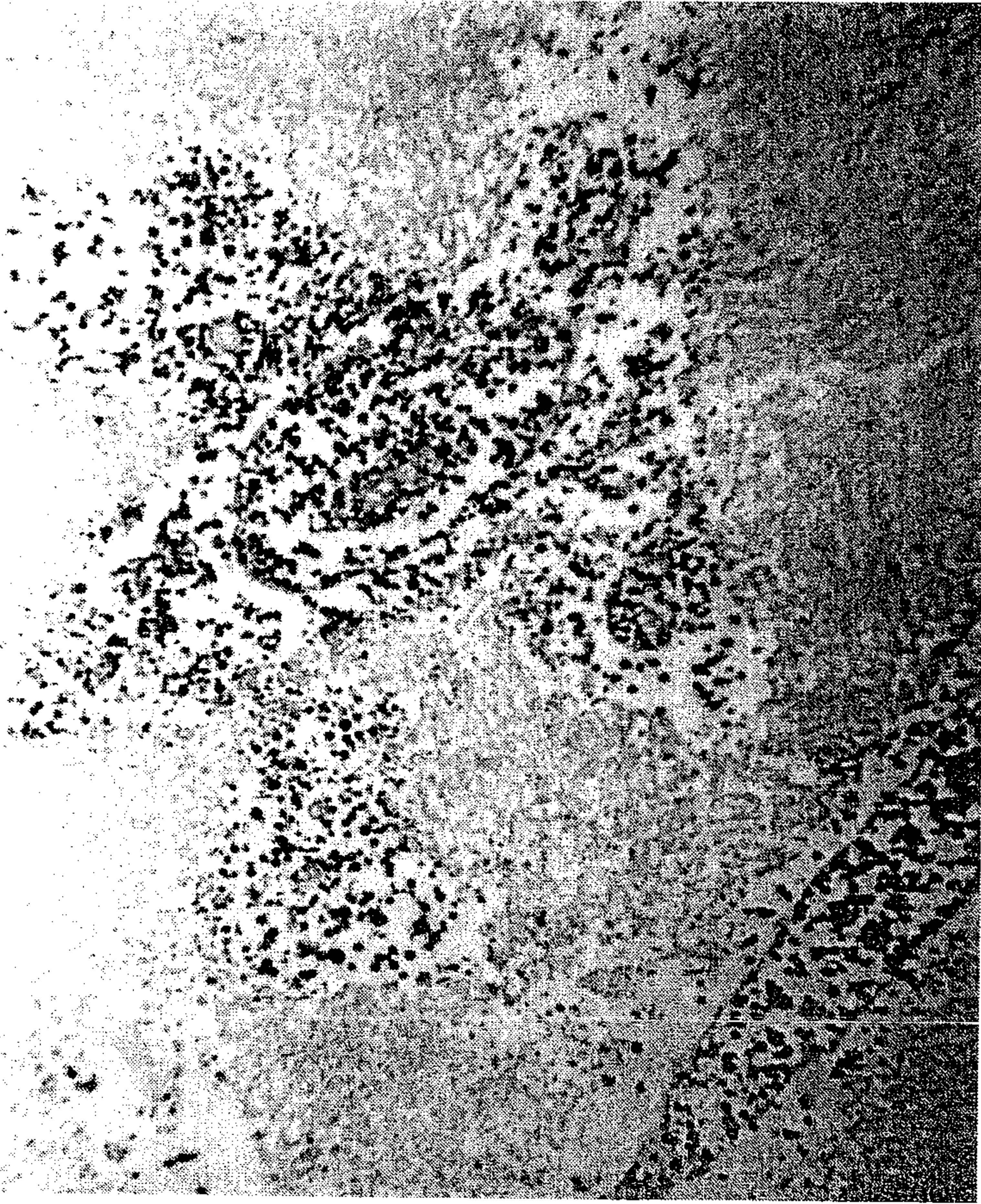


FIG. 13A

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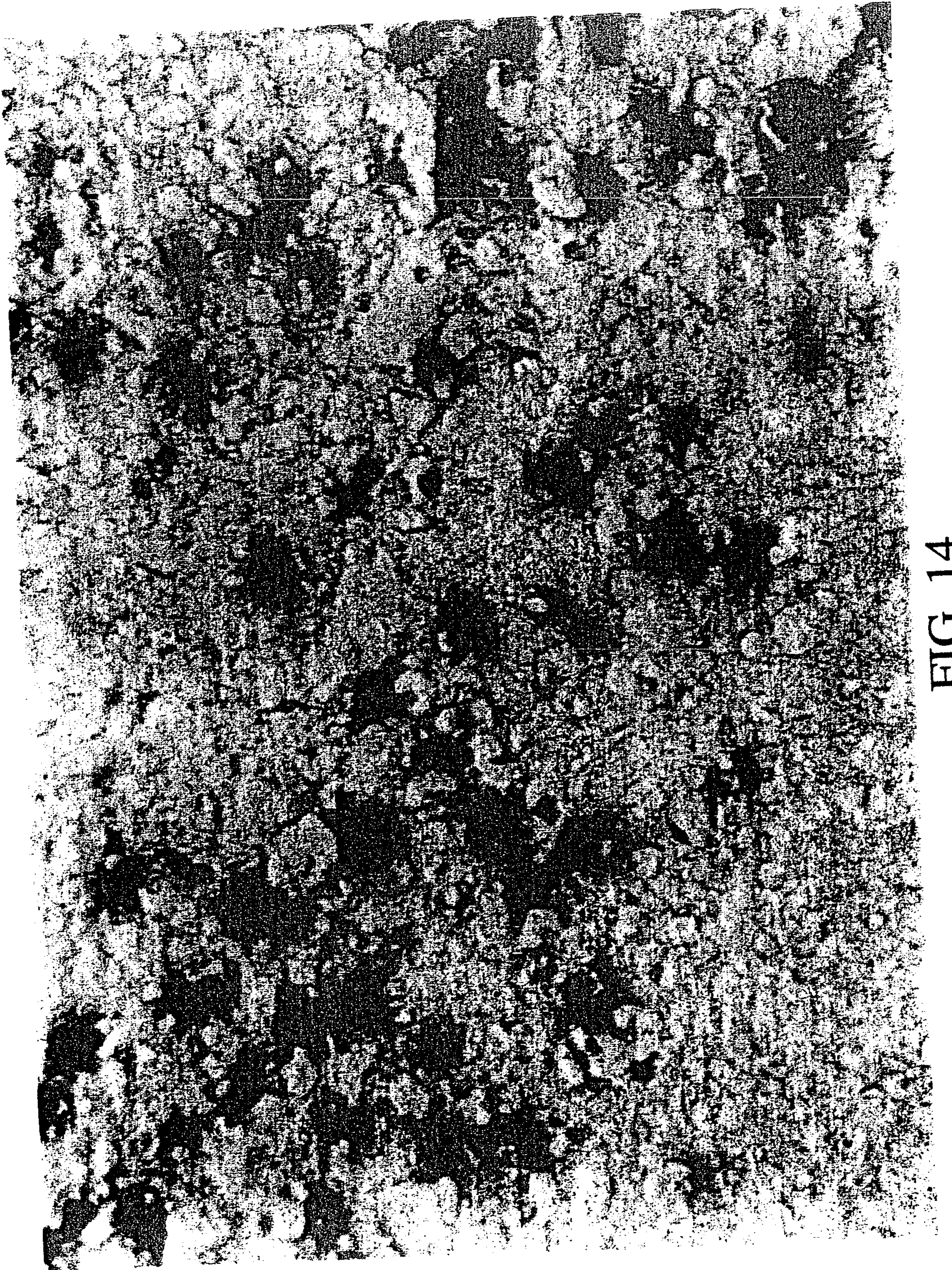


FIG. 14

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FIG. 15B

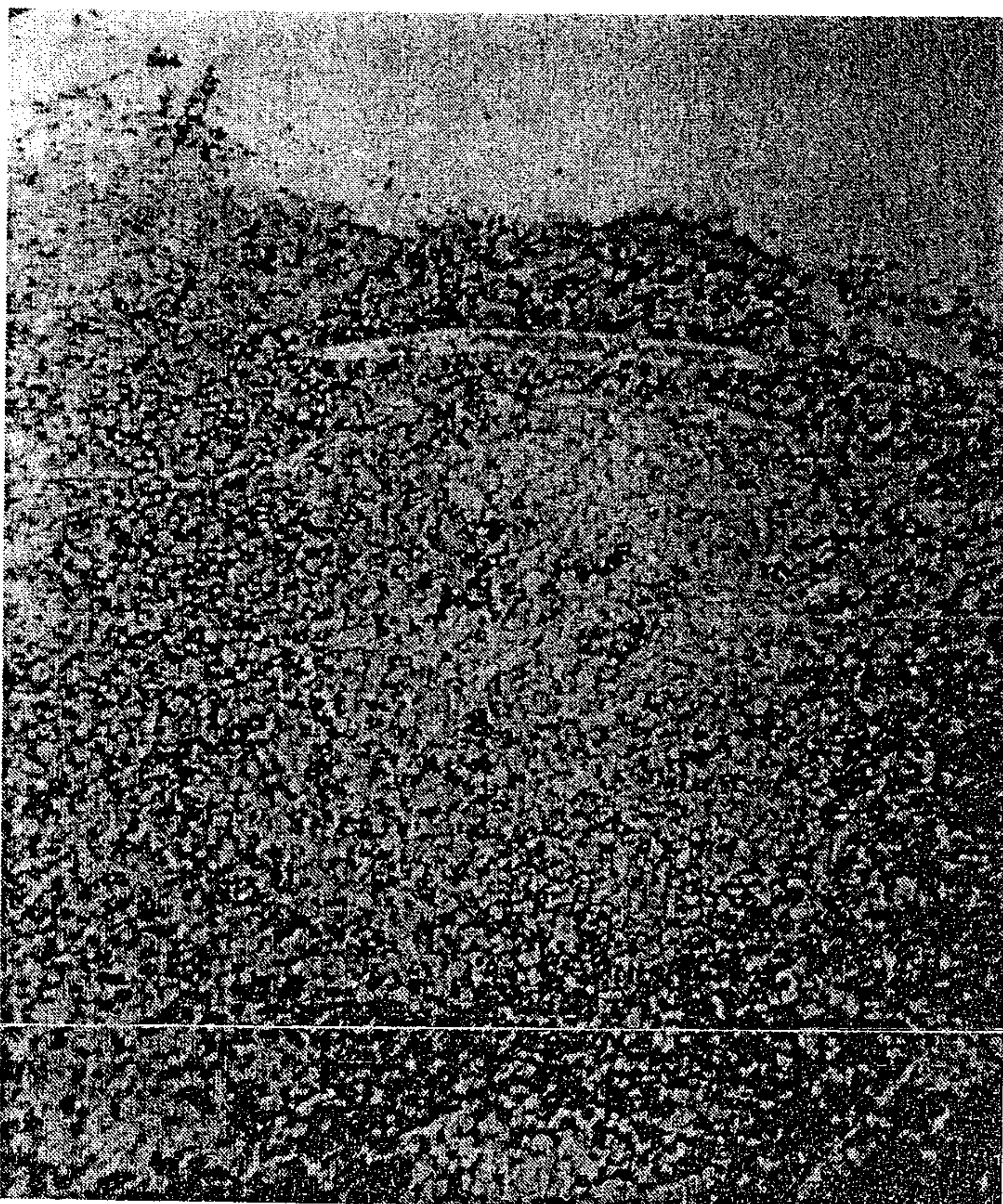


FIG. 15A

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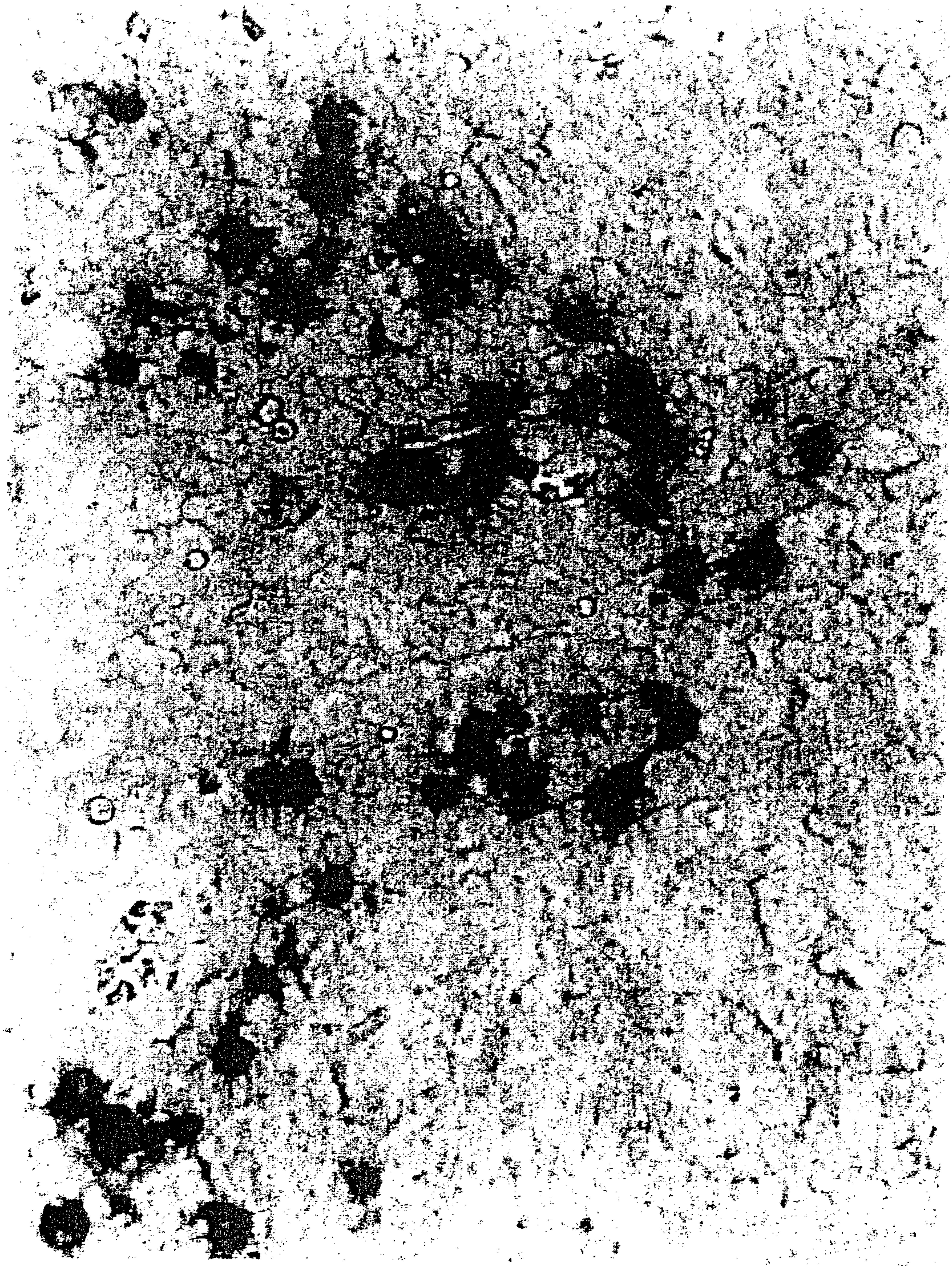


FIG. 16

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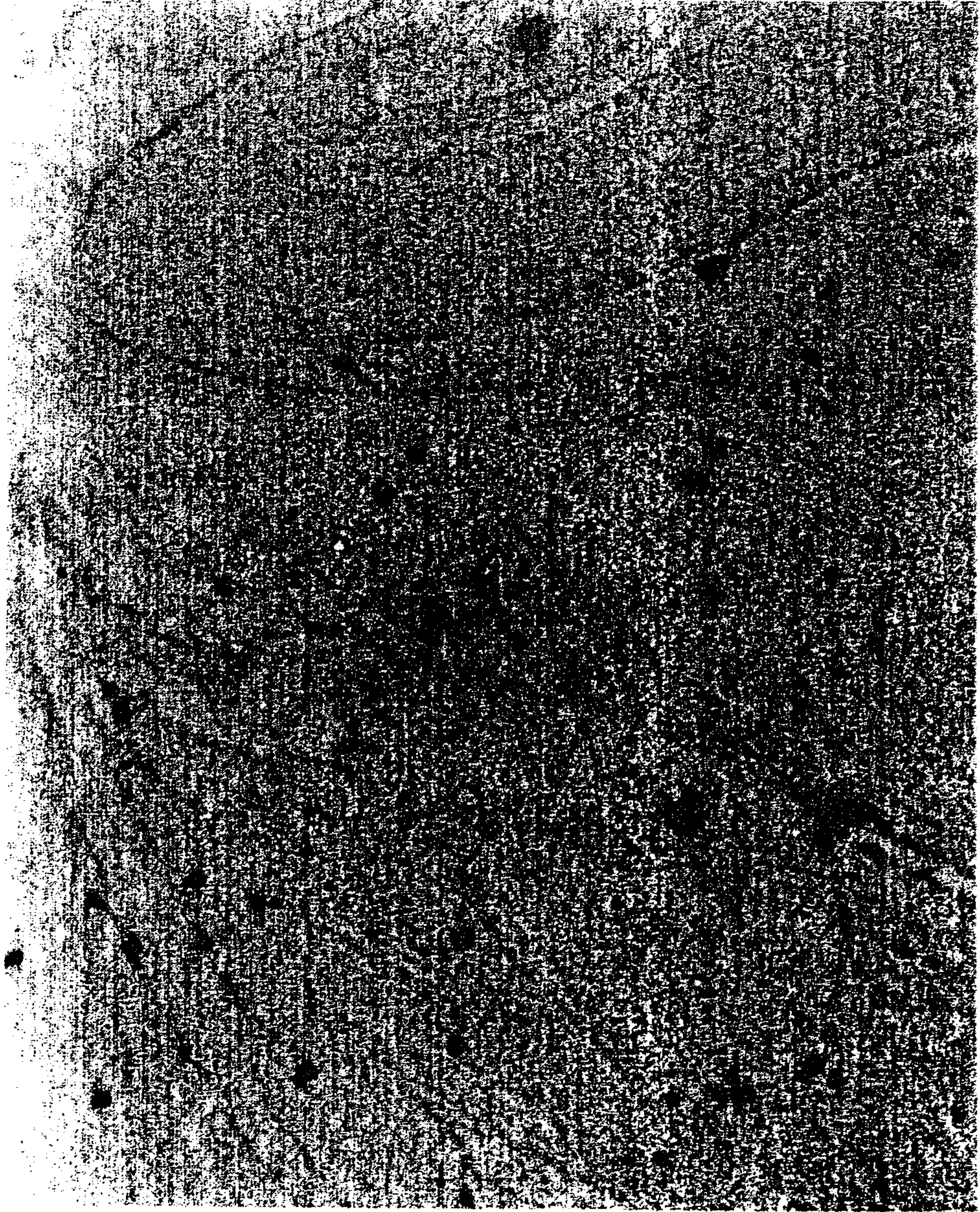


FIG. 17

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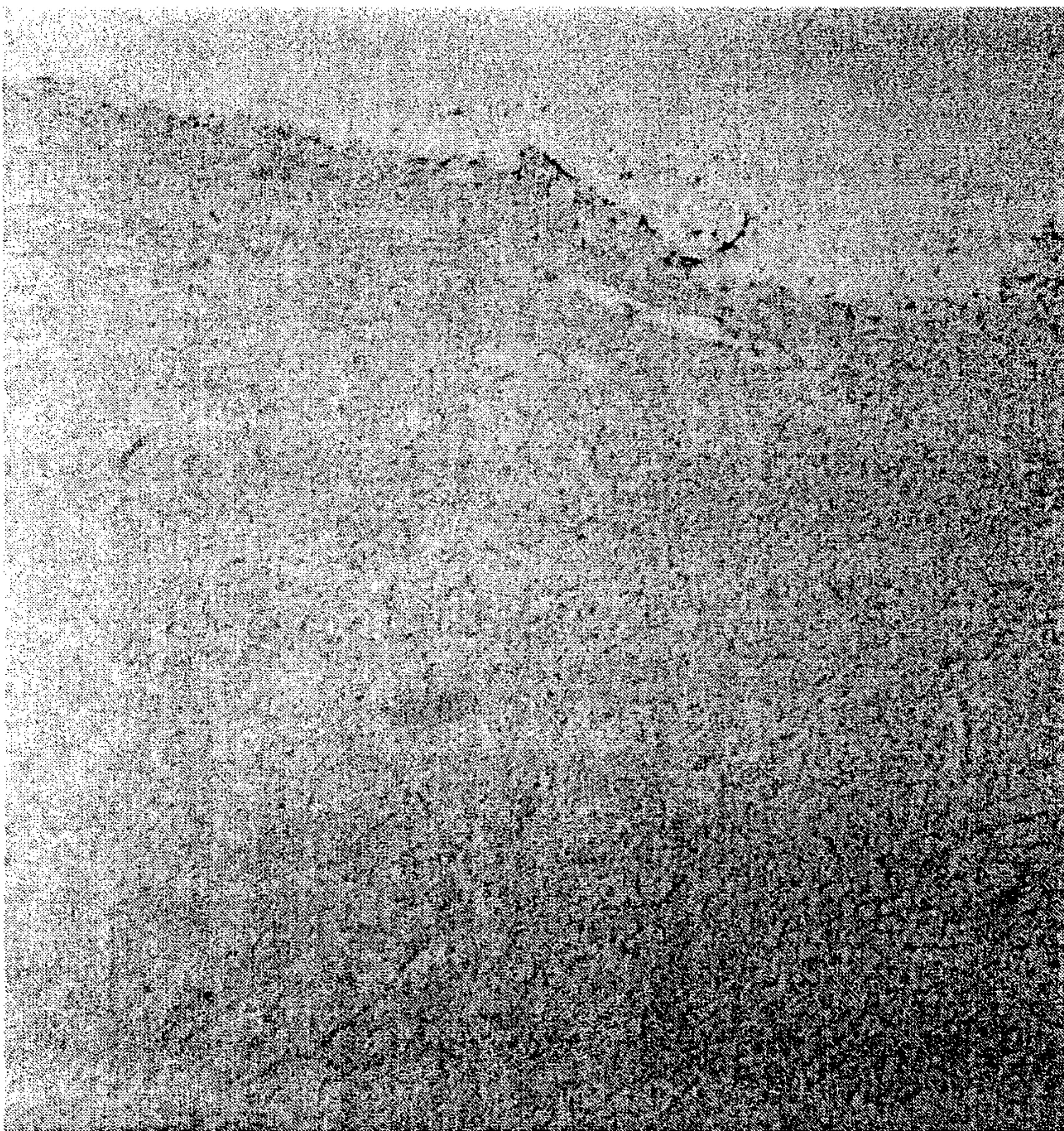


FIG. 18B



FIG. 18A

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FIG. 19

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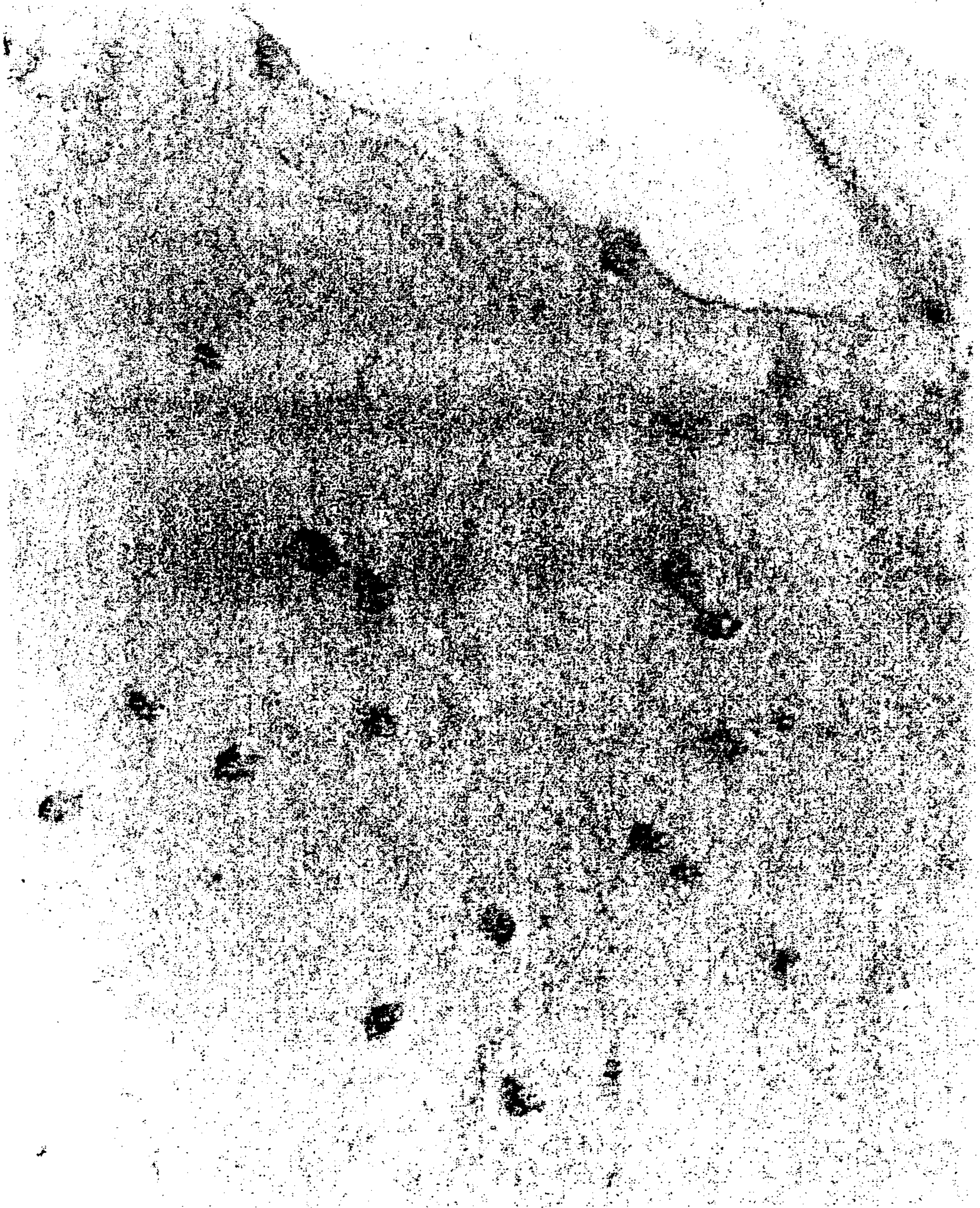


FIG. 20

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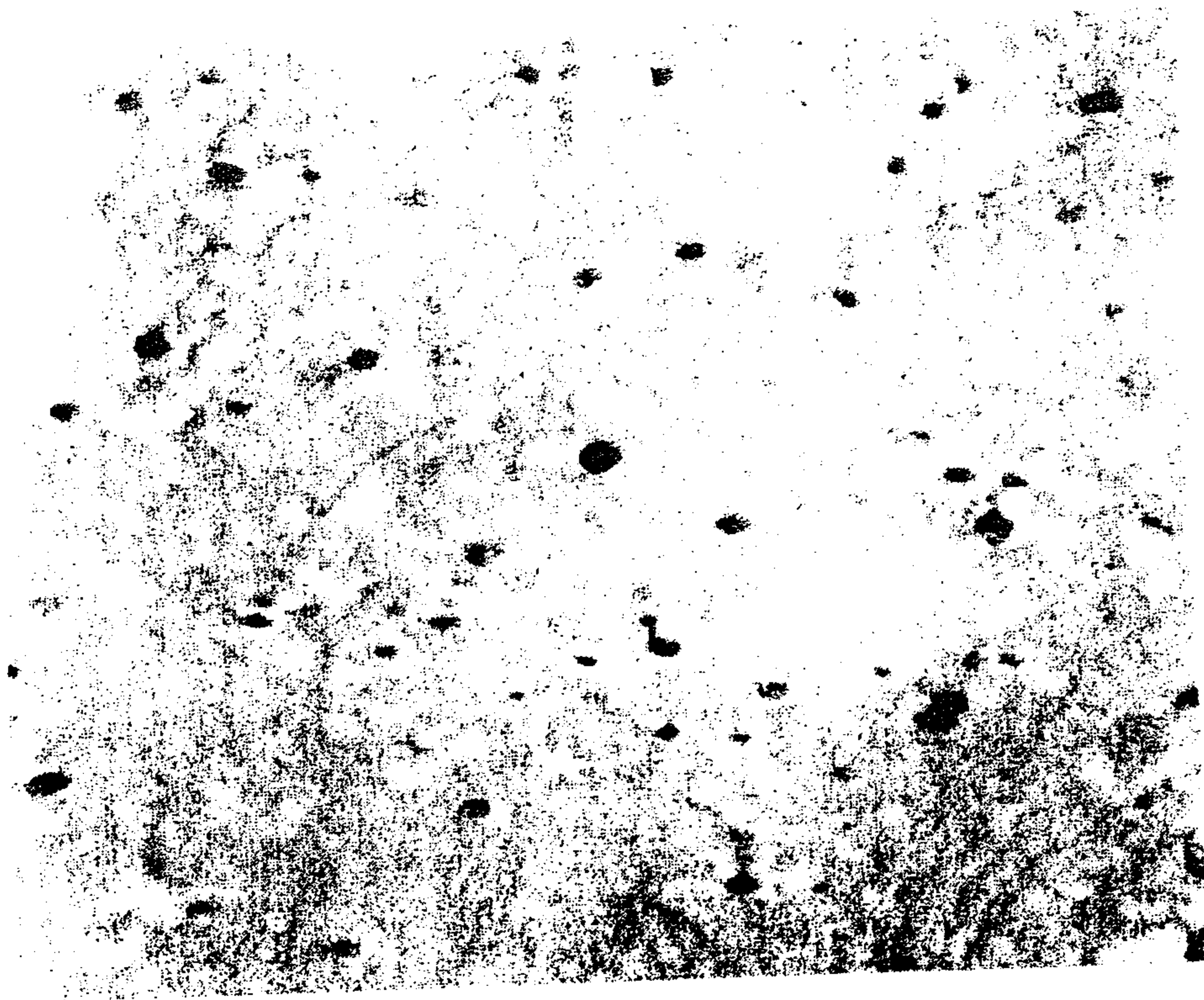


FIG. 21B

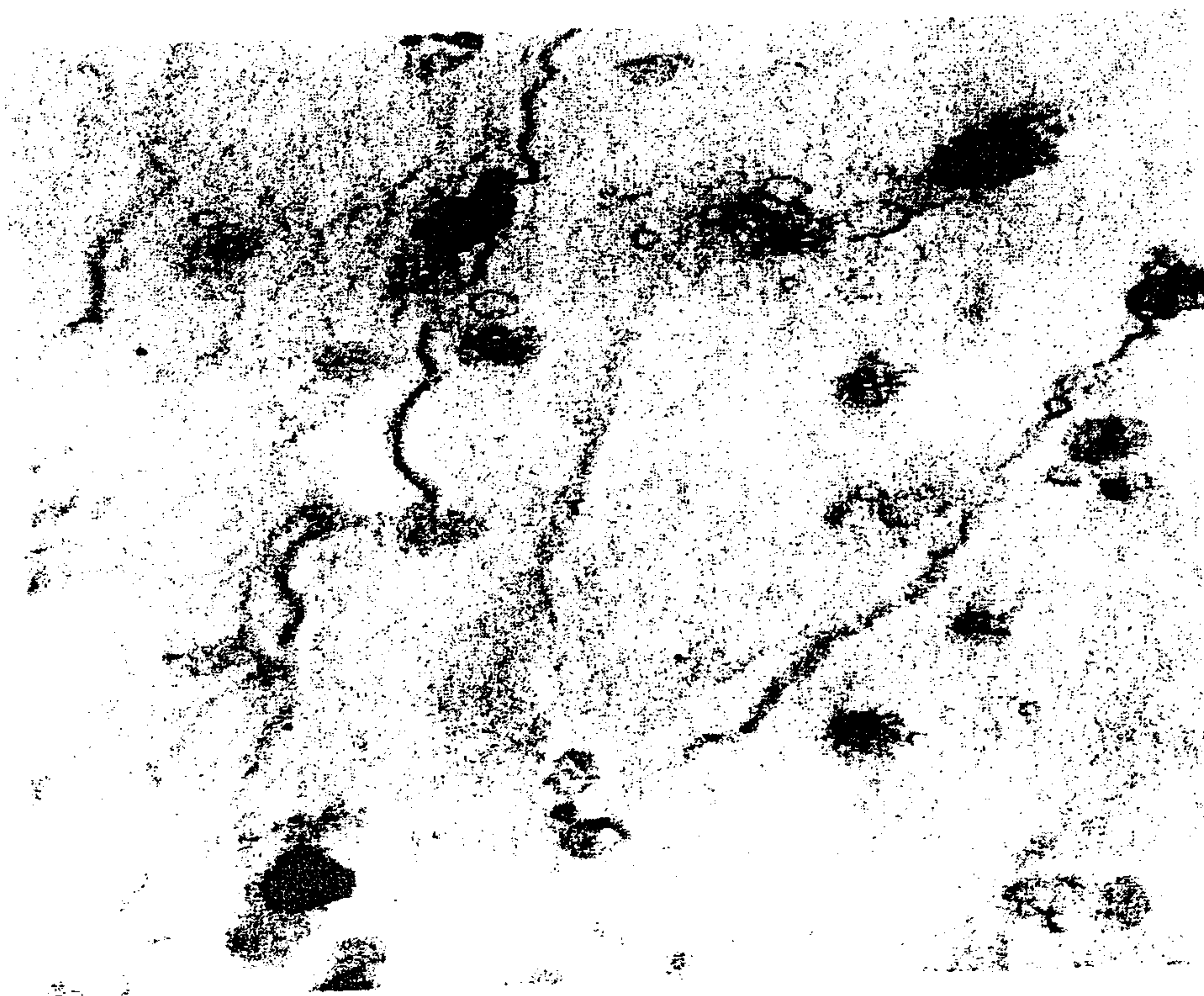


FIG. 21A