Sampling error and development of sampling strategies for biological tissues

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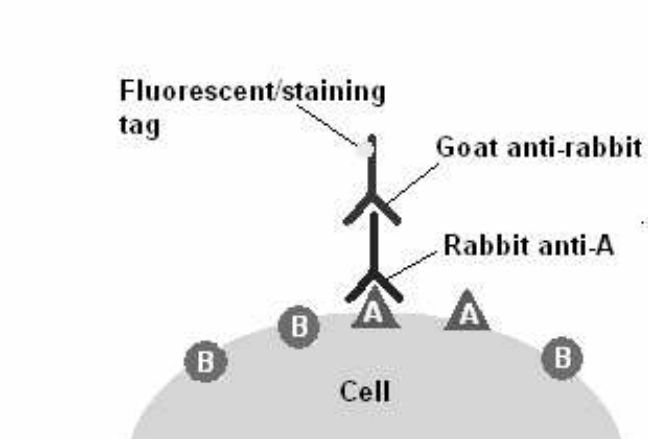
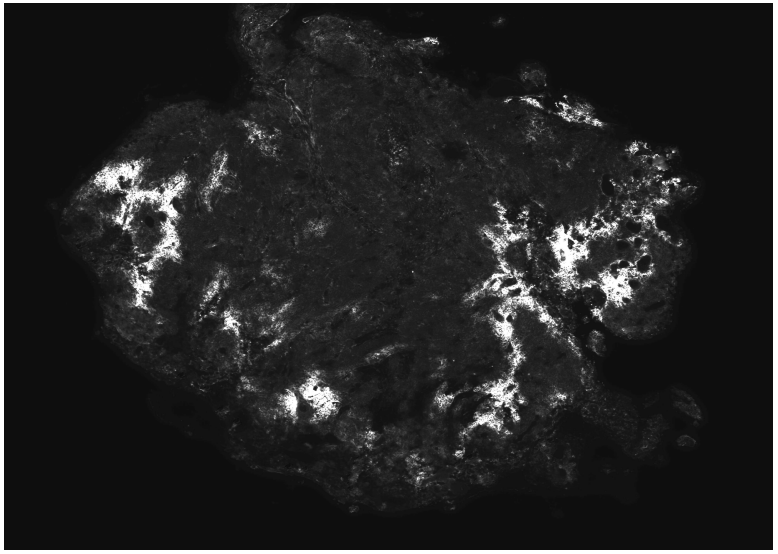
Can we use immunostains as a precise tool?

- **Practice:**
- favourite molecular tool of surgical pathologists
- recent availability of slide scanners and powerful computers
- treatment decisions based on protein expression

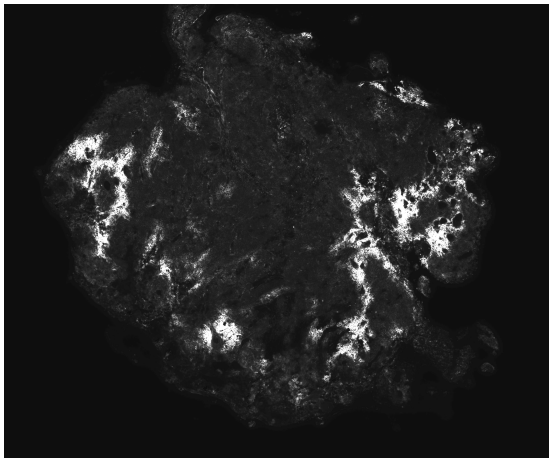
- **Research**
- needs much less tissue comparing to Western blot and RT-PCR -valuable in small specimens like core biopsy
- allows analysis limited to specific areas/compartments
- allows co-localization of several markers – pathway analysis



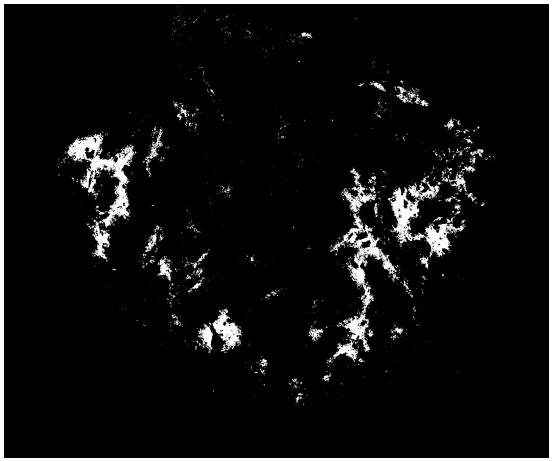


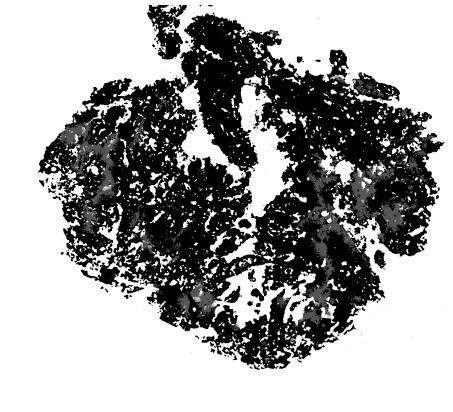
Immunofluorescence image analysis

Image of a fluorescent stain



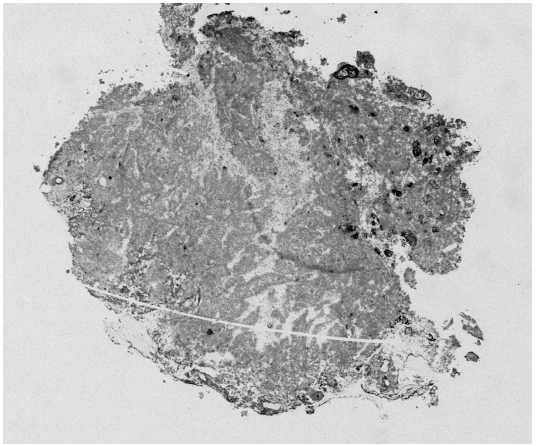
threshold



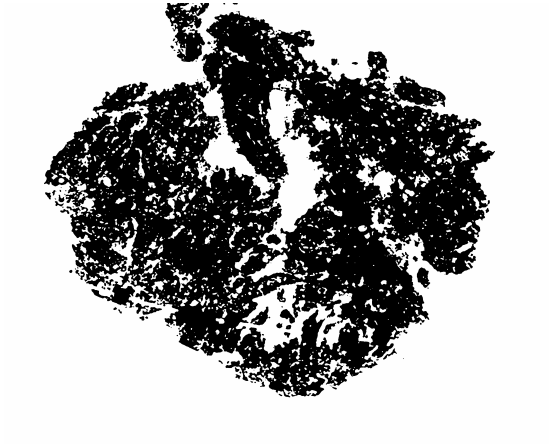


% positive pixel calculation

Histochemical stain to visualize tumor and stroma

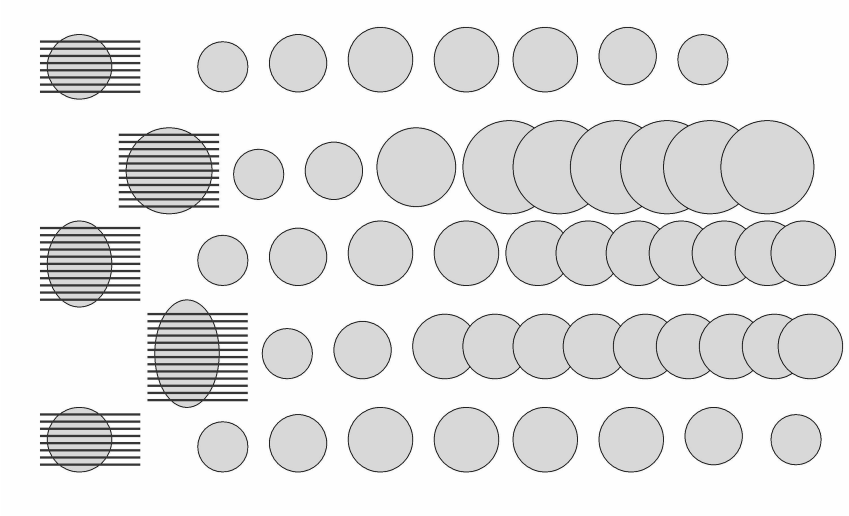


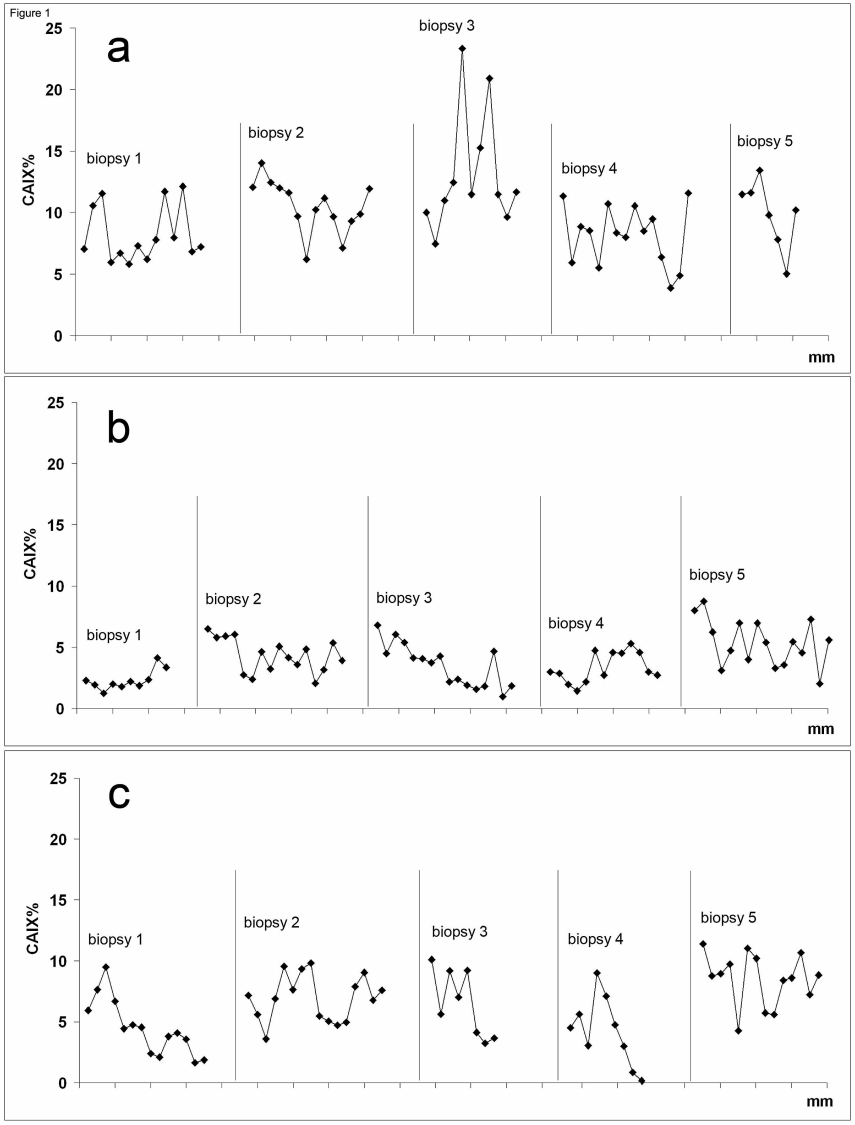
mask



Measurement variation within biopsy, CAIX - hypoxia marker analysis

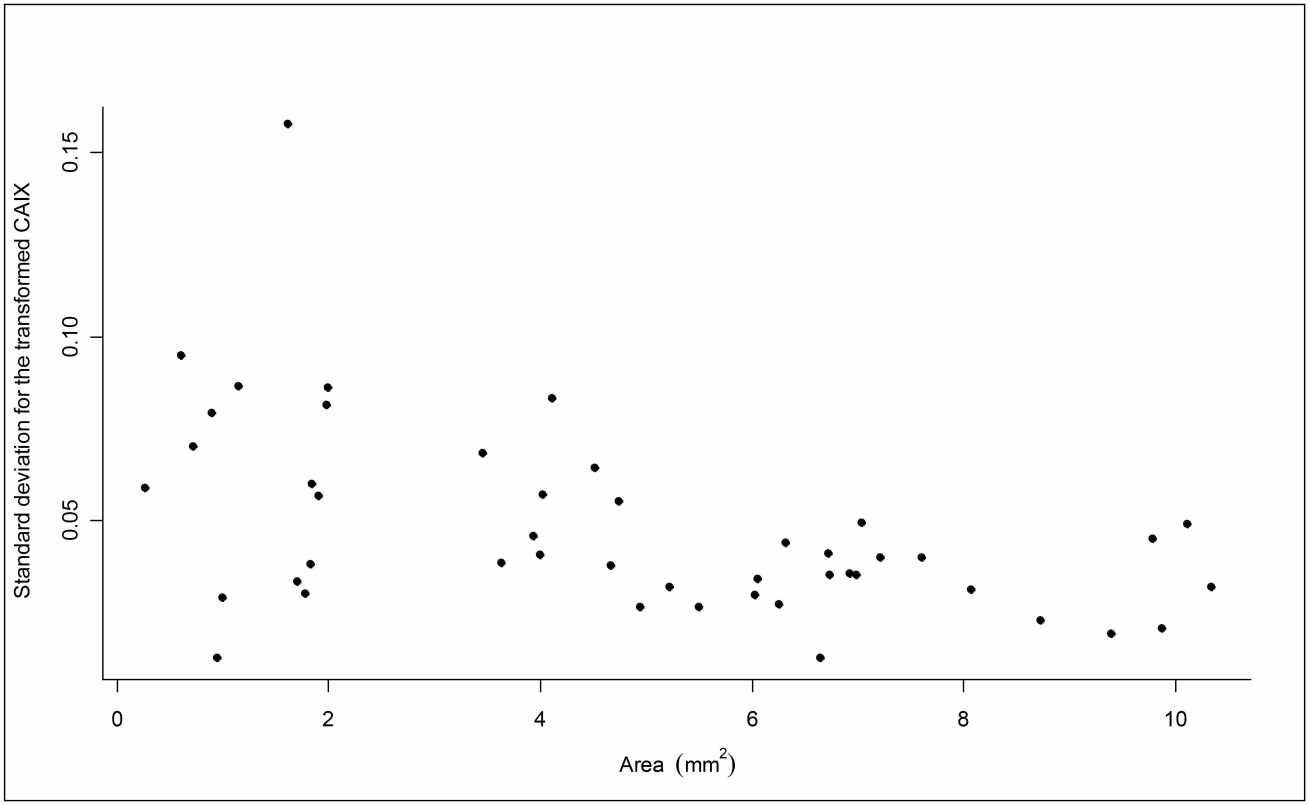
- One panel per tumor (a, b & c)
- 5 biopsies for each tumor
- Each biopsy fully sectioned
- Each datapoint – CAIX% value within a section
- Sections 250 µm apart, shown in a sequence as they were cut



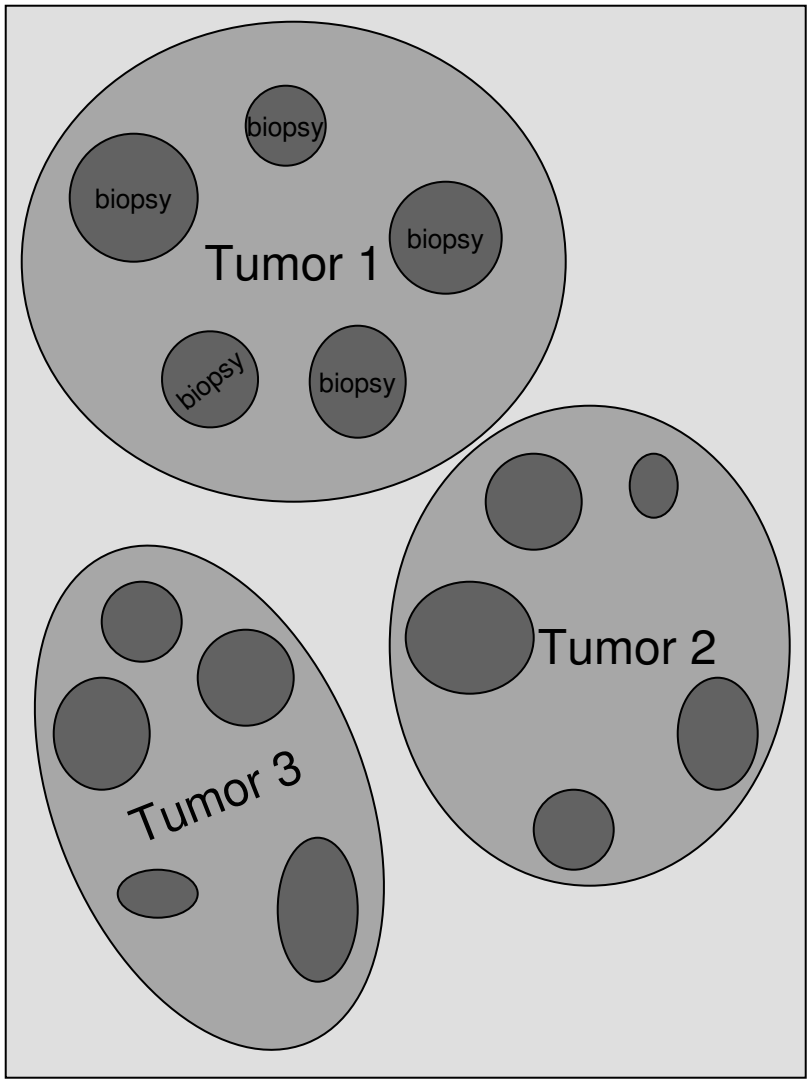


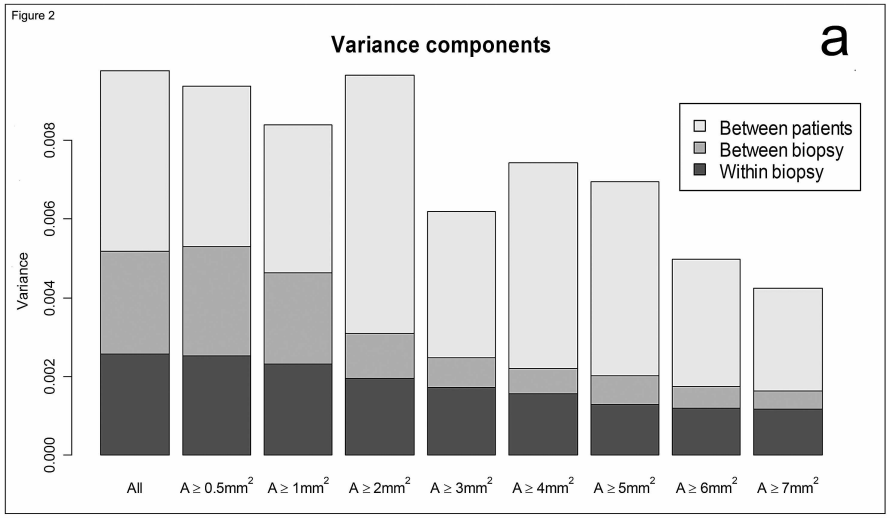
Variability was smaller in larger biopsies

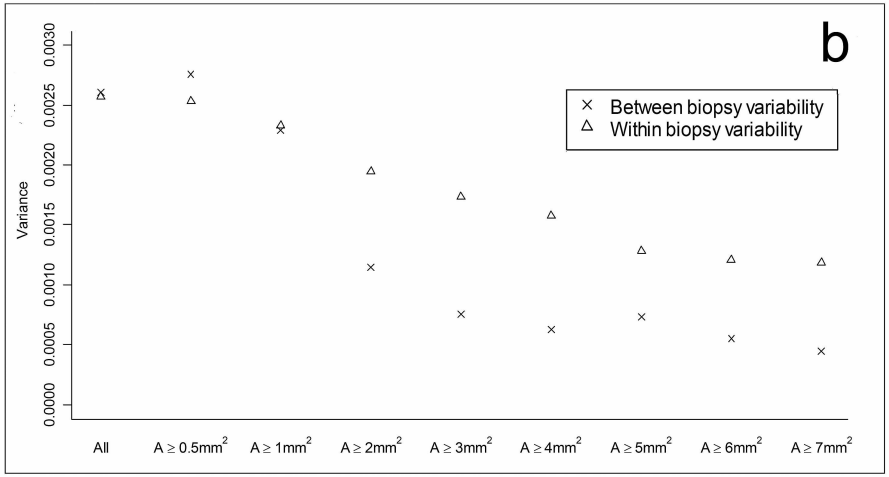
•Each data-point shows standard deviation (transformed) within a biopsy vs. average tumor area

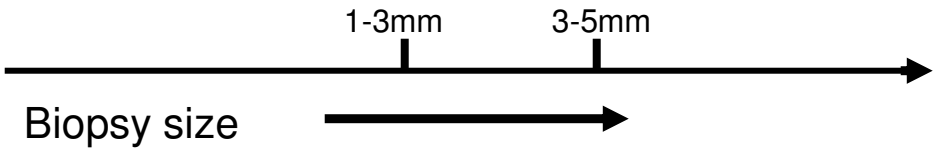


Increasing biopsy size reduces variance between biopsies within tumors and error of the method



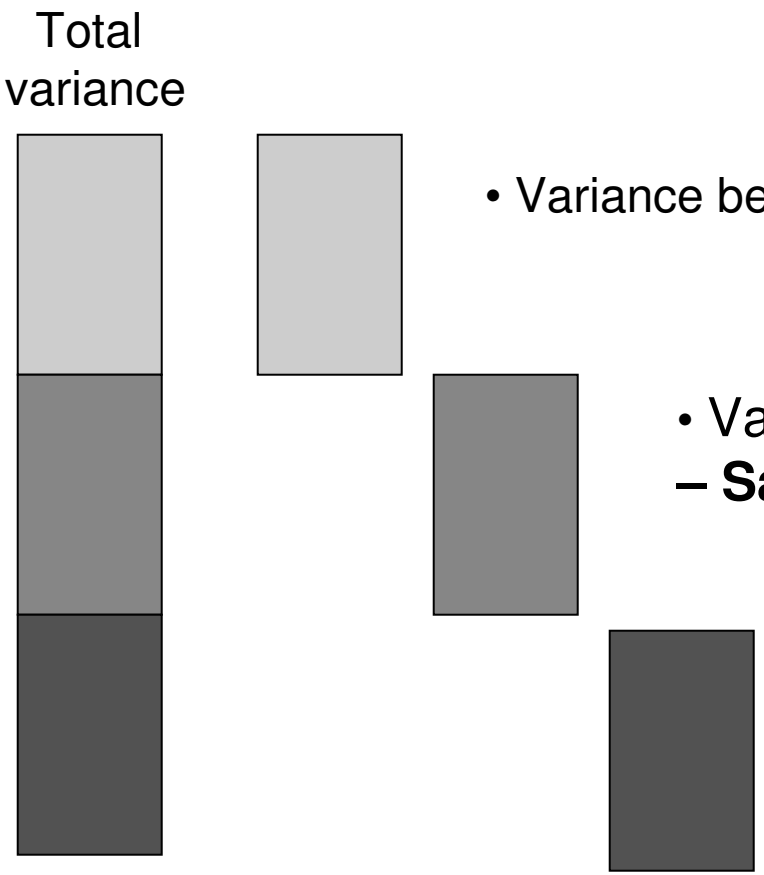






Biopsy size

Variance analysis



• Variance between tumors – **Sought difference**

• Varianace within tumor/between biopsies
– Sampiling error (macro level)

• Variance within biopsy/between individual sections –
Sampling error (micro level) **+**
Analytical error(due to method)

Conclusions from the first set

- Larger biopsies improve both the sampling and analytical errors :

- select biopsies with larger tumor area

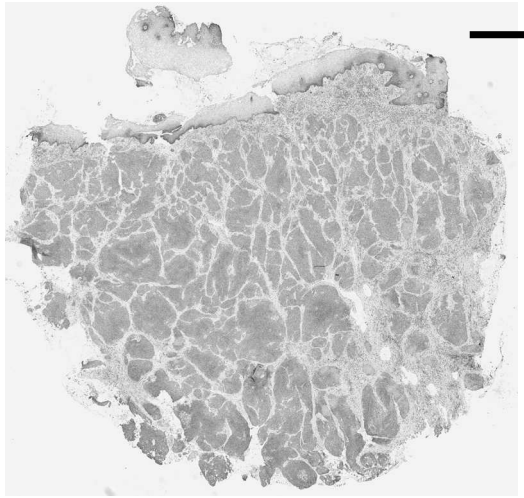
- Steps to reduce the analytical error:

-10 µm sections vs. 5 µm ?

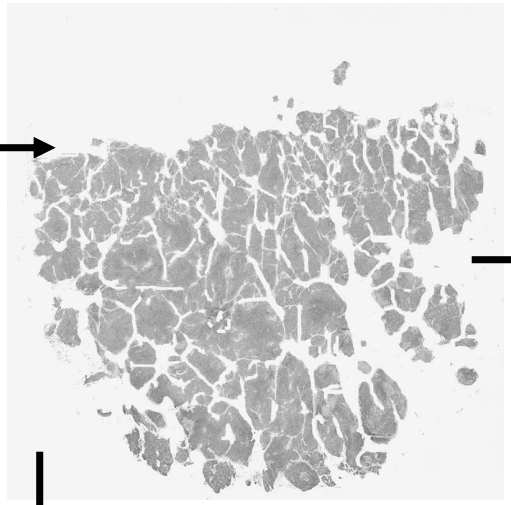
-fluorescent vs. brown stain?

Immunohistochemistry based analysis

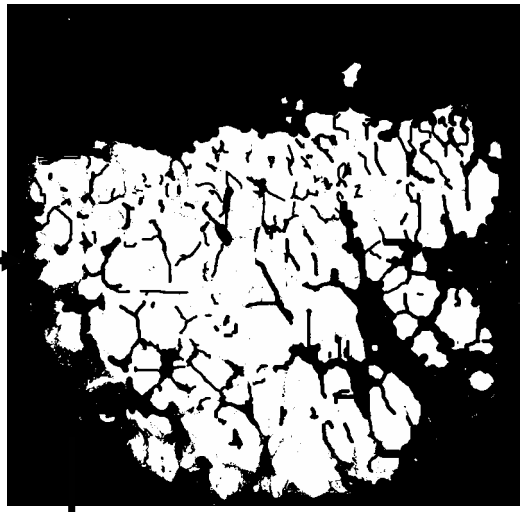
Immunohistochemical stain, scanning in transmitted light scanner



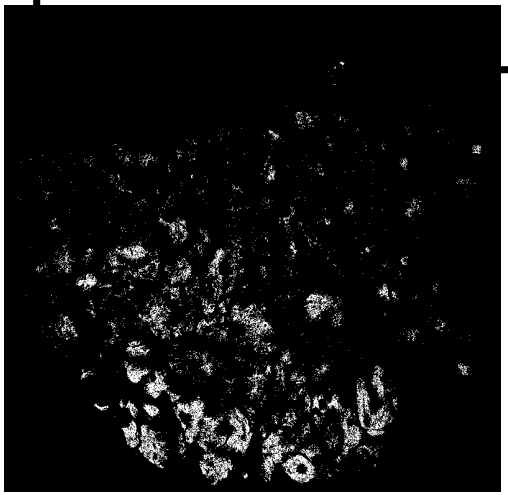
Tumour area selection



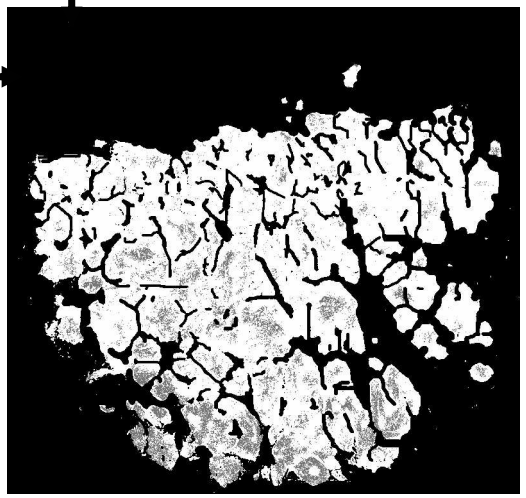
Mask creation



Thresholding on brown stain

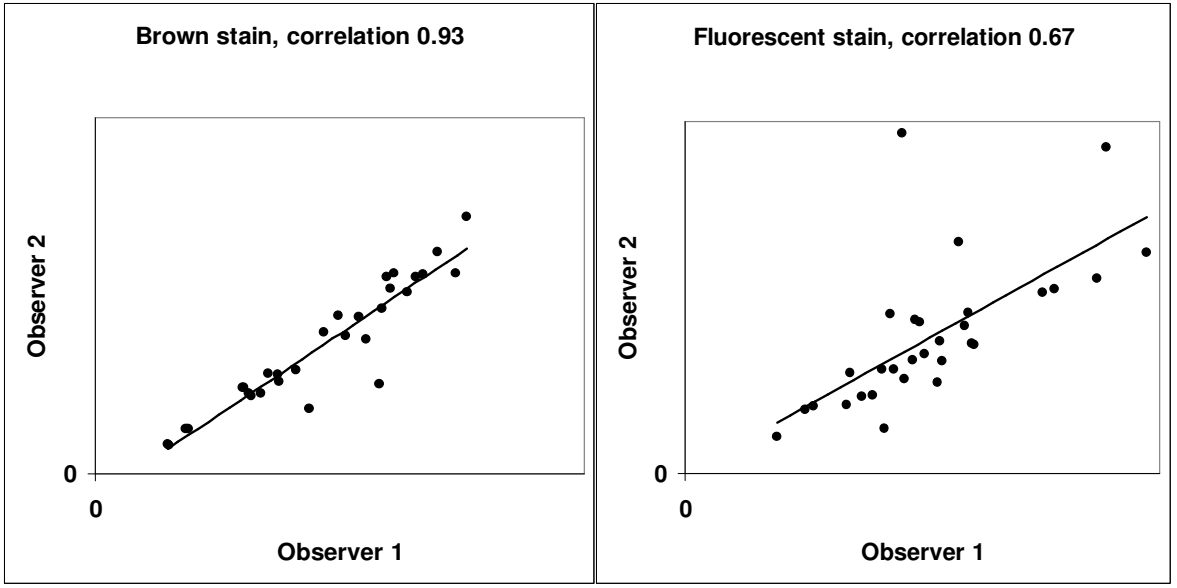


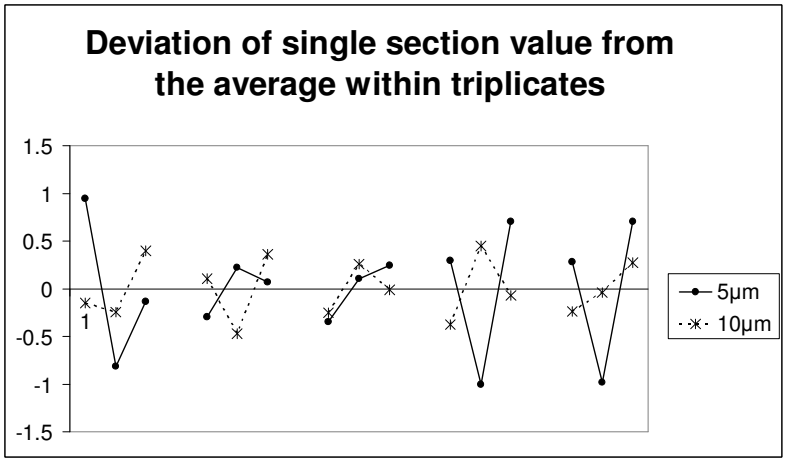
% positive area calculation



Reduction of the analytical error

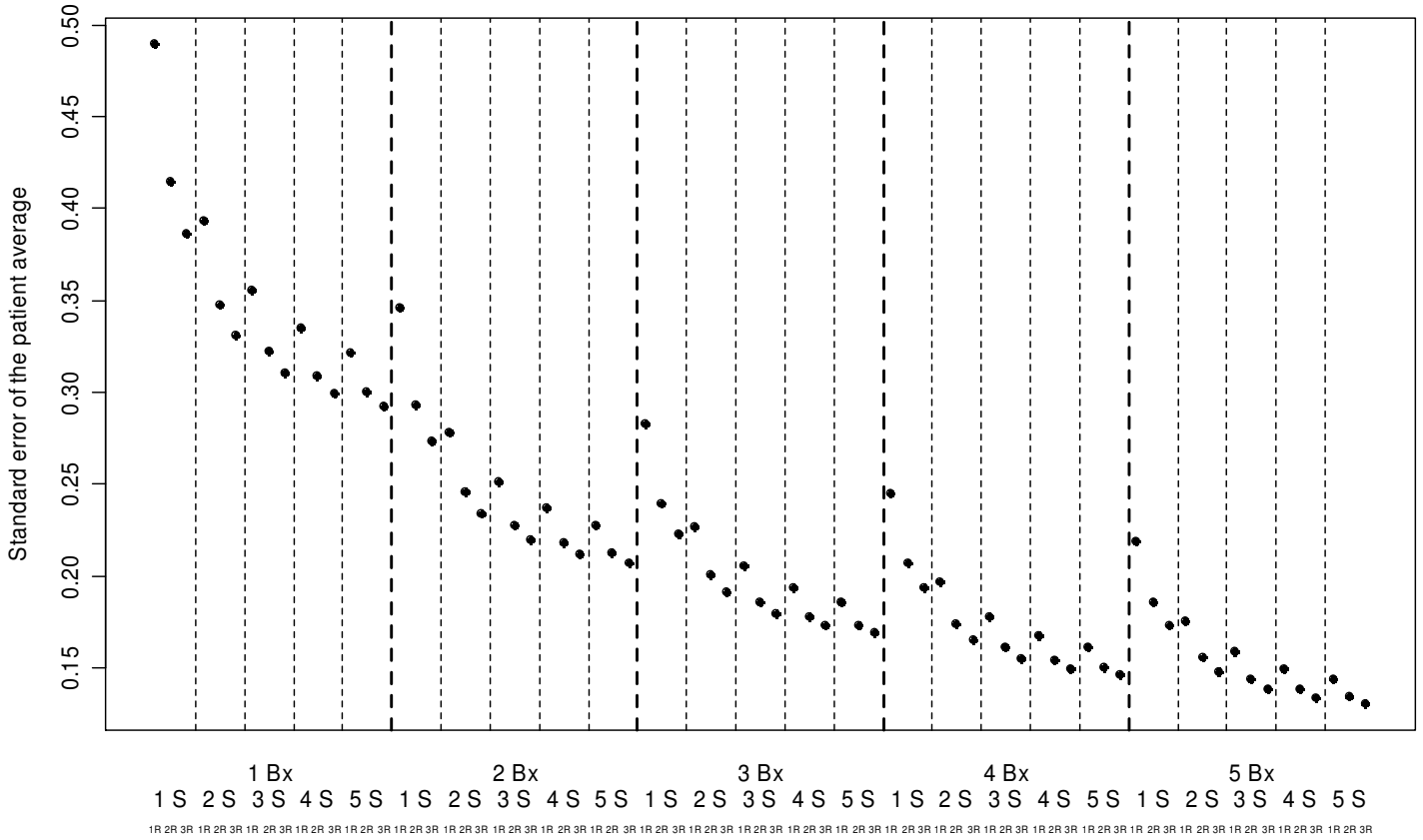
Assessment of coloured images is more reproducible than greyscale ones





Variation of tissue advancement in the microtome is more pronounced at thinner sections

How much is enough?

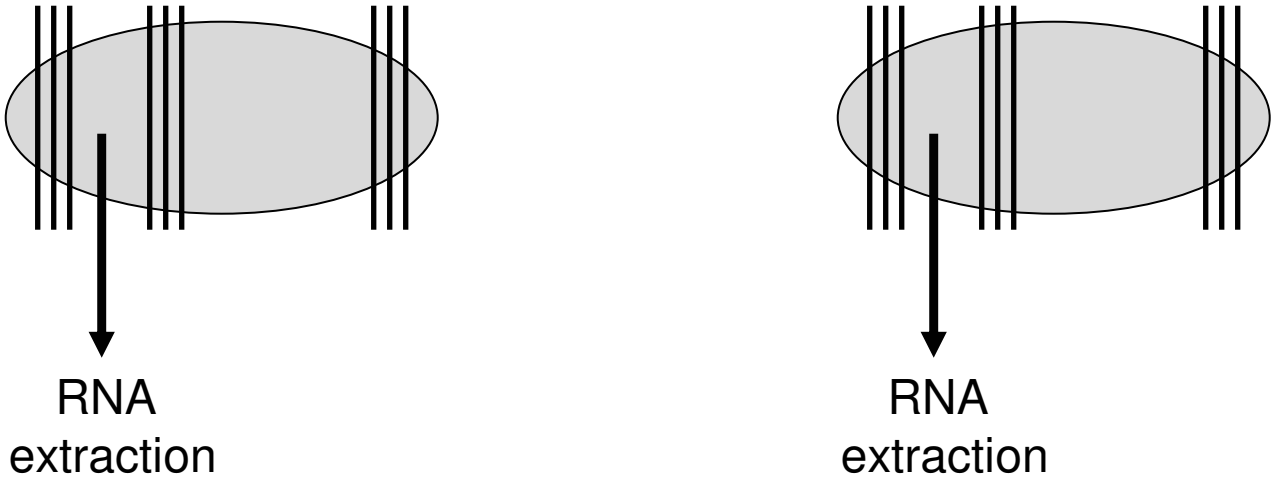


Sampling + analytical error

Combinations of increasing number of biopsies, sections and repeat analysis

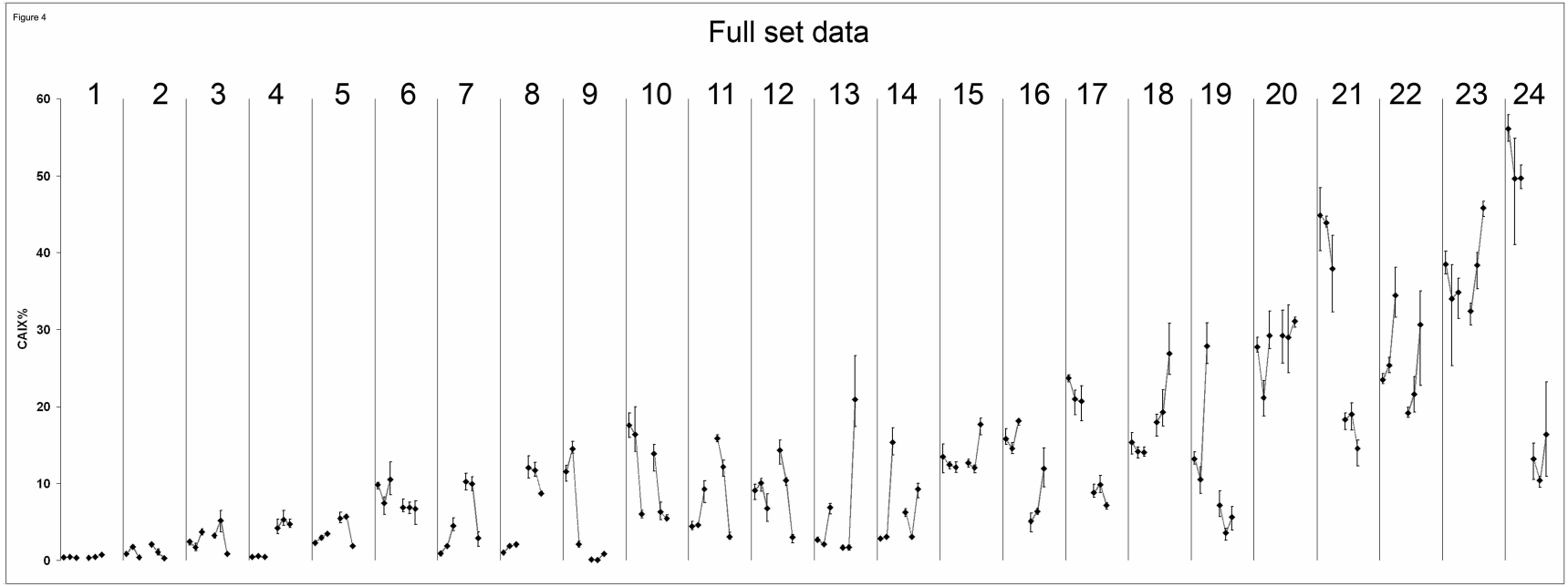
CAIX assessment

- 24 patients with invasive SCC of the cervix
- 2 biopsies sectioned at 3 levels:
  - 1st and 2nd 250 µm apart, tissue between collected for RNA extraction
  - tissue flipped and 3rd level cut from the opposite side
  - each level cut in triplicate serial sections

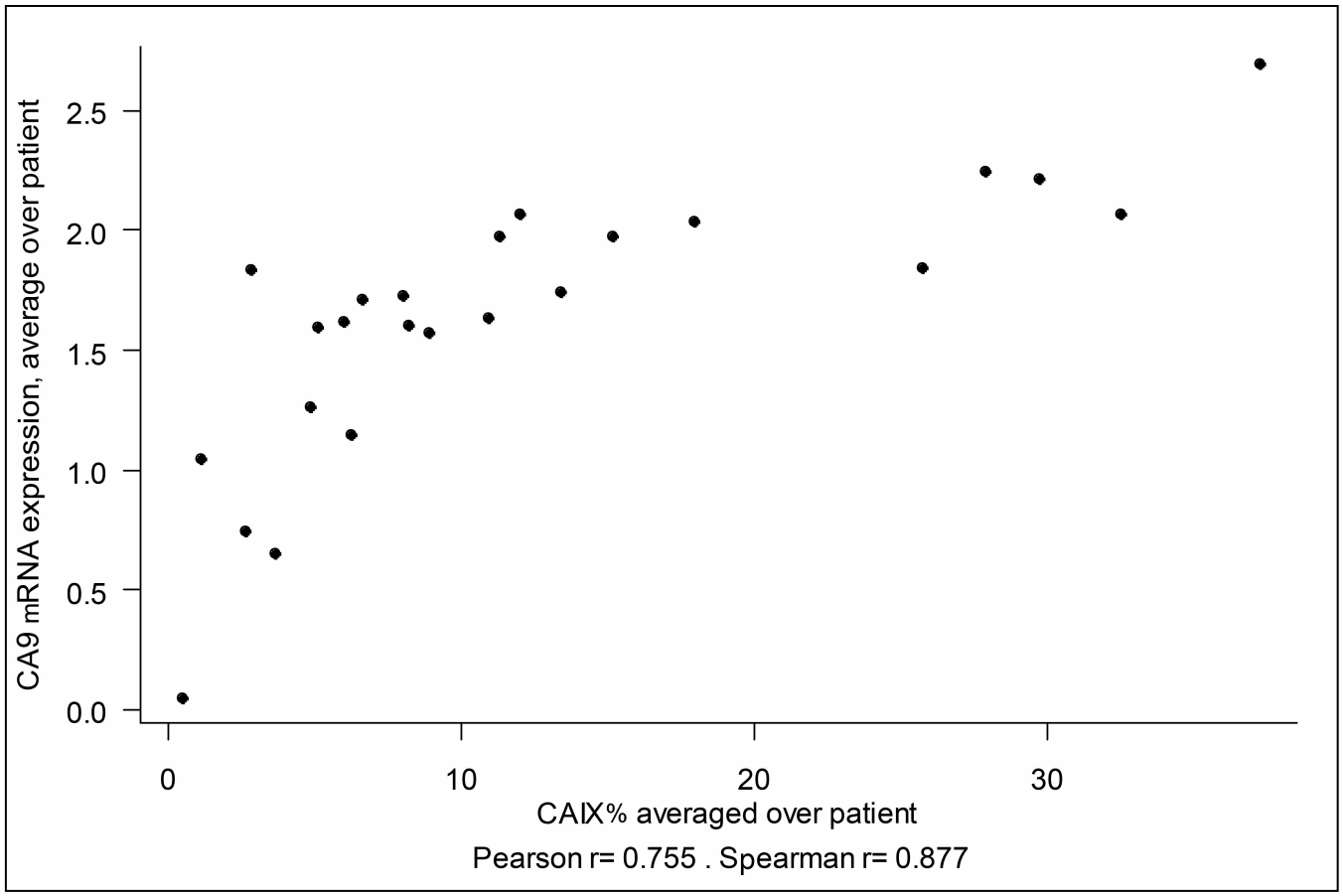


Variance of measurement reflects heterogeneity of CAIX expression

• Individual points are averaged triplicate sections (min and max shown as a bar)

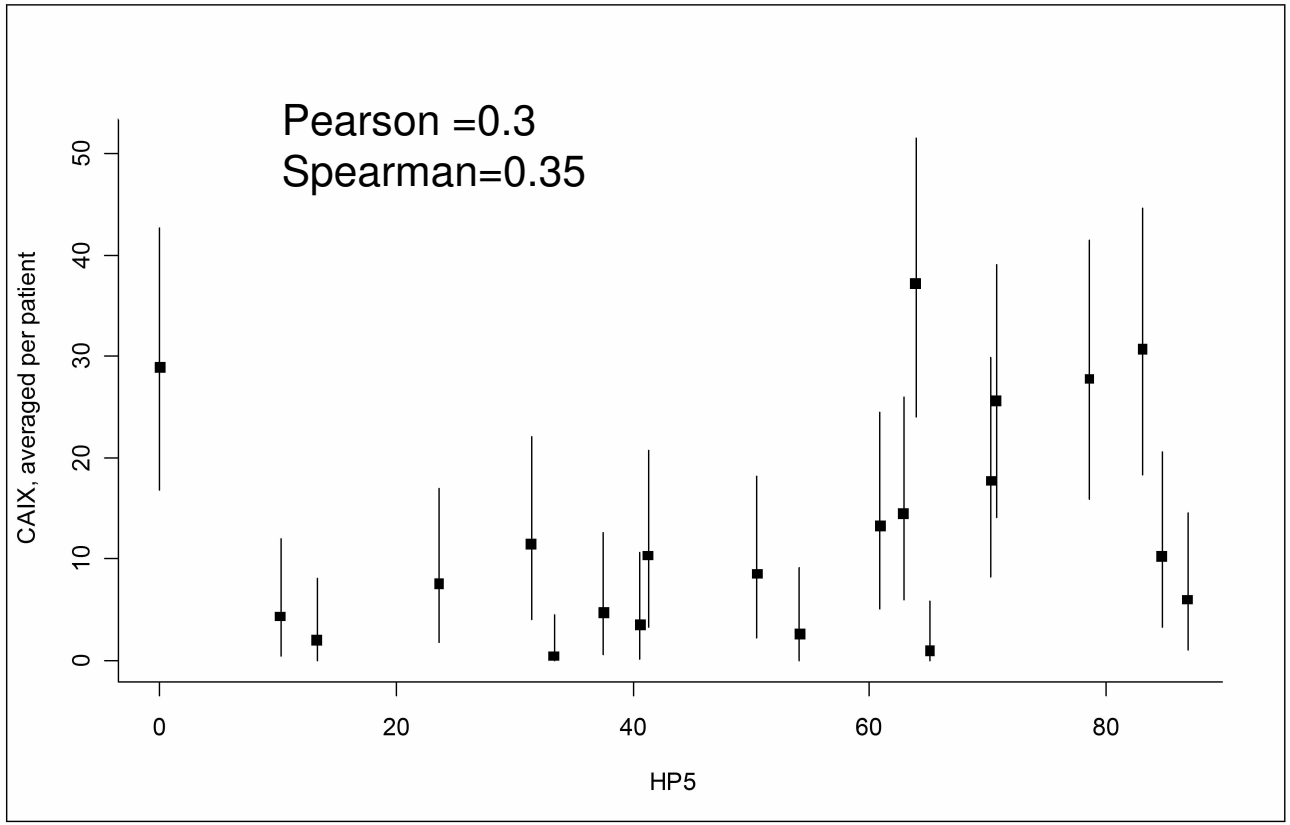


Protein vs. RNA expression

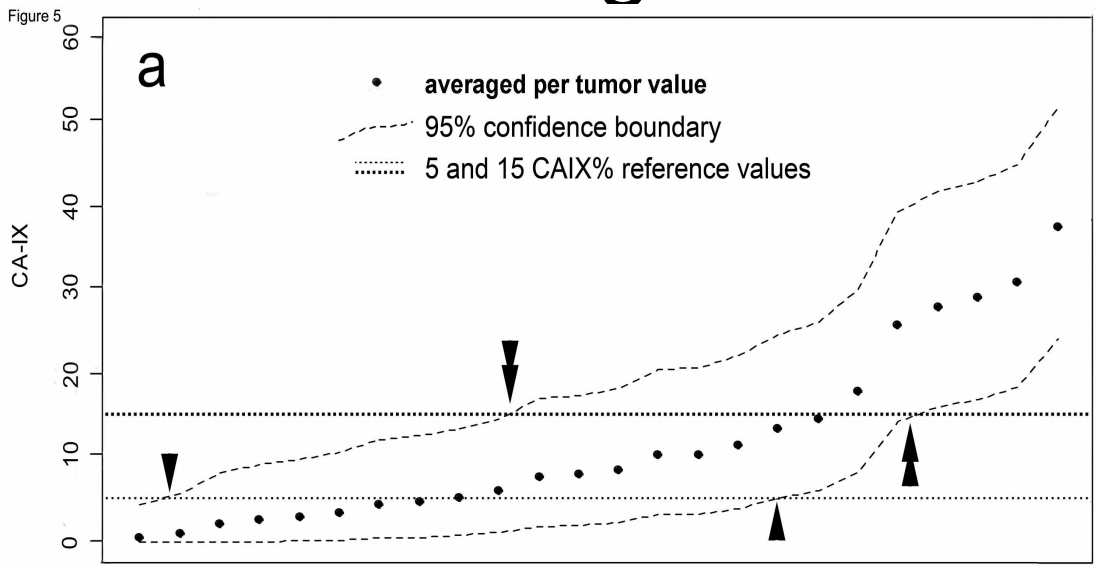


Correlation with direct pO2 measurements

•CAIX% values (shown with 95% CI) plotted against percentage of Eppendorf probe readings less than 5 mm Hg (HP5)



Effect of the measurement accuracy on tumor categorization



- If 5 CAIX% is set as a hypoxia threshold (single arrows), 15 tumors (62%) cannot be assigned to either category with 95% confidence
- Steep segments of the distribution – biological "ON-OFF" switch, reduces the number of uncategorized samples (double arrow)
- Threshold through zero has only a half of the error, however it cannot be applied when all samples express a protein

Tissue microarray simulation

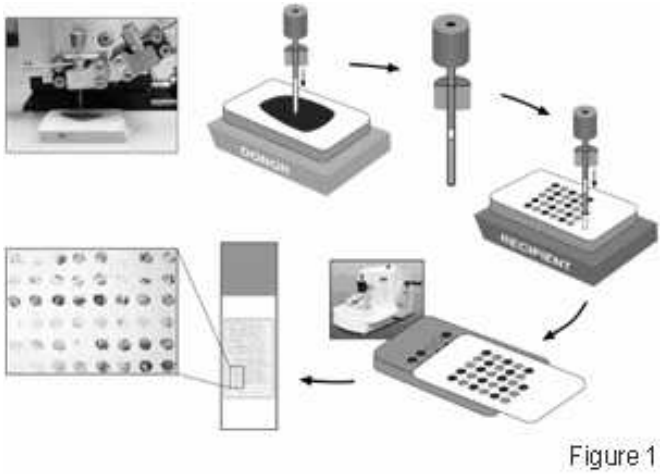


Figure 1

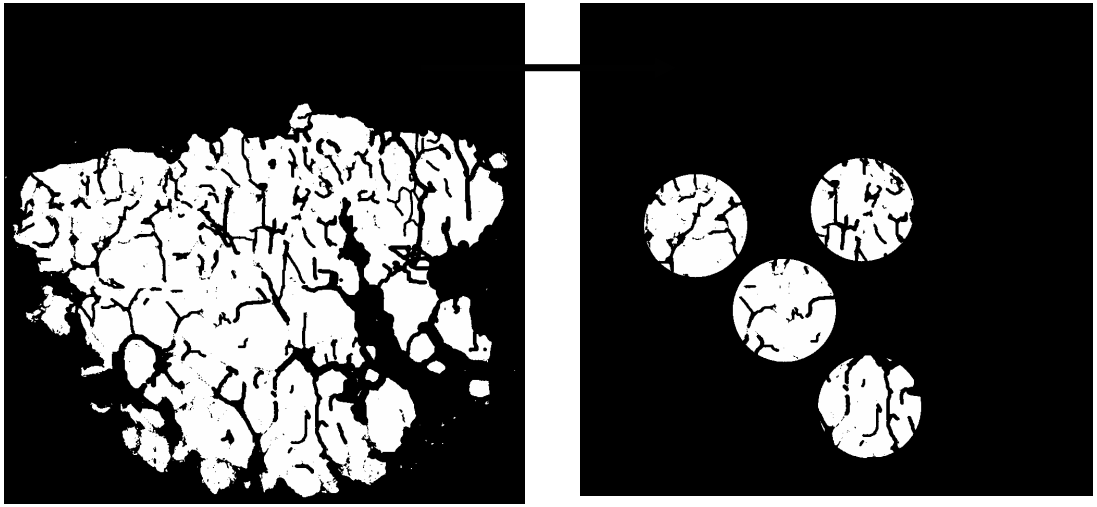
http://tissuearray.org/yale/

Tissue microarray technology is aimed at screening of large numbers of histological samples

Needle cores of multiple tumors are transferred into a single paraffin block

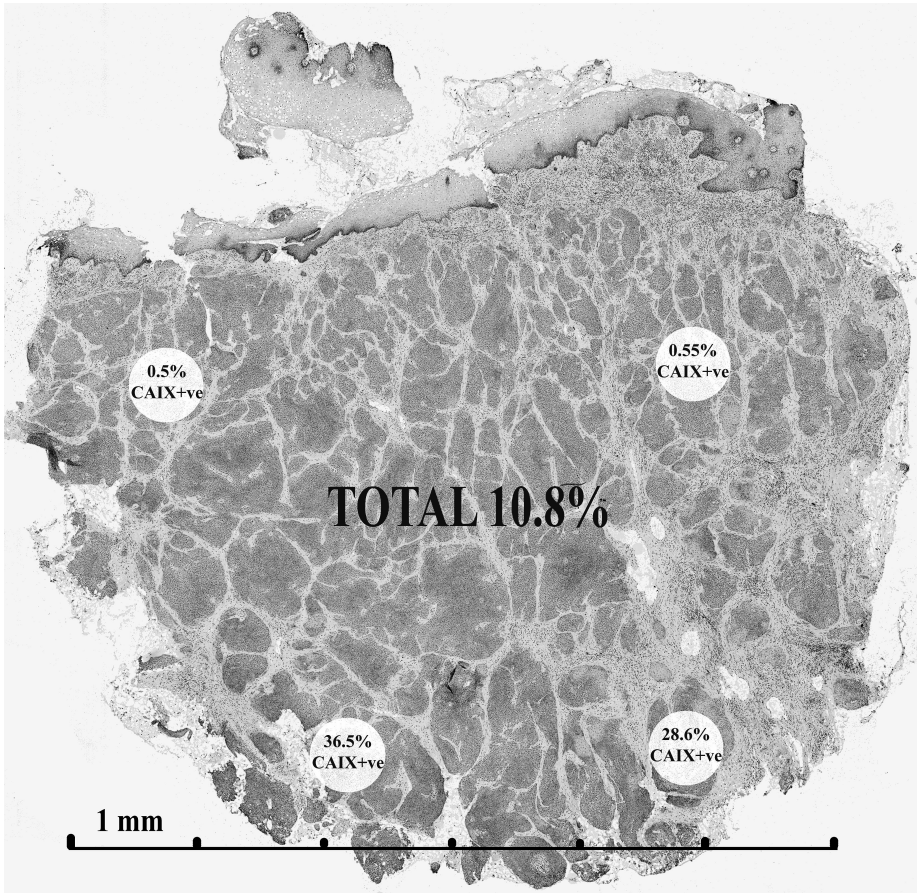
- 145 sections
- Tumor masks limited by circles of 0.6 and 1.5mm in diameter
- Sets of 1 or 2 biopsies; combinations of 1, 2 and 4 cores per biopsy

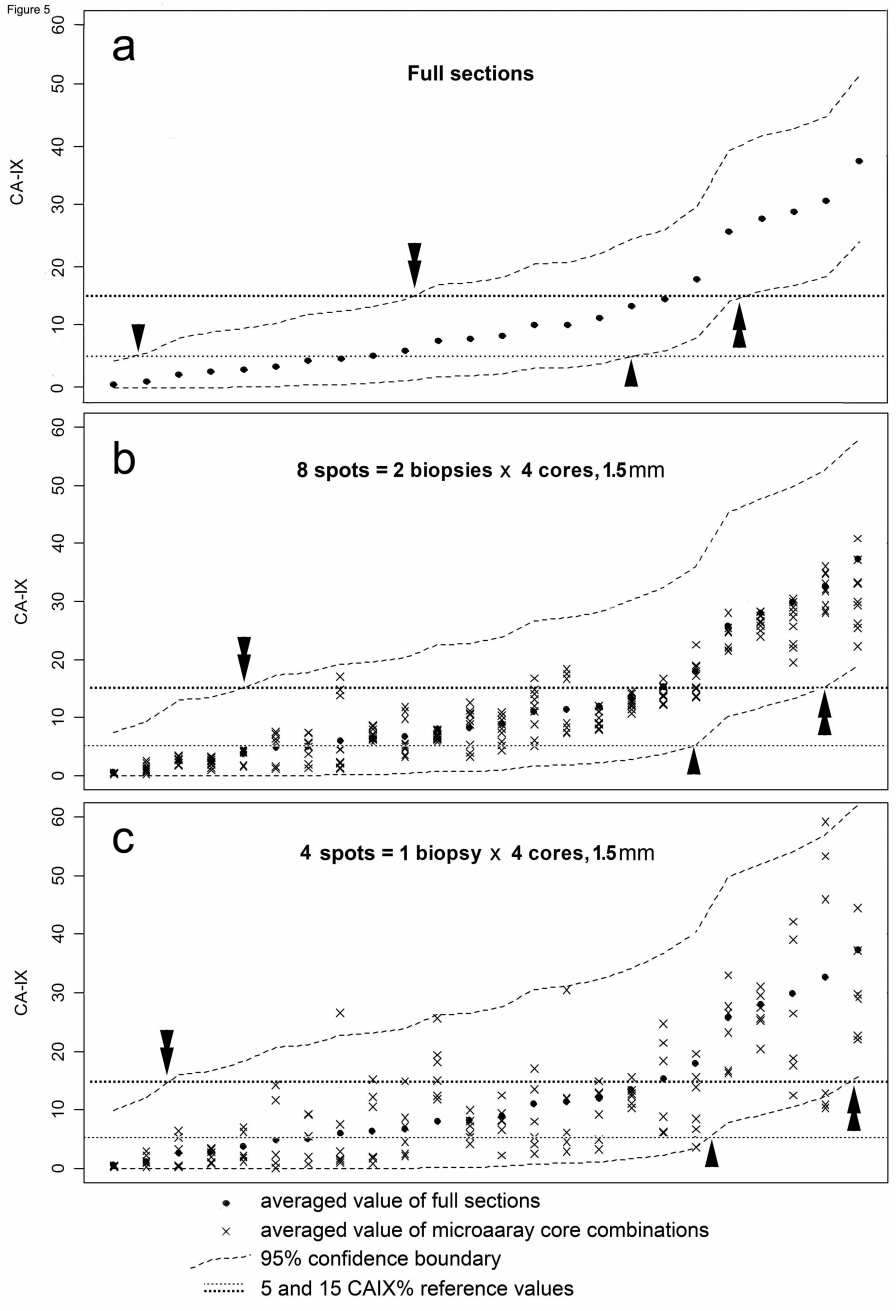
Simulation



Tissue microarray simulation

Reduction of the size of a histological sample widens the sampling error





Can we calculate how much is enough?

Sampling theory of Gy

Two types of heterogeneity

- Distributional – focal absence or presence of a substance
- Constitutional – relative proportion of the substance where it is present

Gy P. Sampling for analytical purposes. Wiley, New York, 1998

Sampling theory of Gy

$$s^2 = (1/MS - 1/ML)\, d^3 f g c \beta$$

- s^2 - fundamental variance of sampling
- *MS* - the mass of sample (section/level)
- *ML* - the mass of a lot (tumor/biopsy)
- *d* – size of the largest (upper 95%) positive foci
- *f*- a shape factor (a cube *f*=1, sphere *f*=0.52, irregular *f*=0.25)
- *g* – size distribution factor (all particles the same size *g*=1, wide size distribution *g*=0.25)
- c – constitutional factor ~ density */ percentage*
- *β* – liberation factor (adherence of particles to the matrix)

Gy P. Sampling for analytical purposes. Wiley, New York, 1998

In case of 4-10μm thick histological sections the tumor mass cancels itself due to a large size:

$$Sampling\ error = \frac{d^3 f g c \beta}{\text{Mass of sample}}$$

- d – size of the largest foci
- f - shape factor
- g – size distribution factor
- c – constitutional factor ~ density */ percentage*
- β – liberation factor

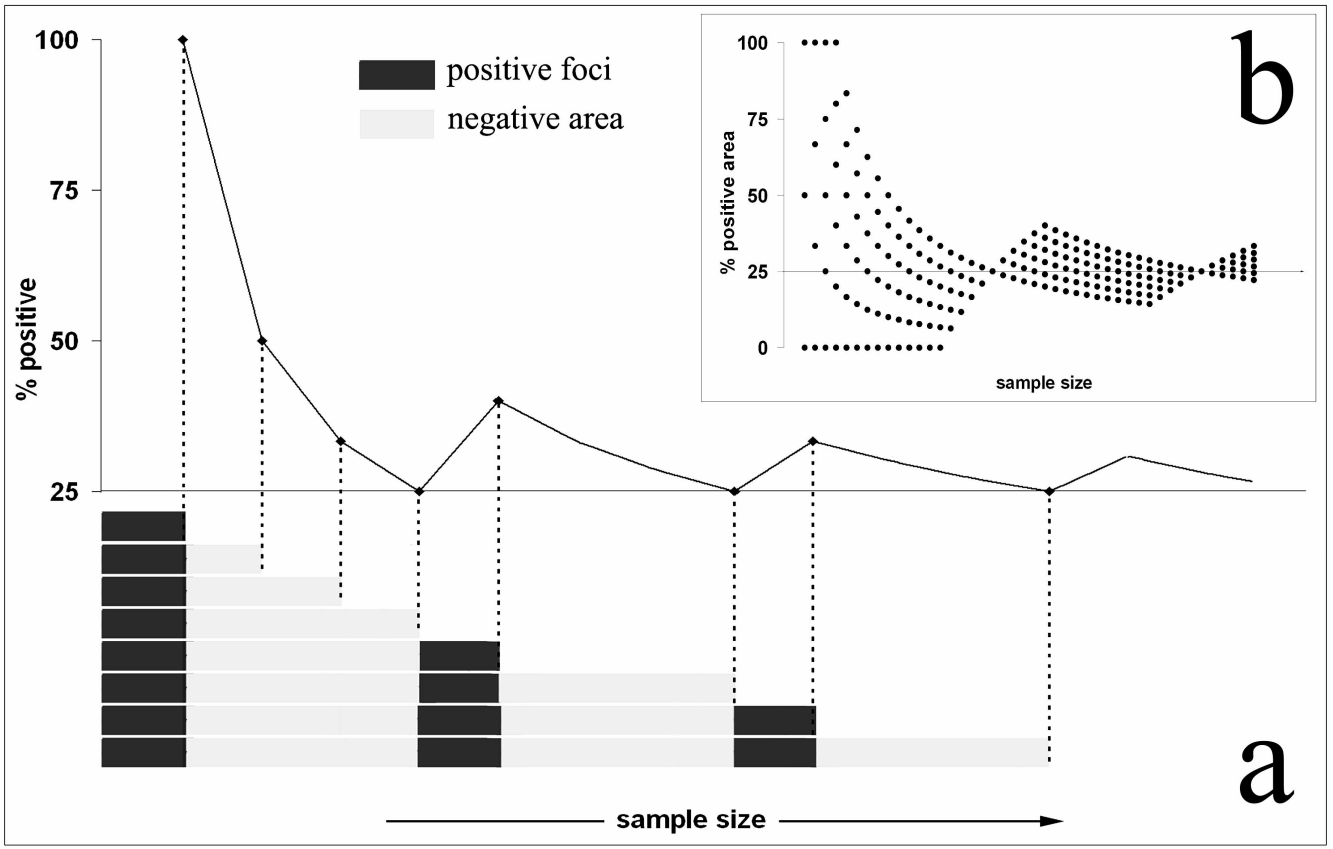
$d^3fgc\beta$

Positive focus size

$D^3fgc\beta$

and size variation

A “core biopsy” of a tumor model with regularly spaced foci



a -Each core starts with a positive focus

b - Random core placement

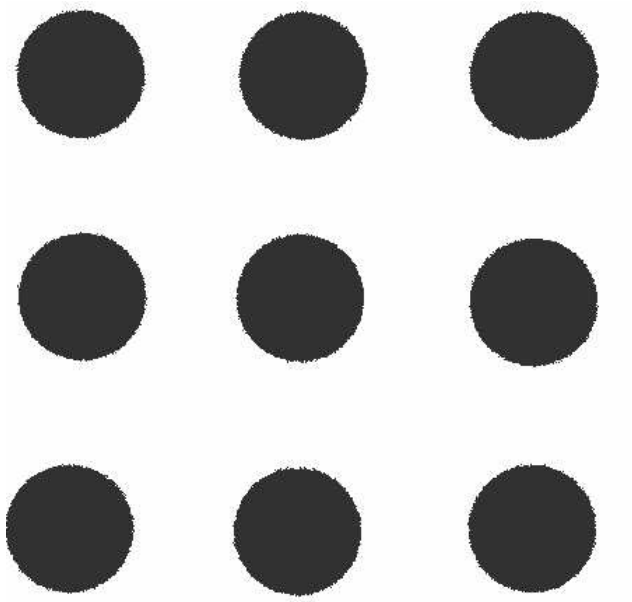
- Each focus with a negative area forms a distribution bell
- When the number of foci is 20 - the height of the bell is 5% (1/20) of the value placing all values within 95% confidence of the total.

$$d^3 f g c \beta$$

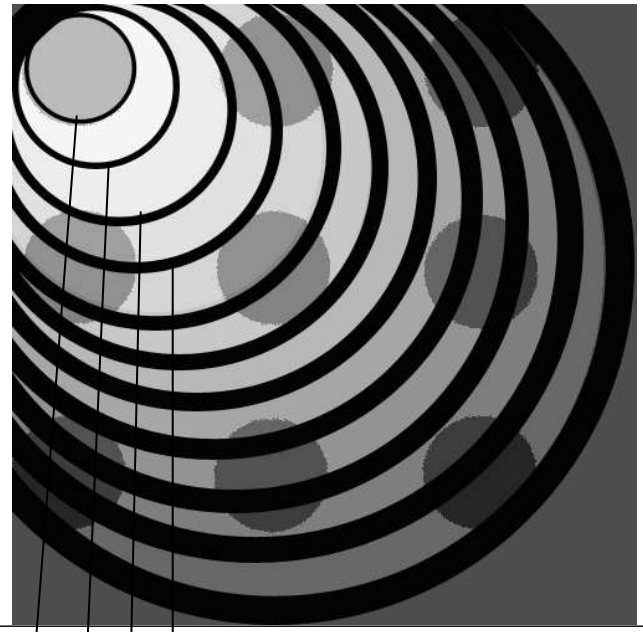
Shape factor

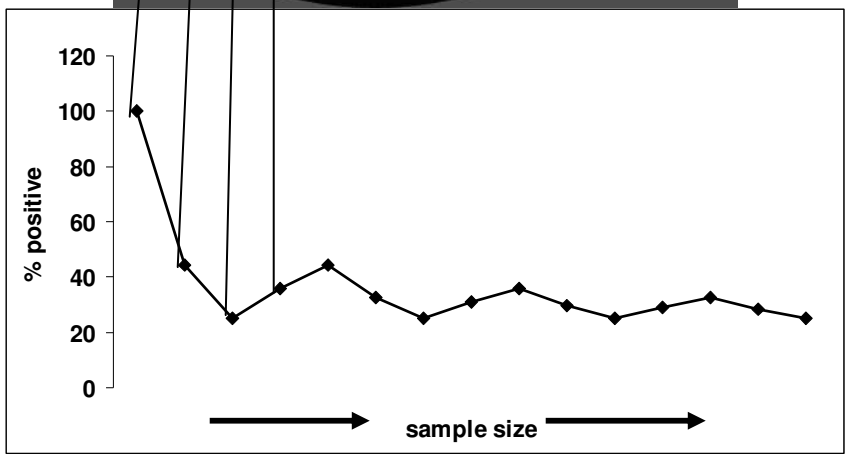
Effect of increasing sample size with round foci

A tumor with focally expressed marker (round foci)



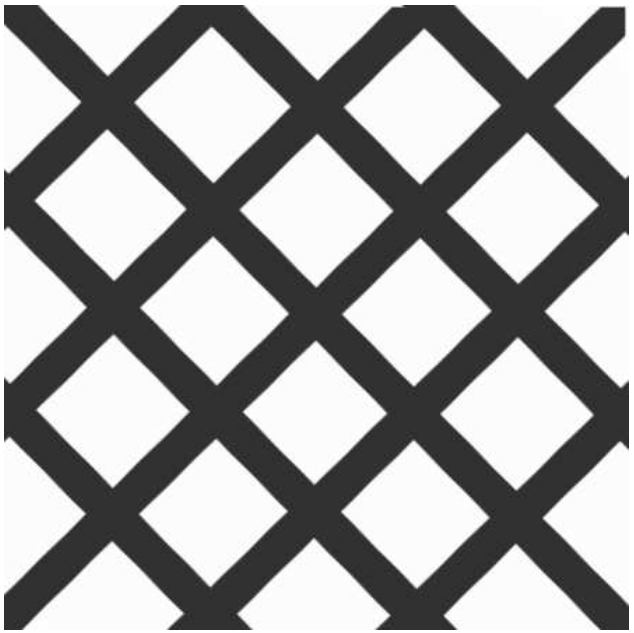
Sampled with increasing biopsy size



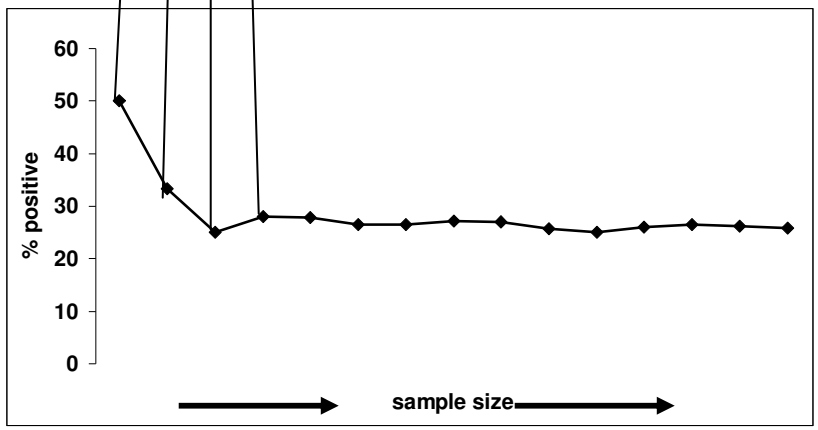


Effect of increasing sample size with a grid-like distribution

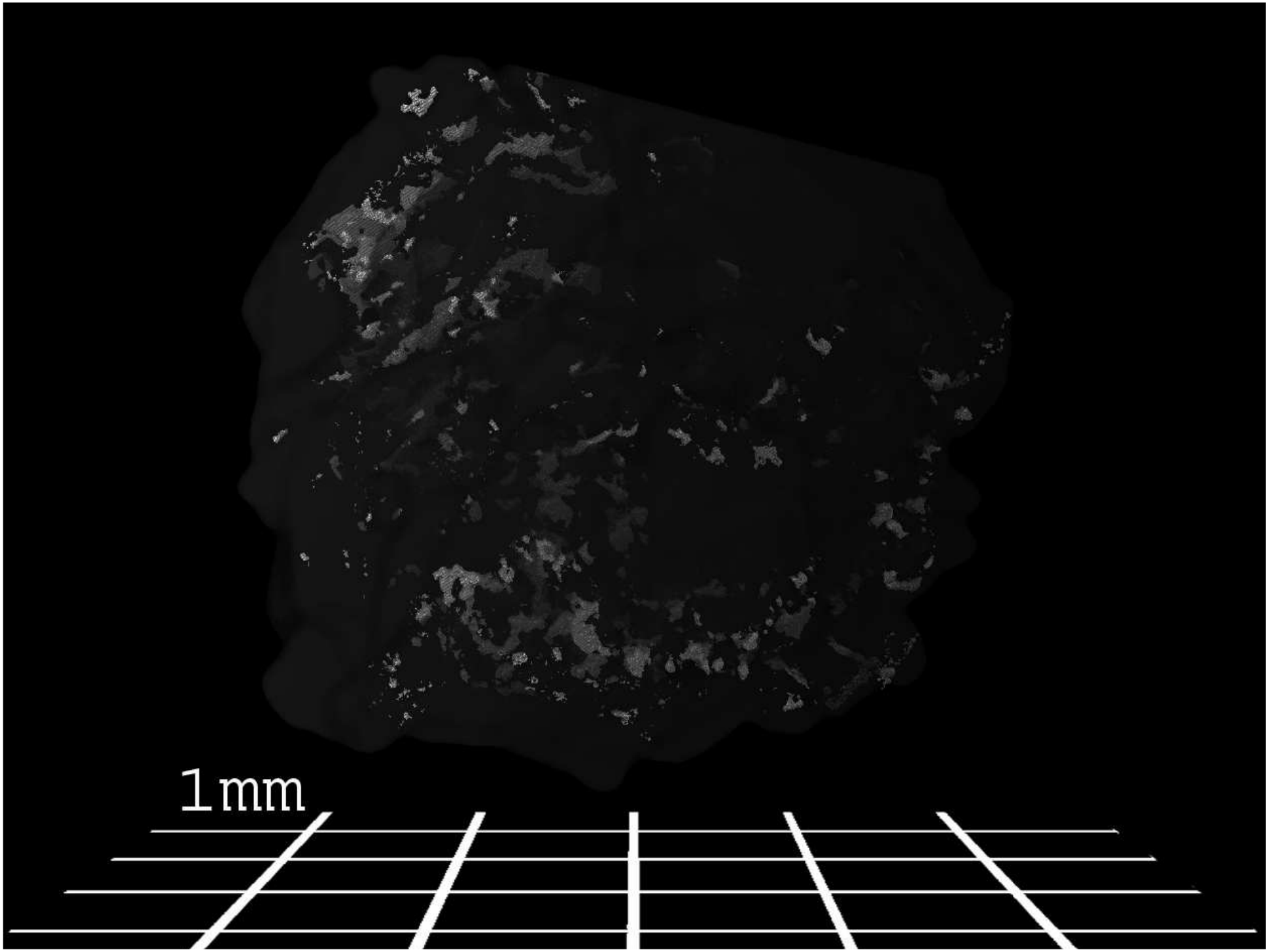
A tumor with focally expressed marker (grid-like distribution)



Sampled with increasing biopsy size



From a model to a real sample: 3D restoration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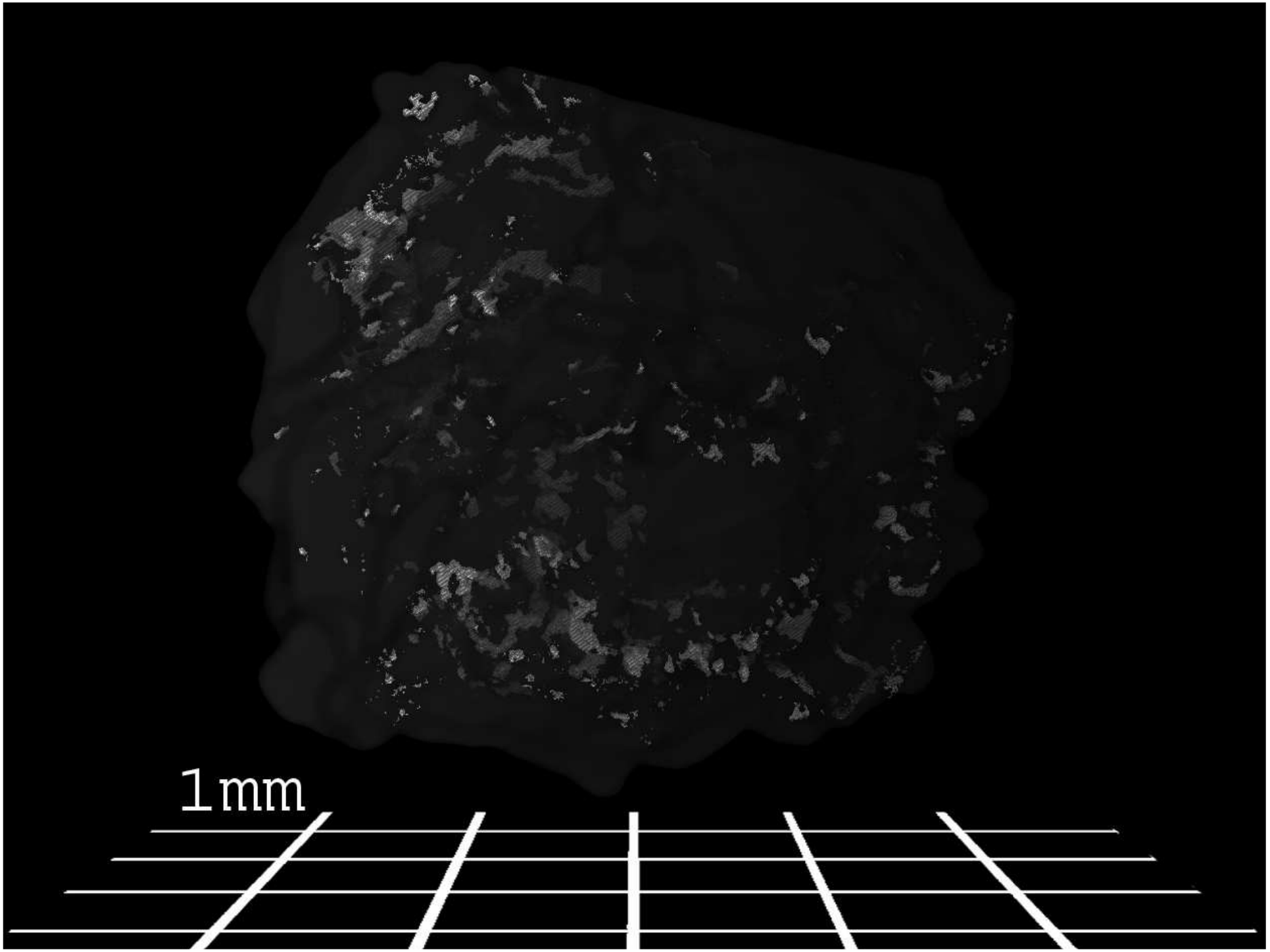
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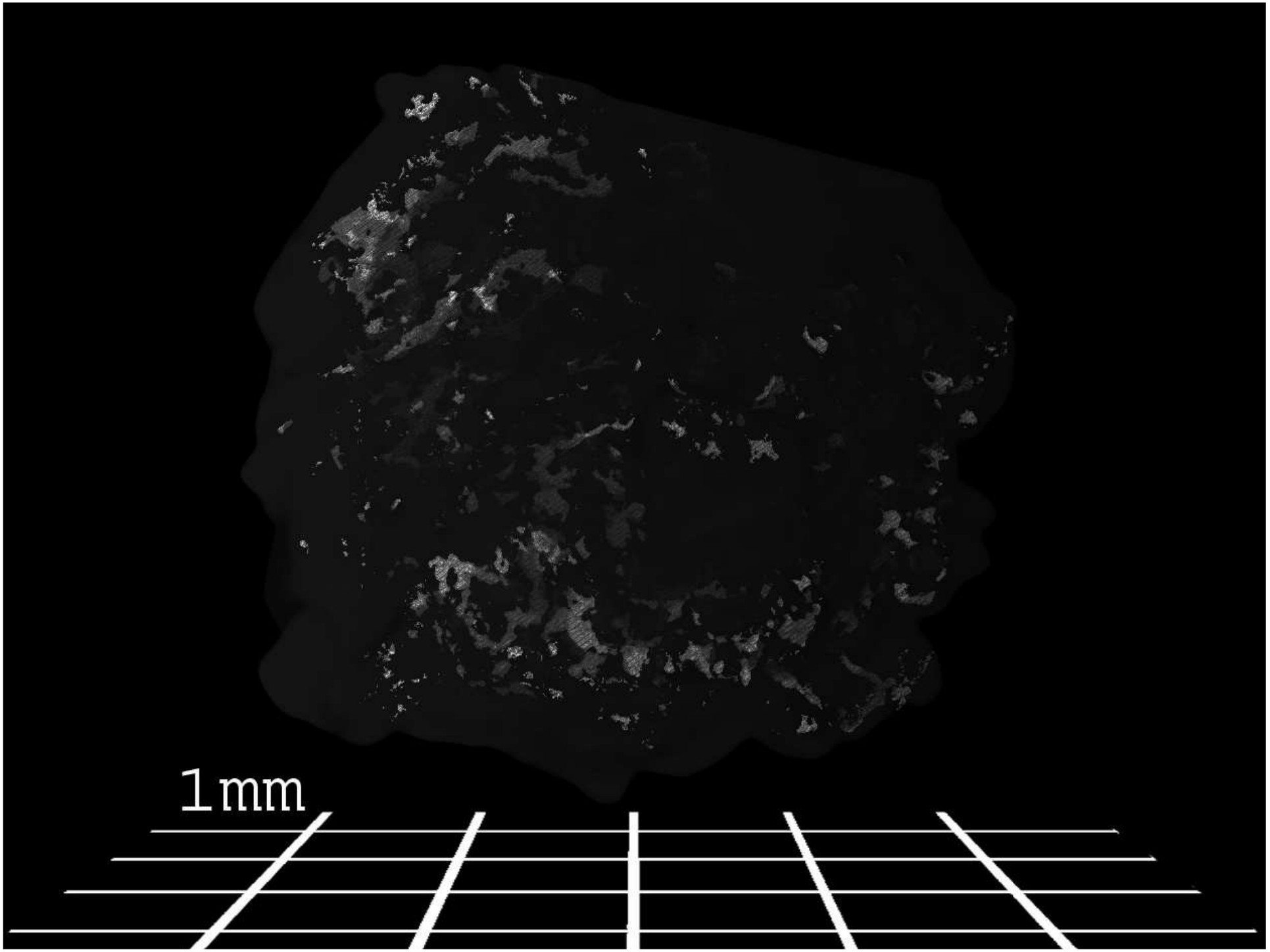
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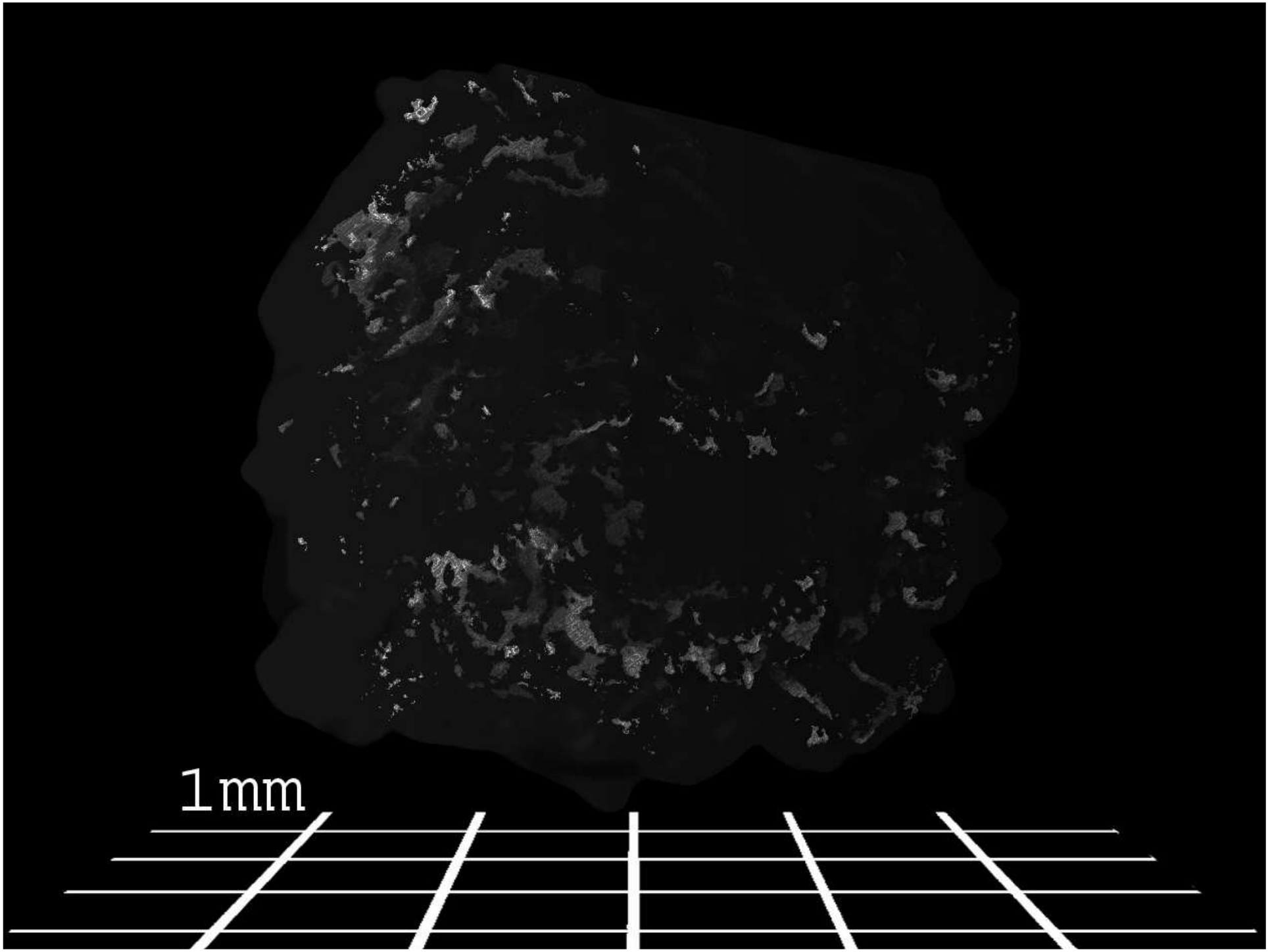
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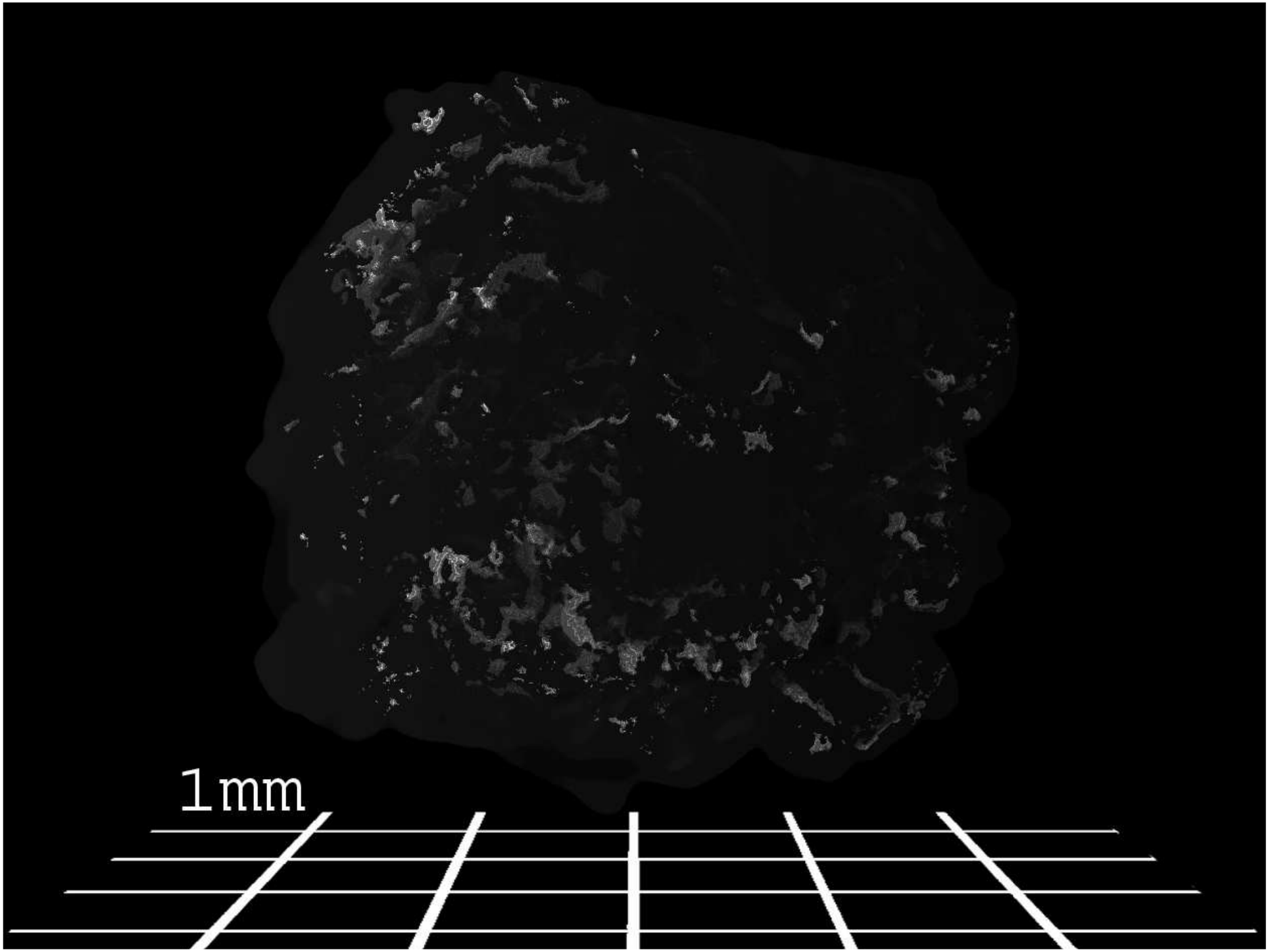
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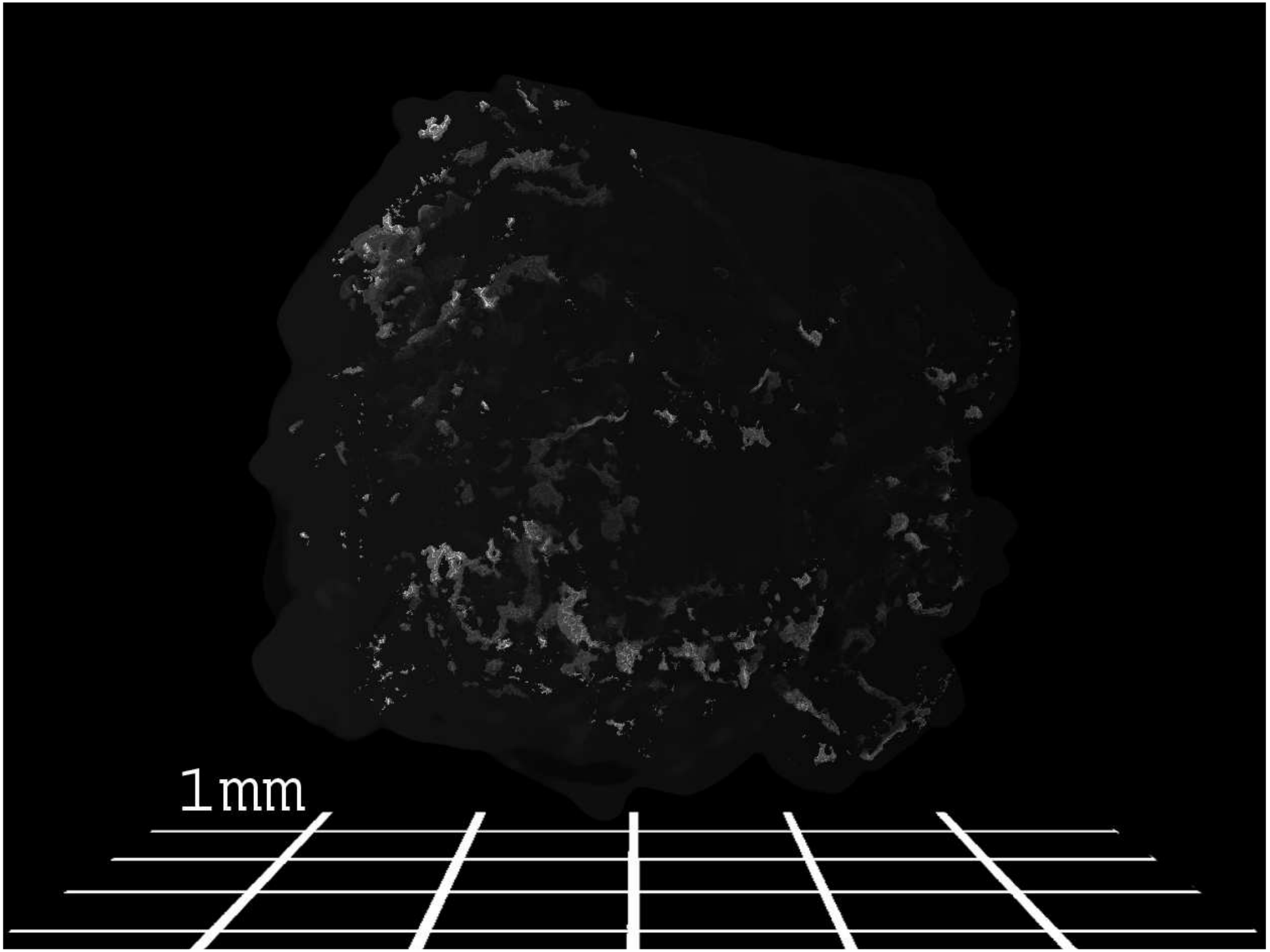
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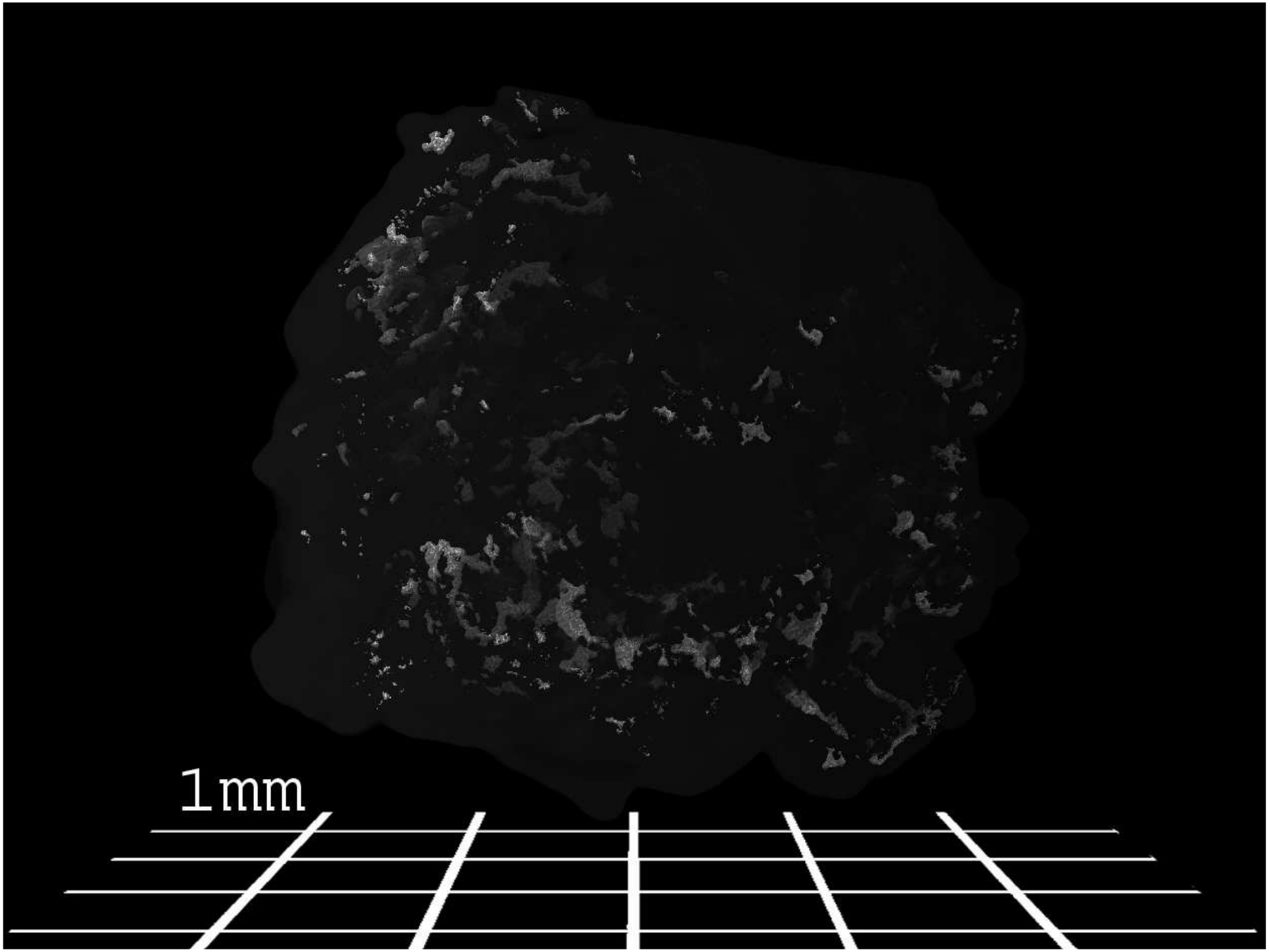
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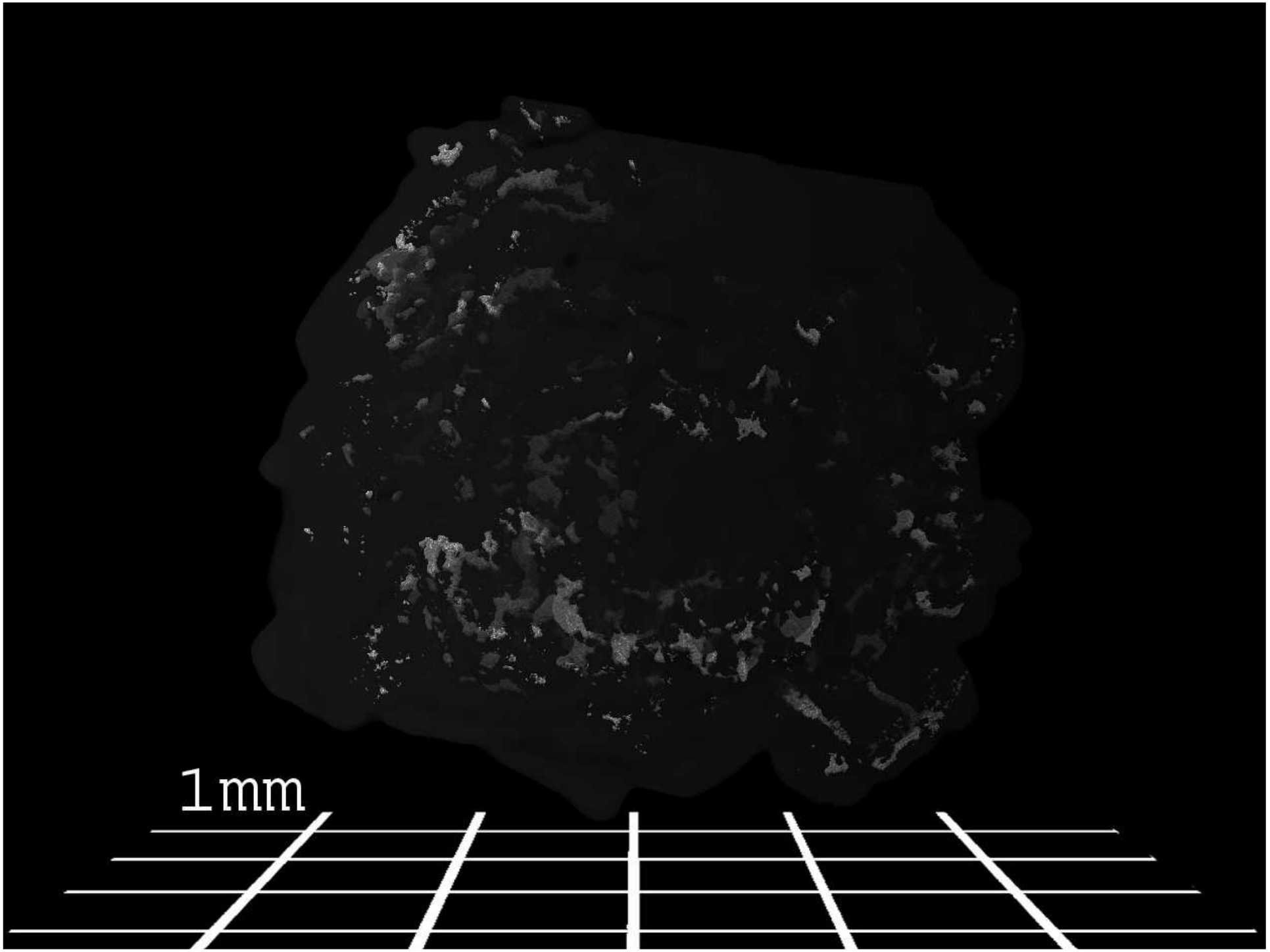
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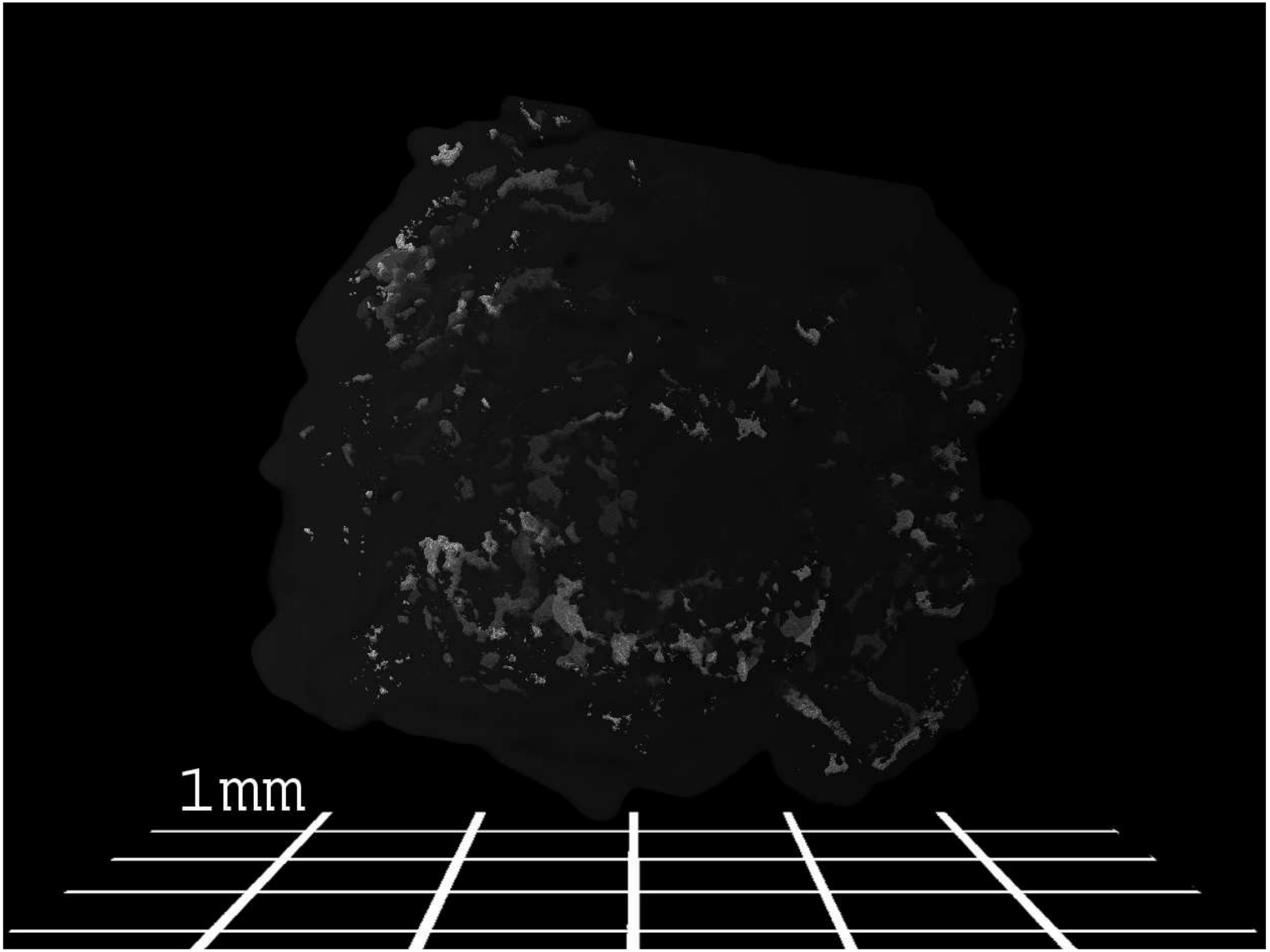
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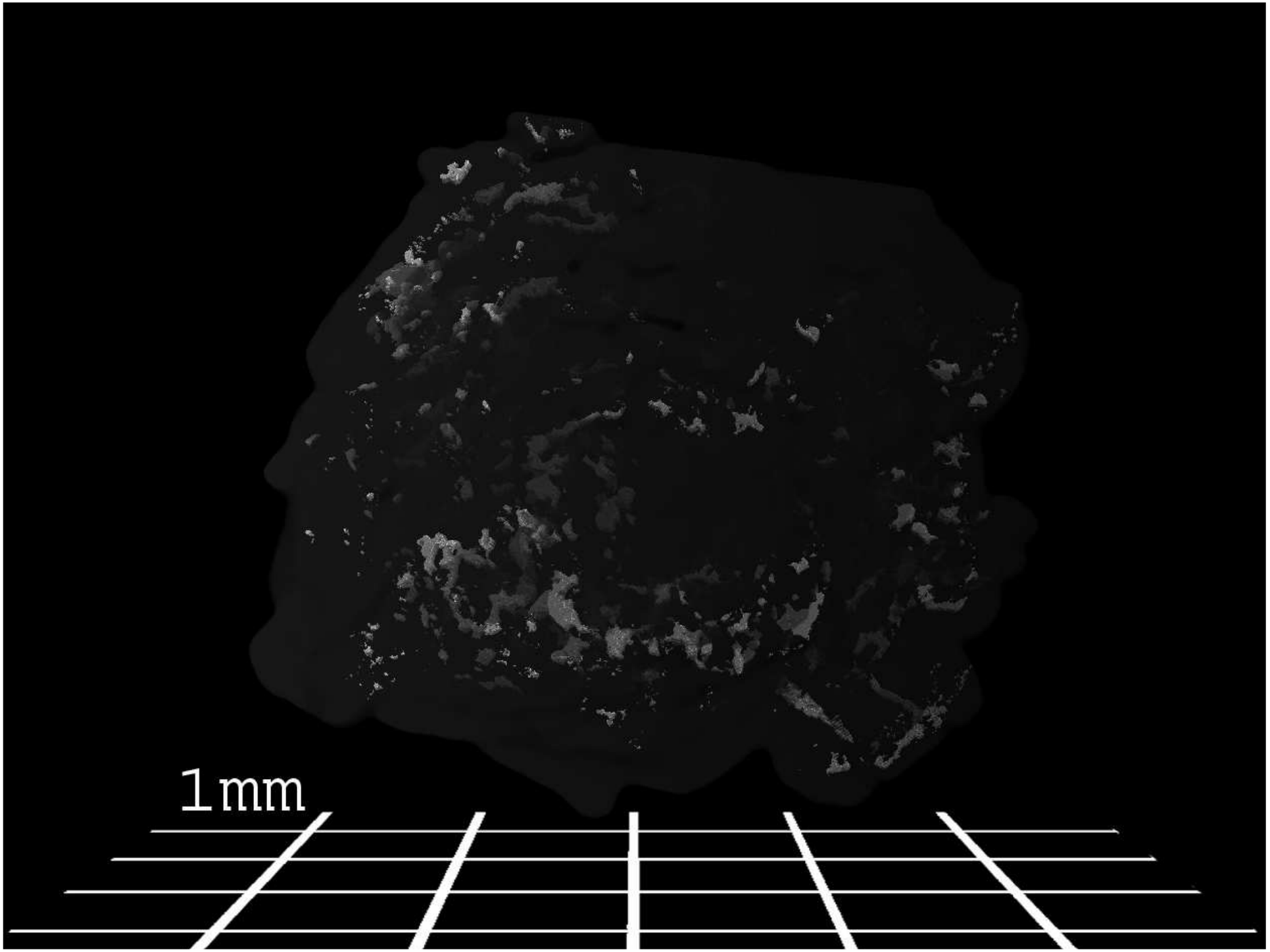
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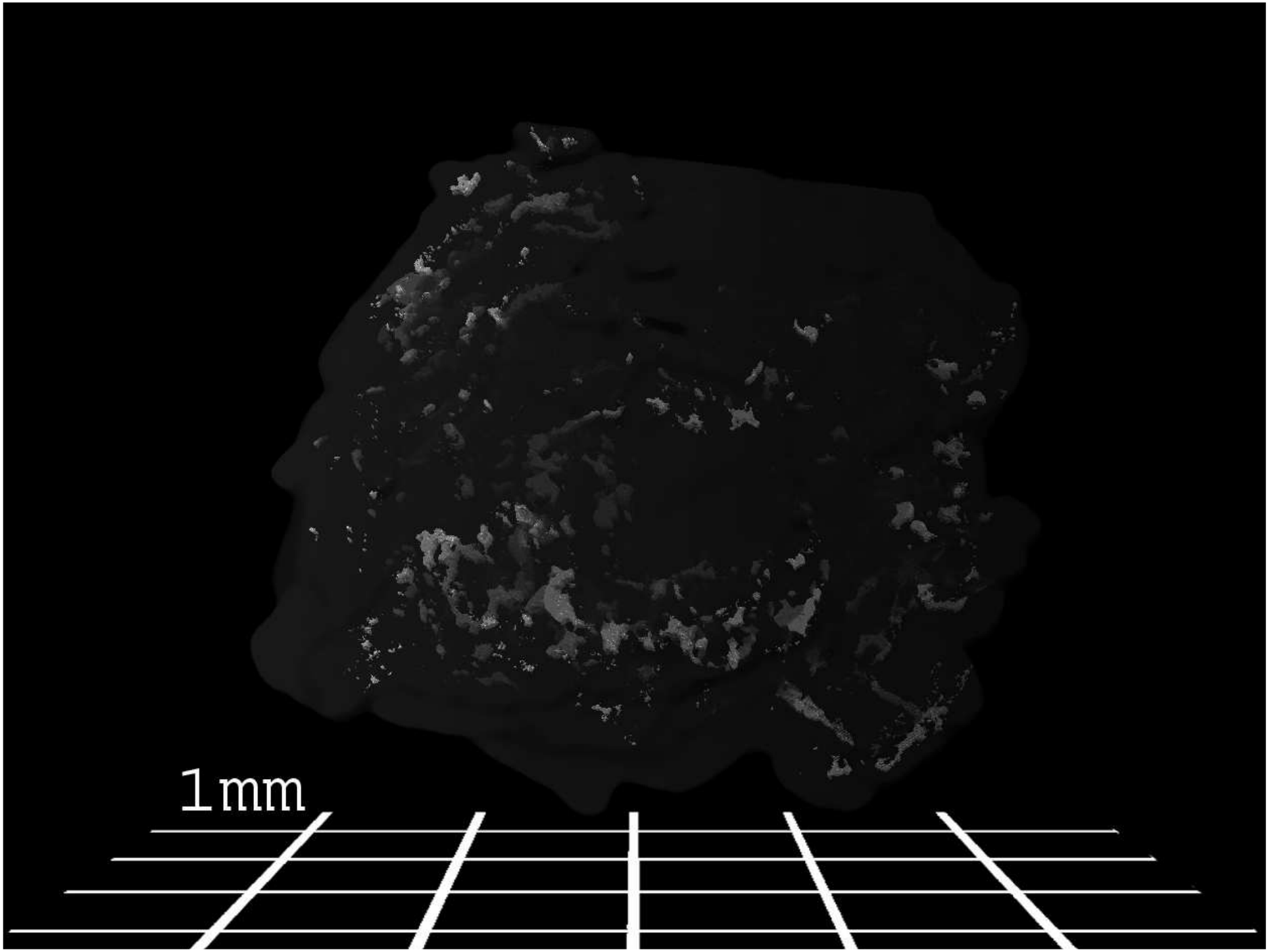
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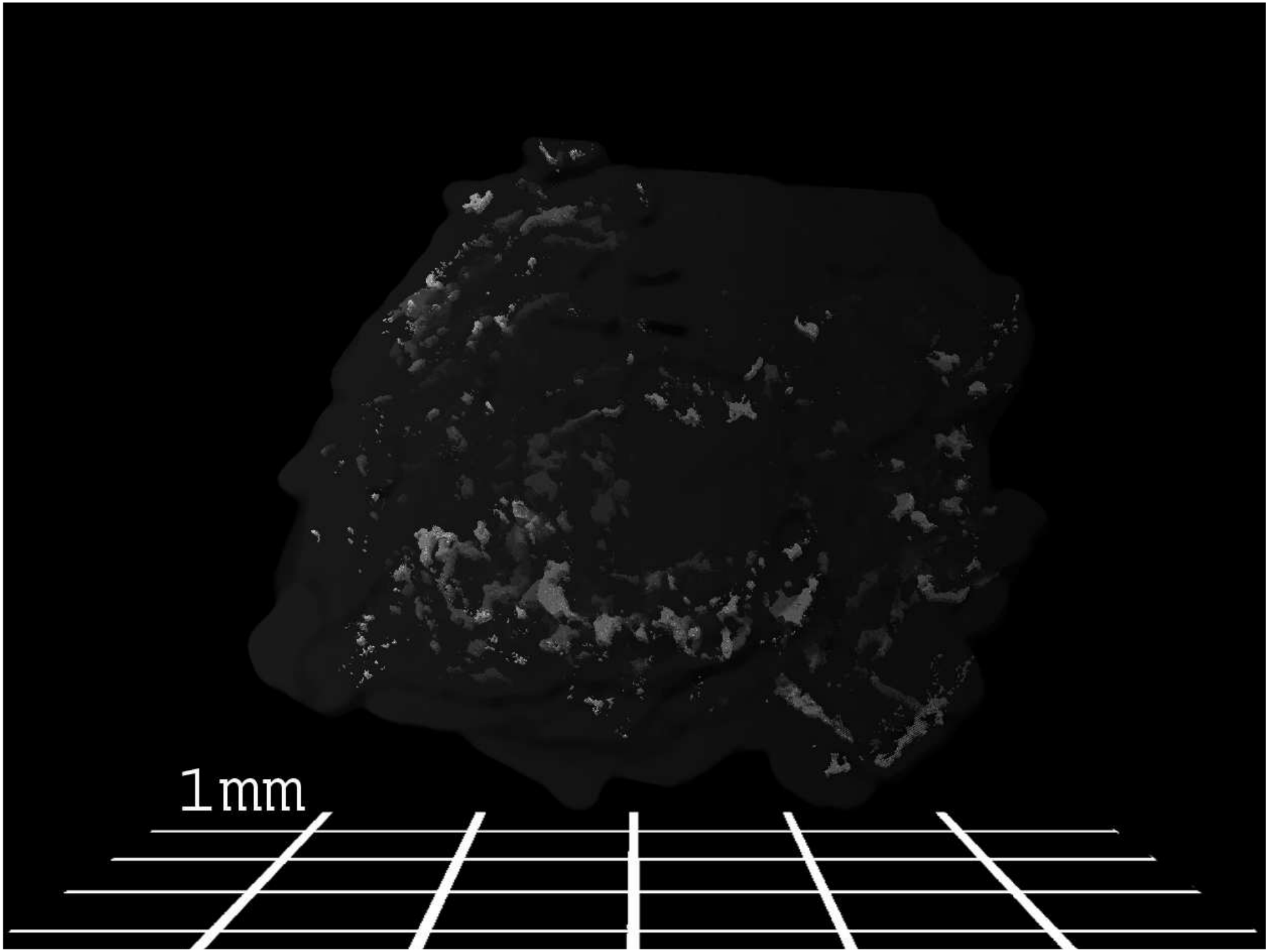
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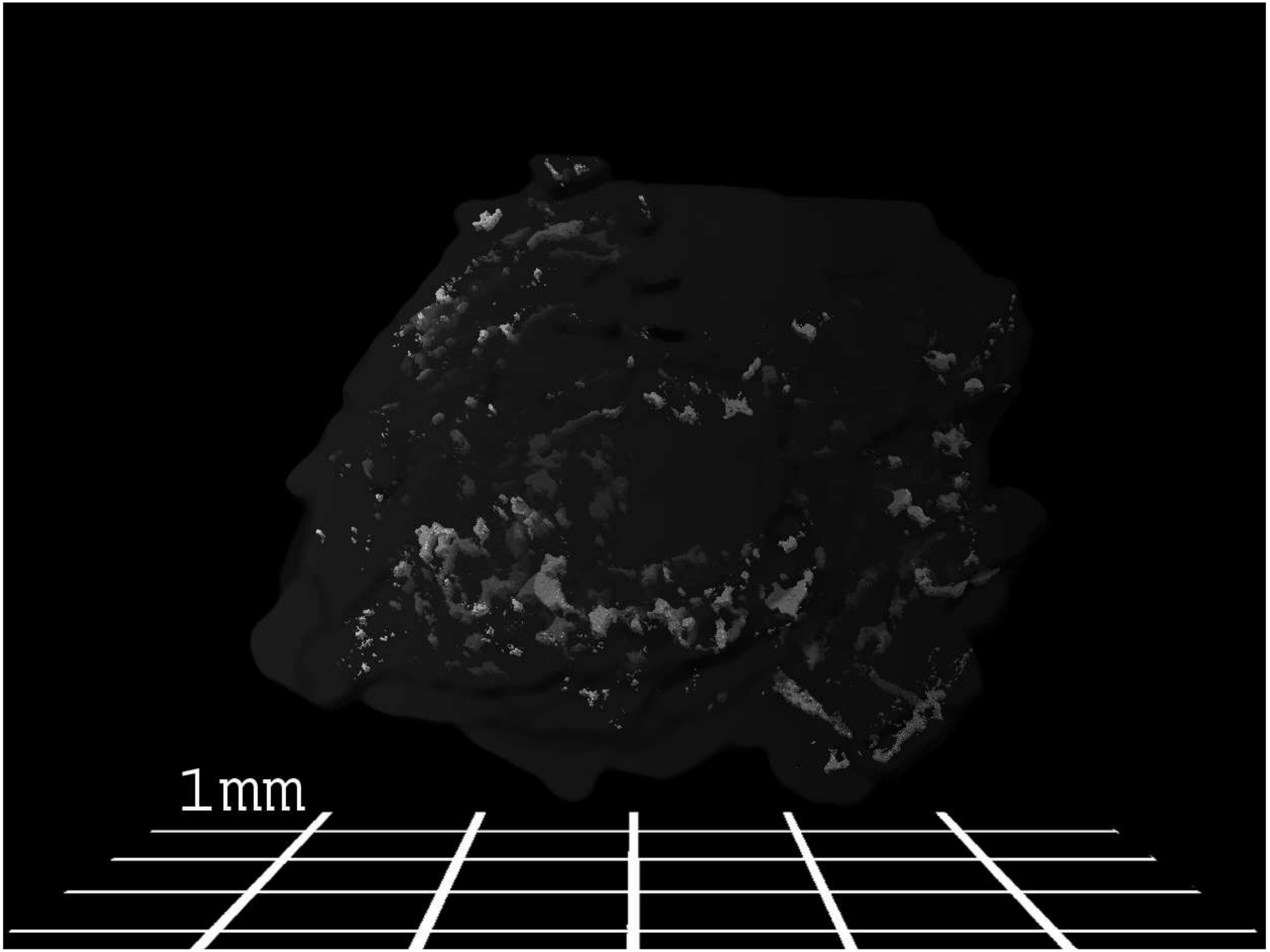
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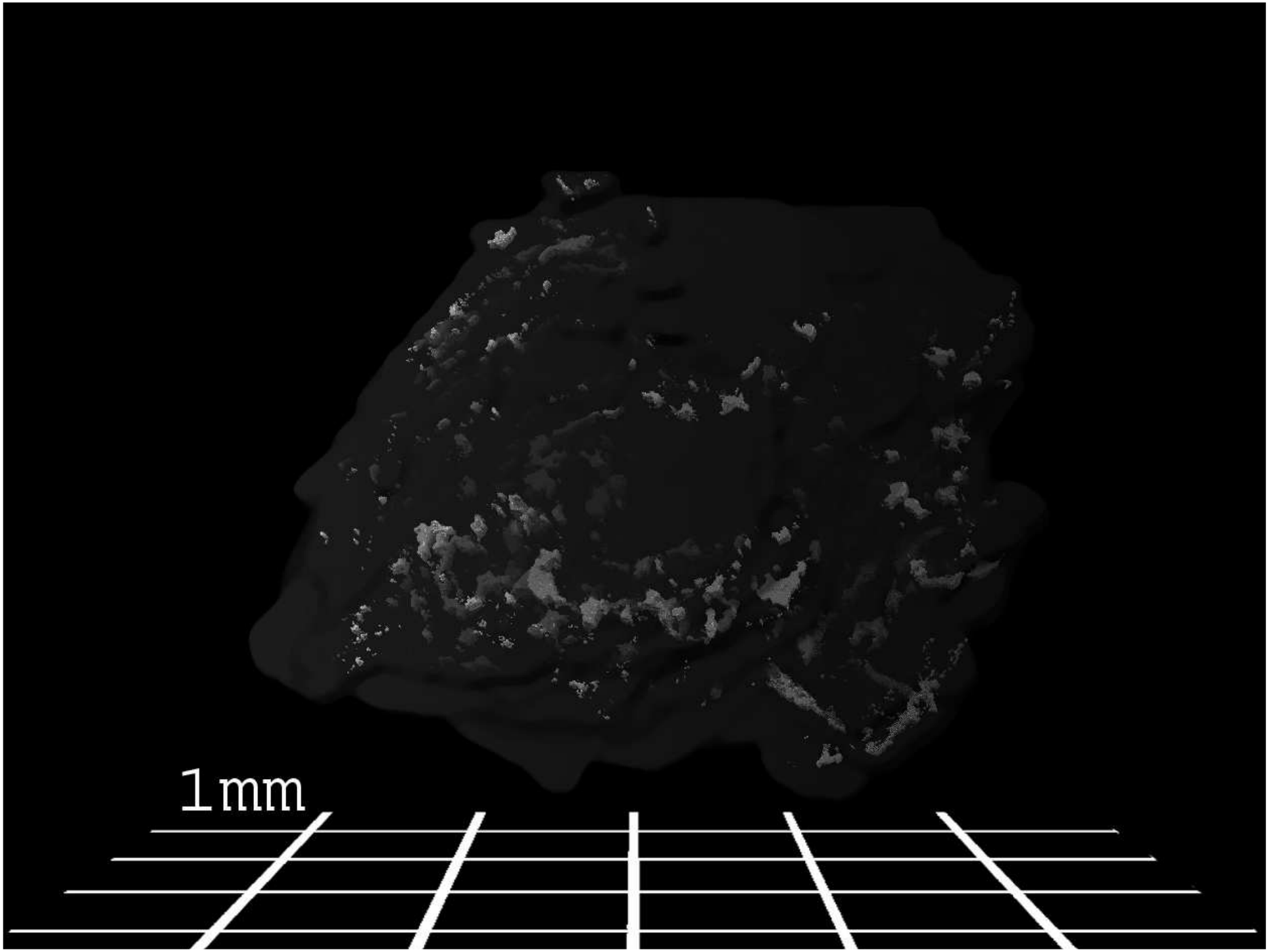
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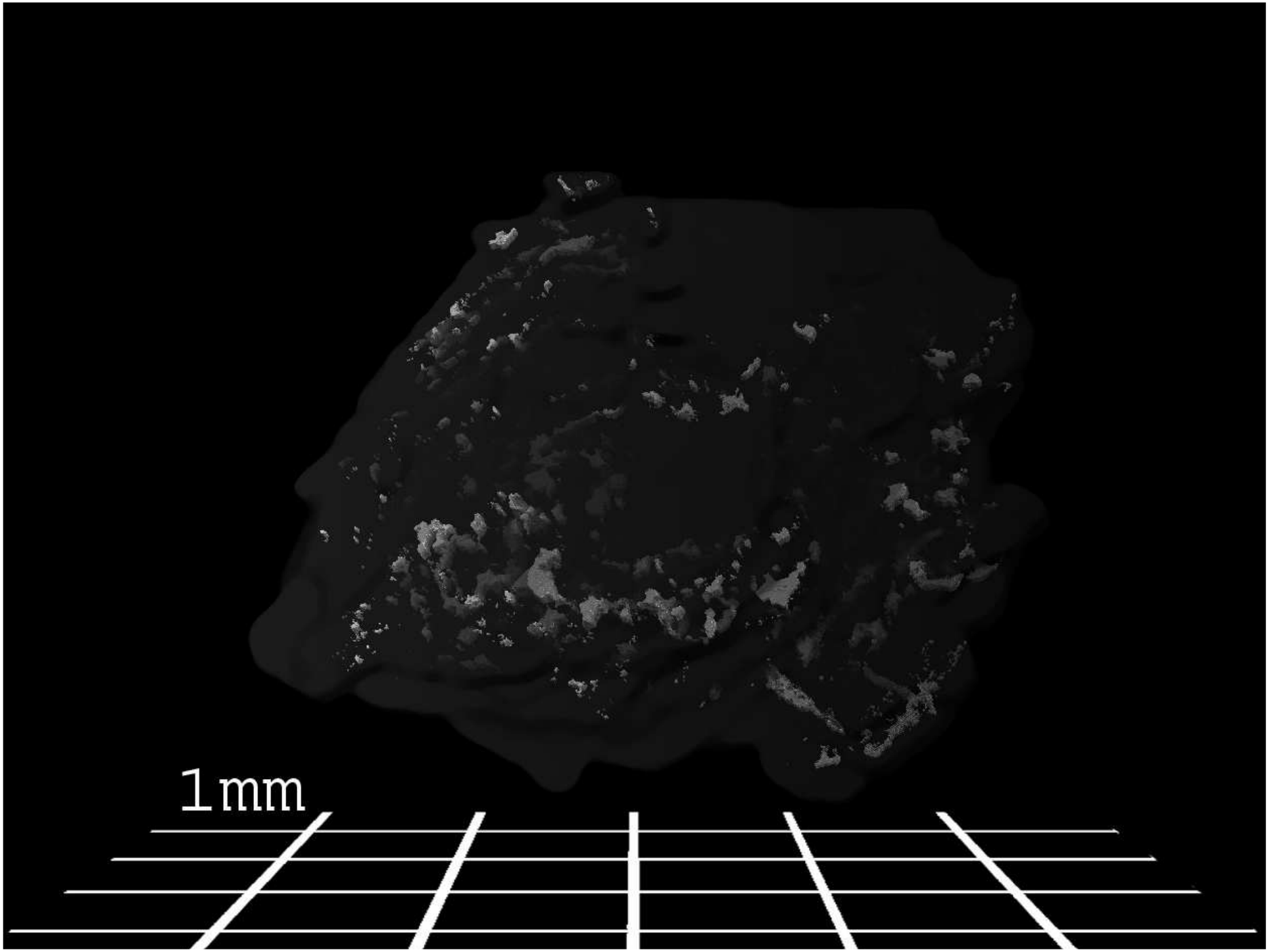
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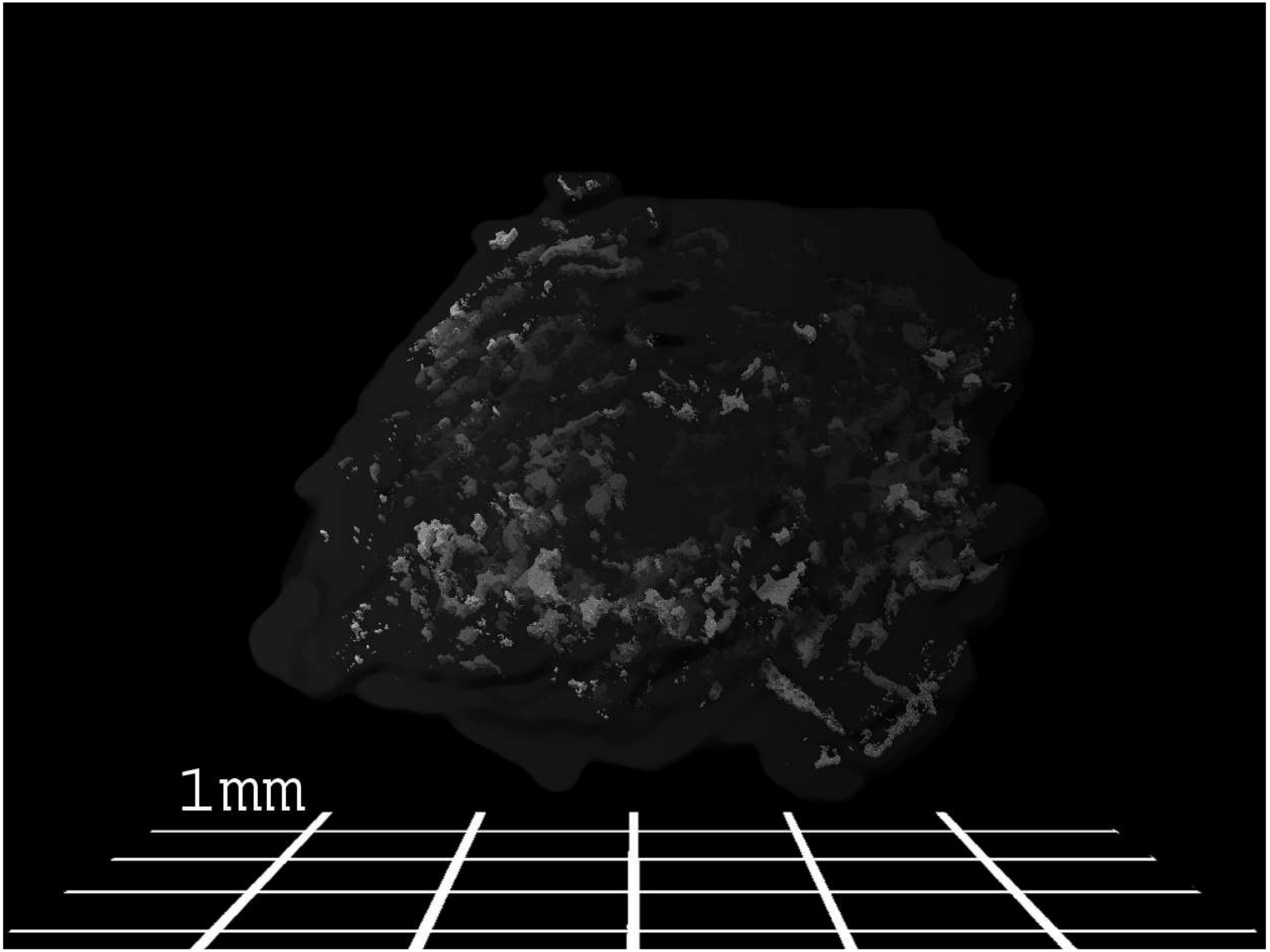
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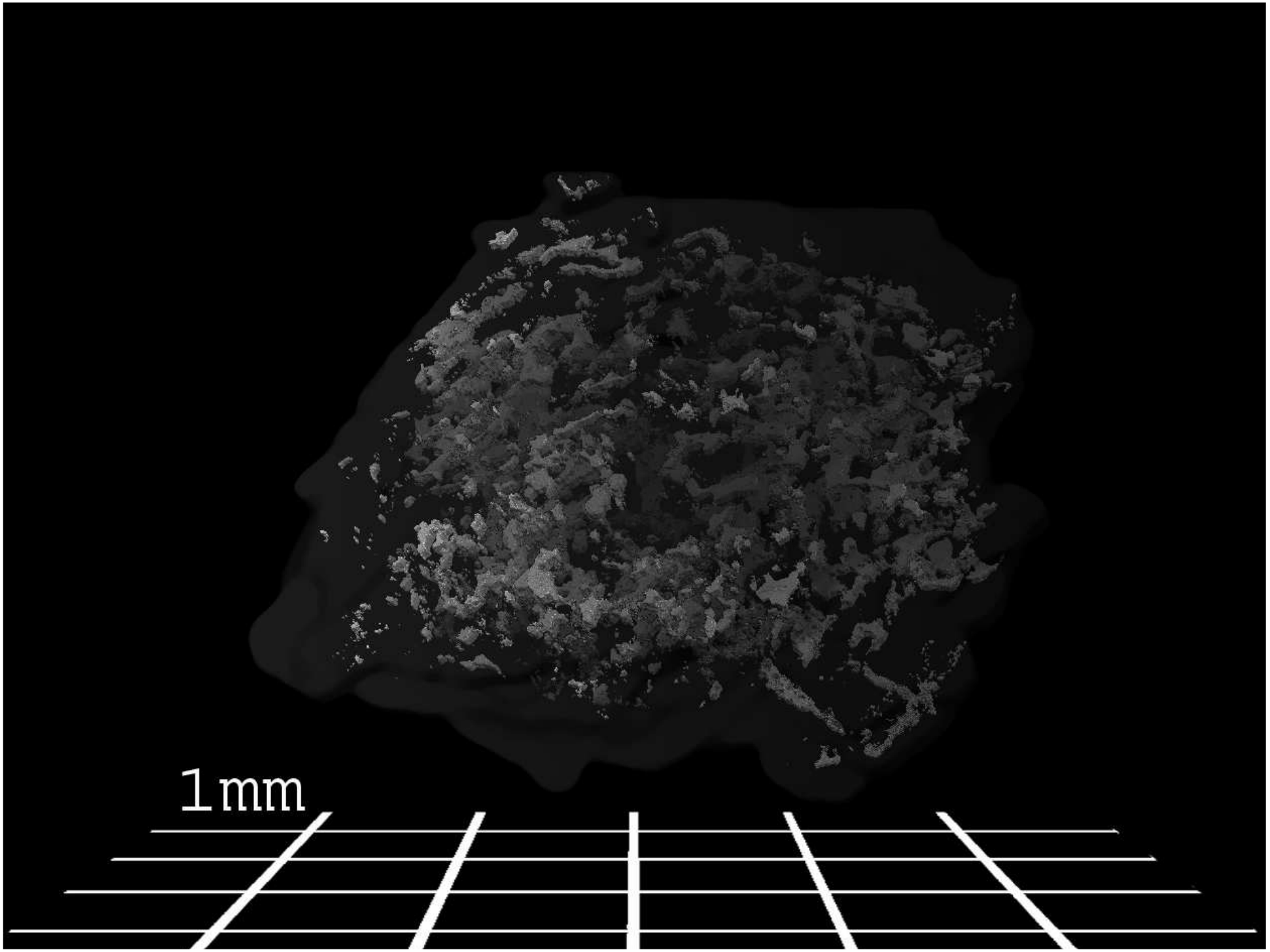
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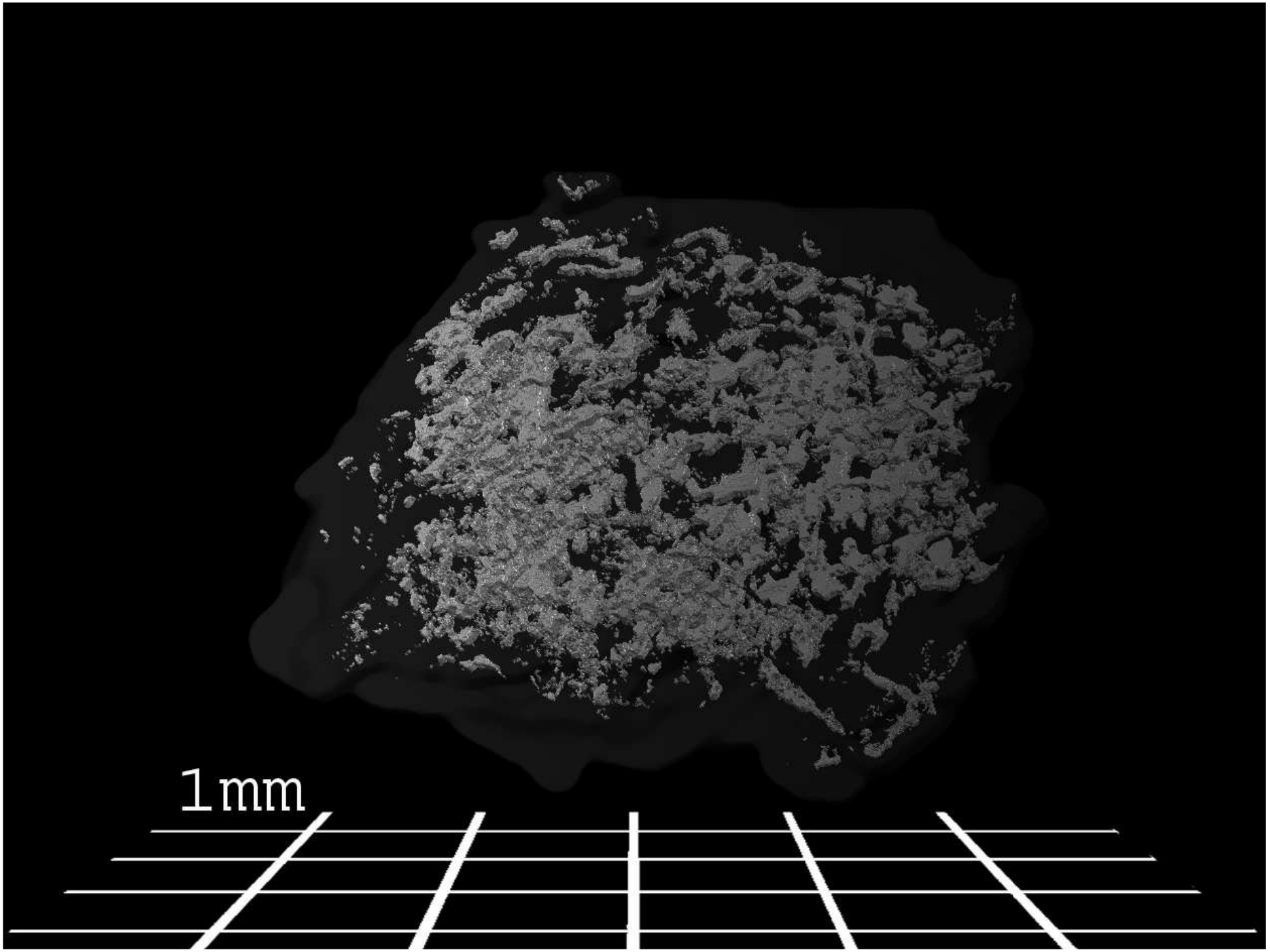
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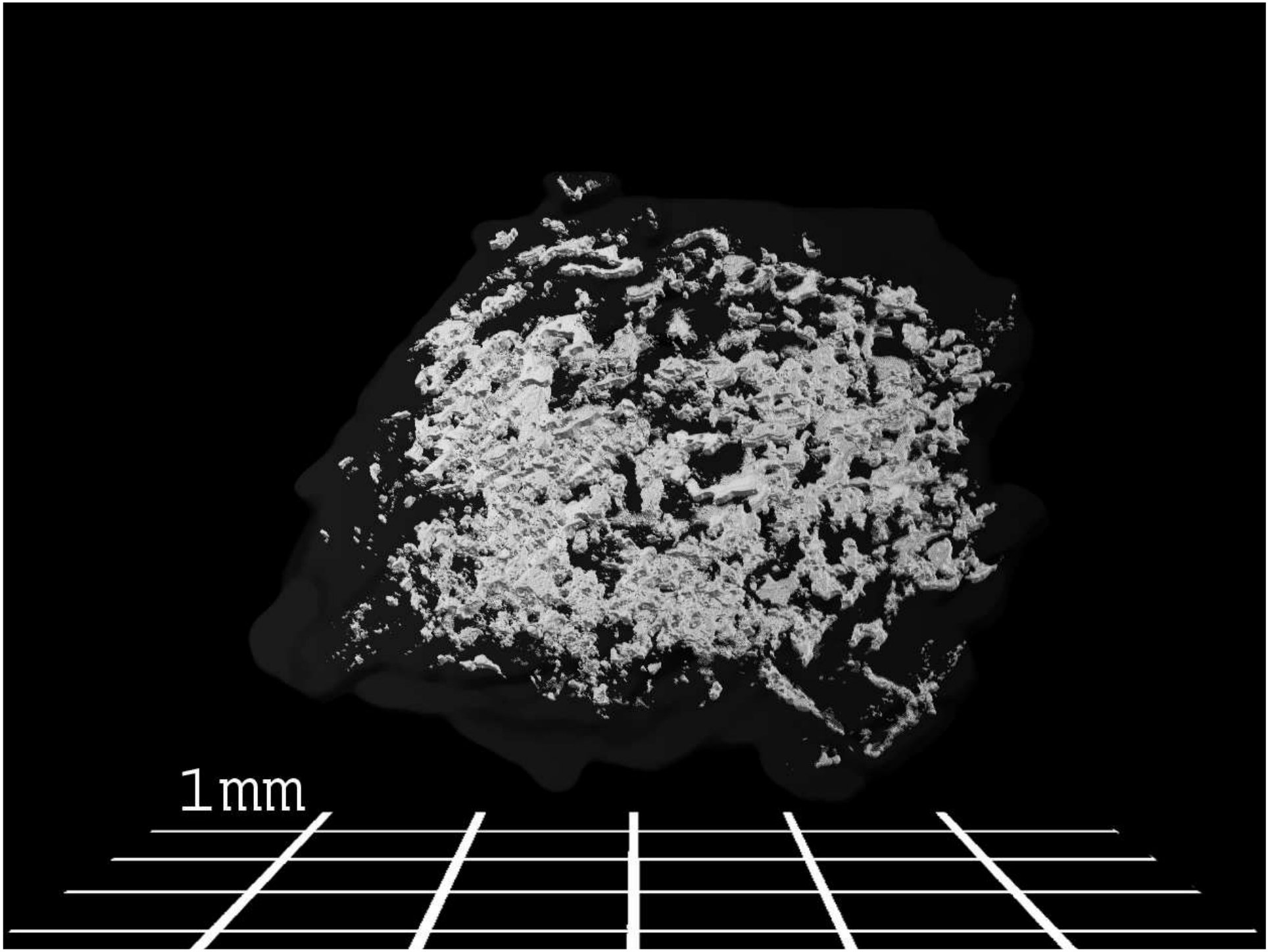
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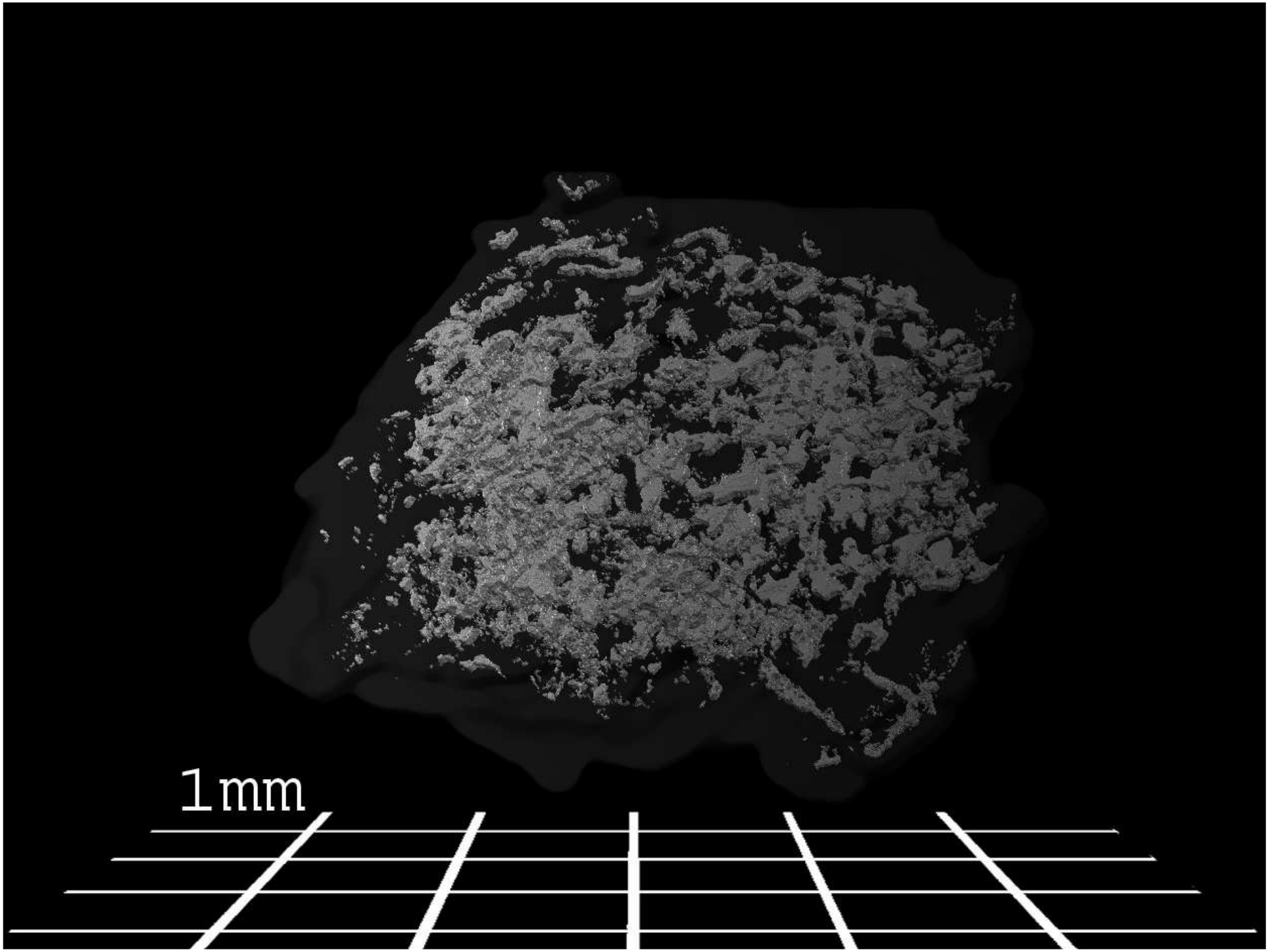
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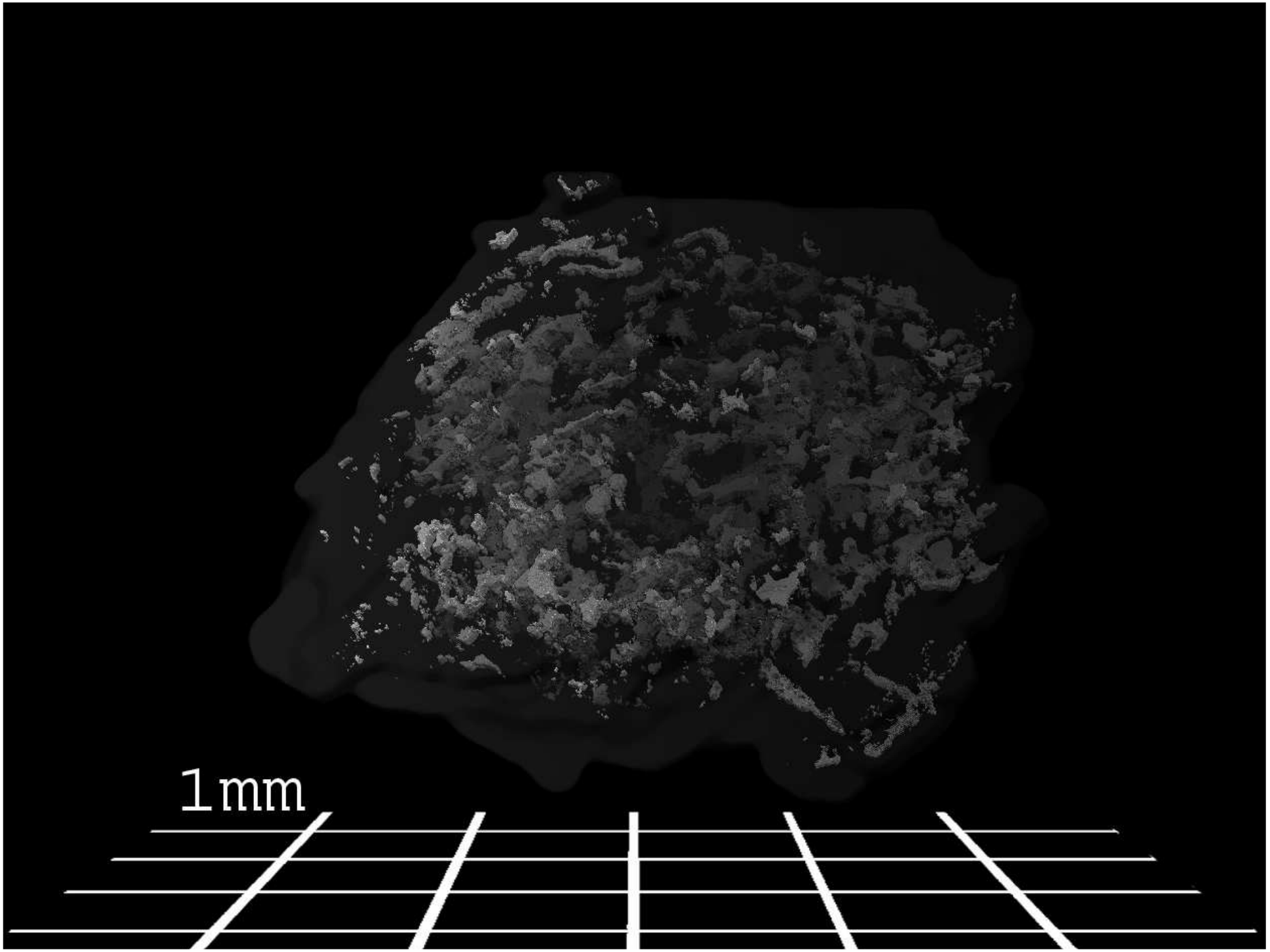
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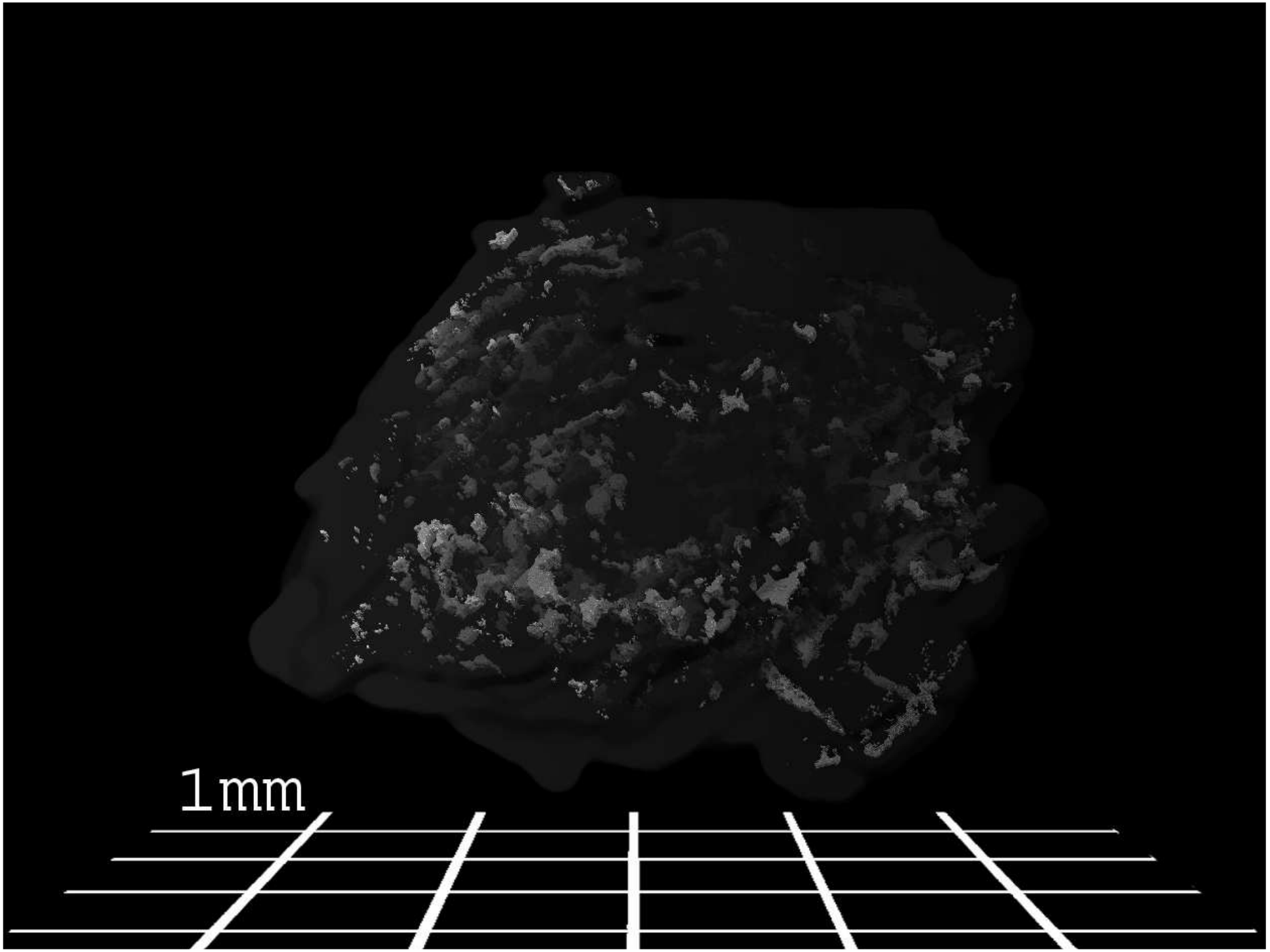
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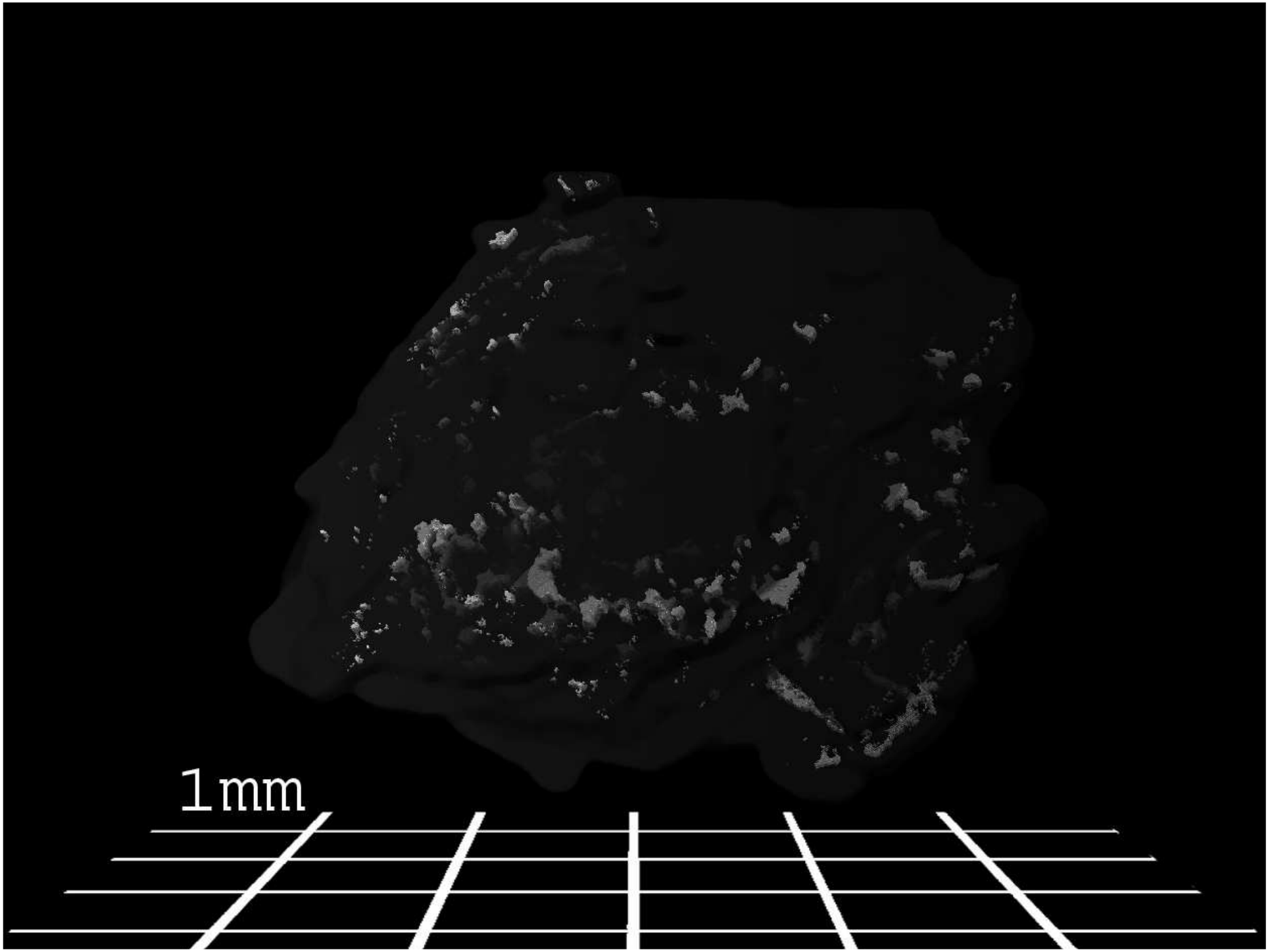
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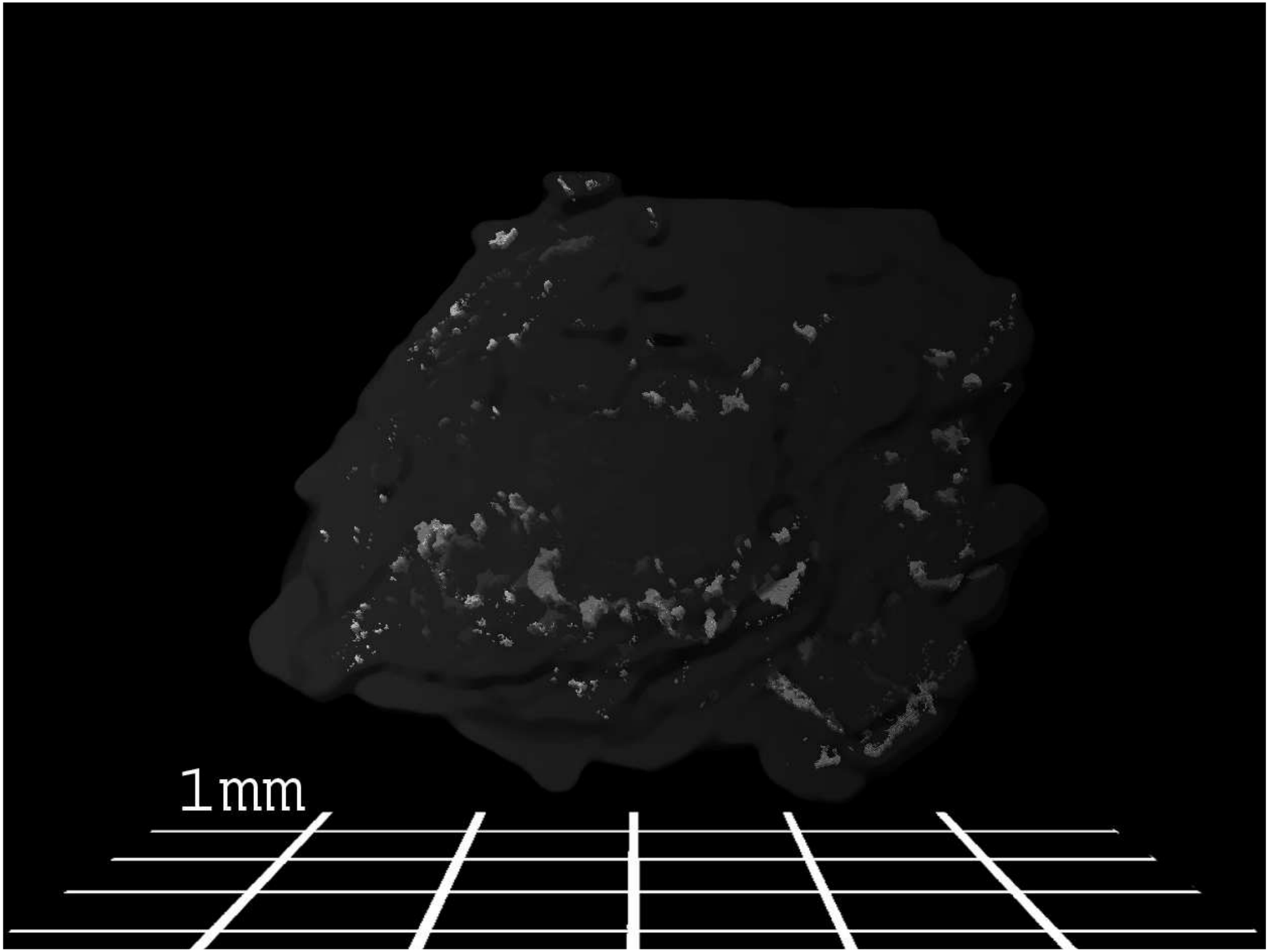
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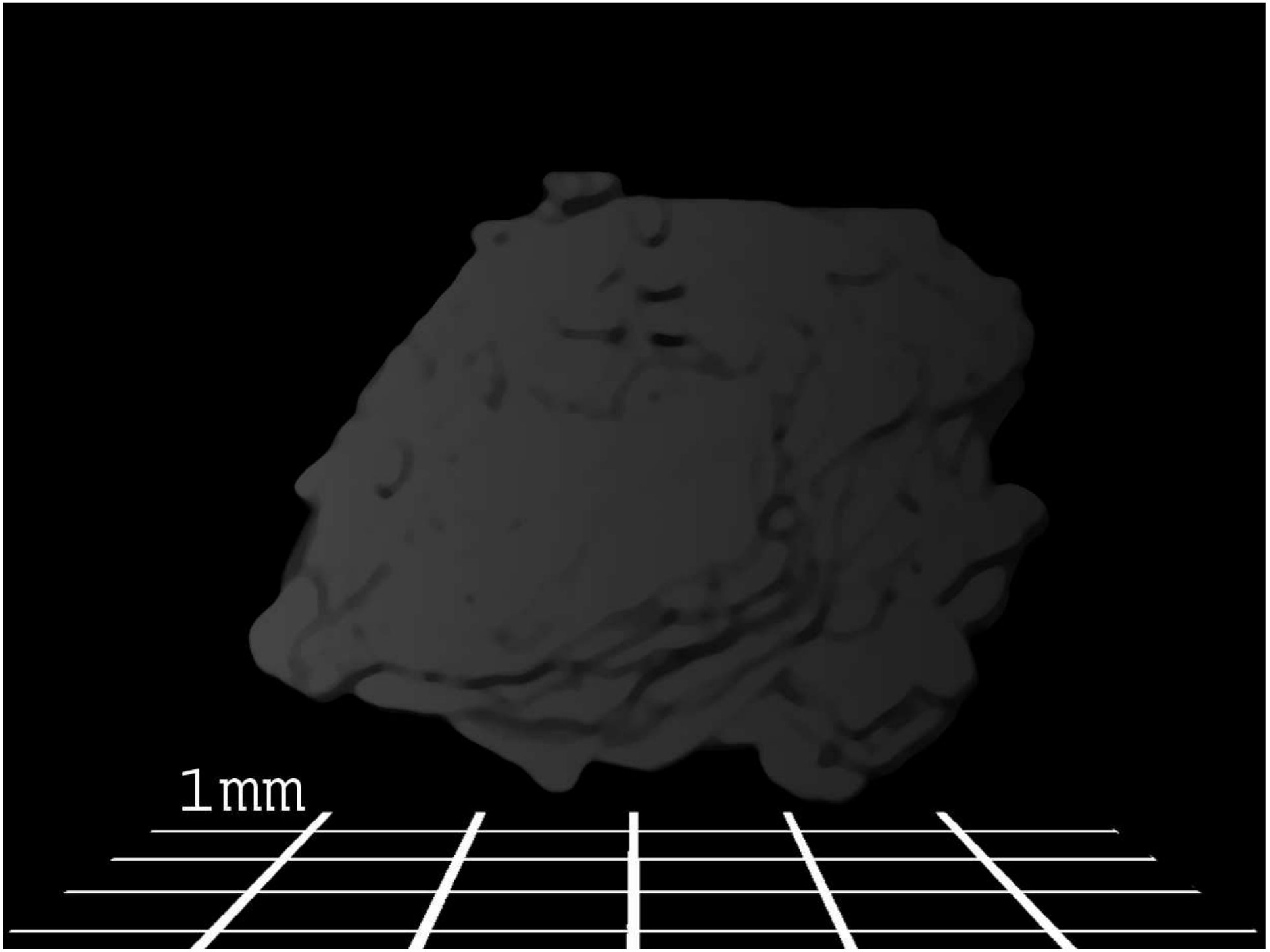
Focus, cluster or tumor lobe?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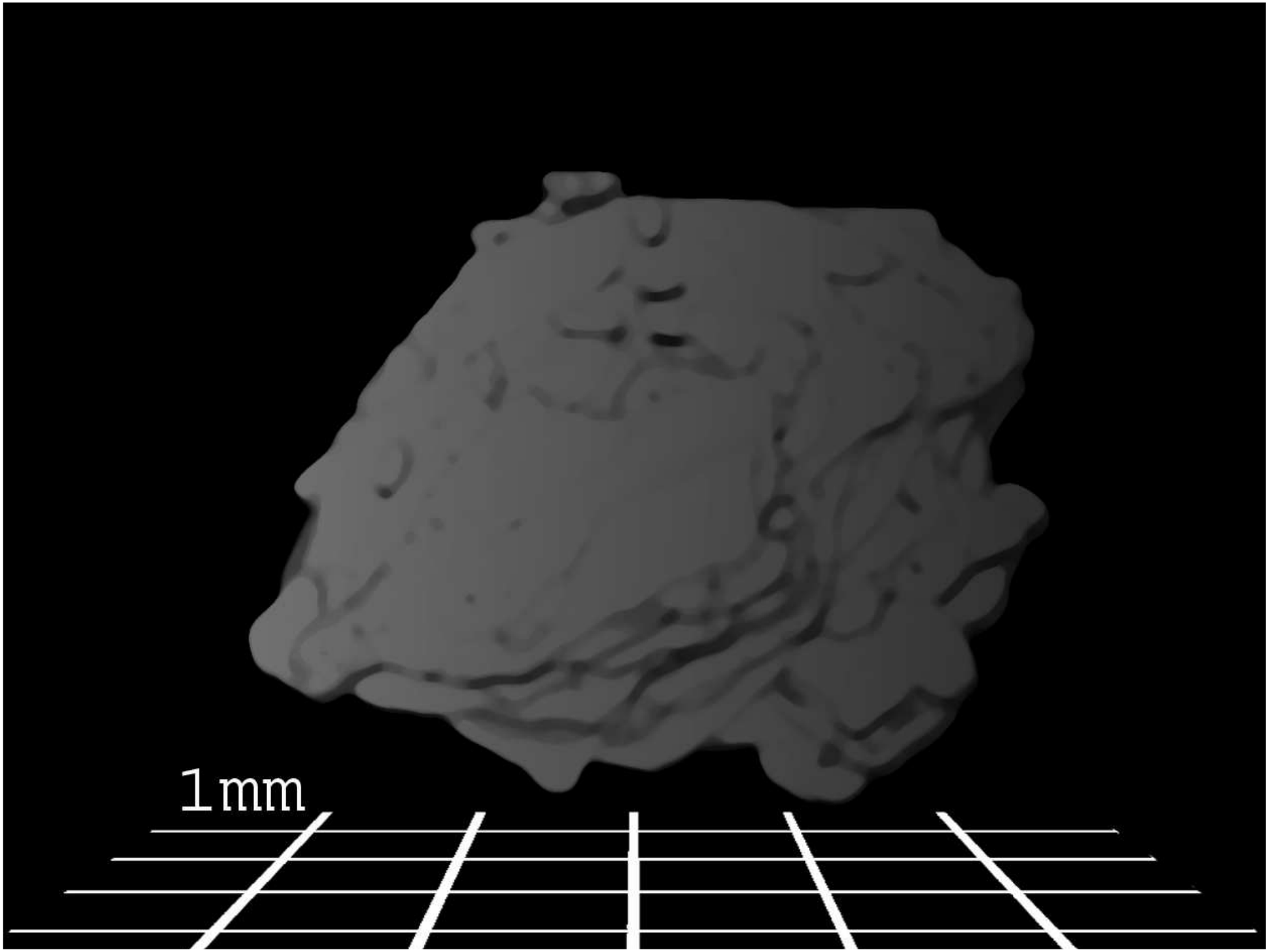
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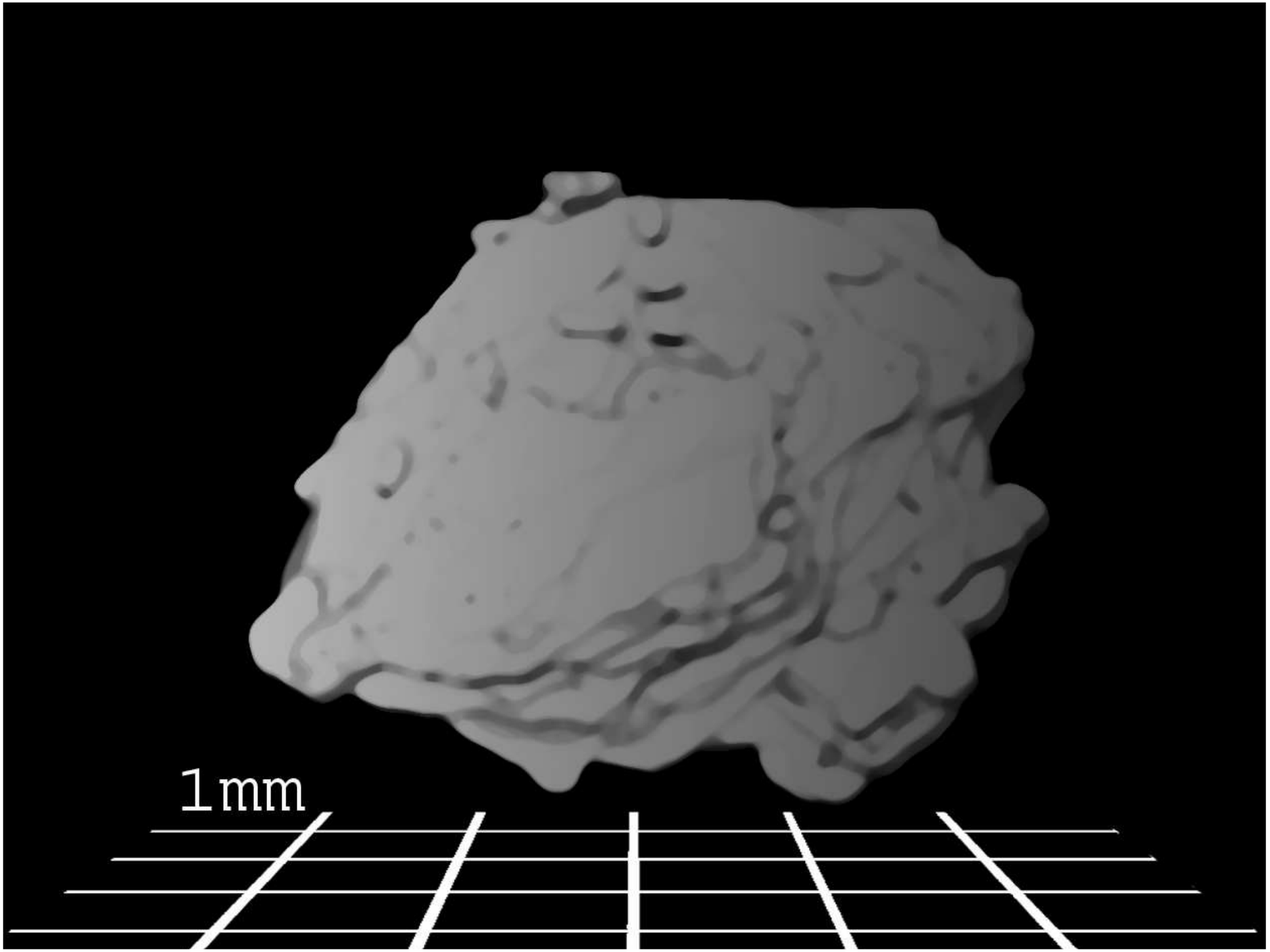
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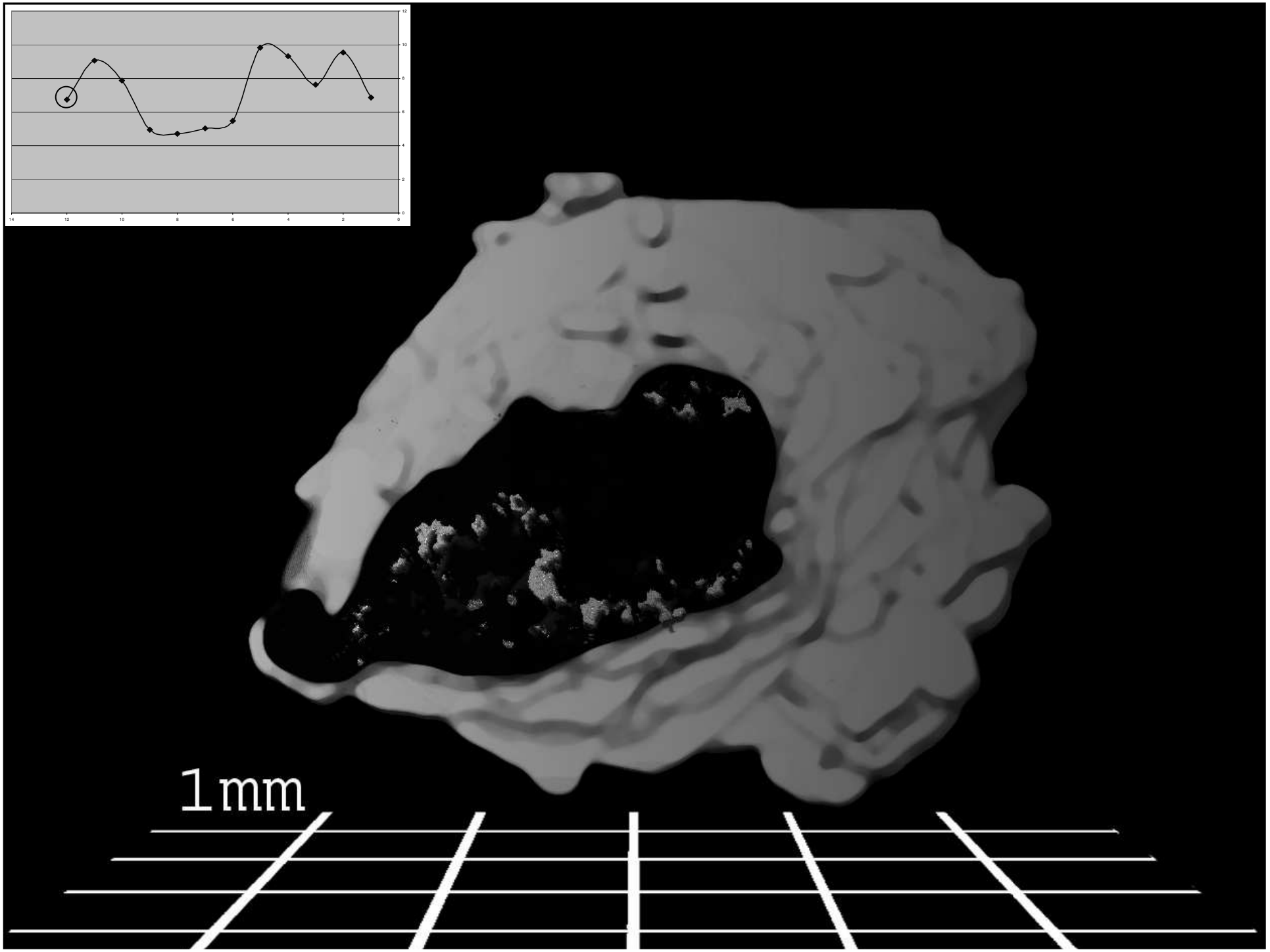
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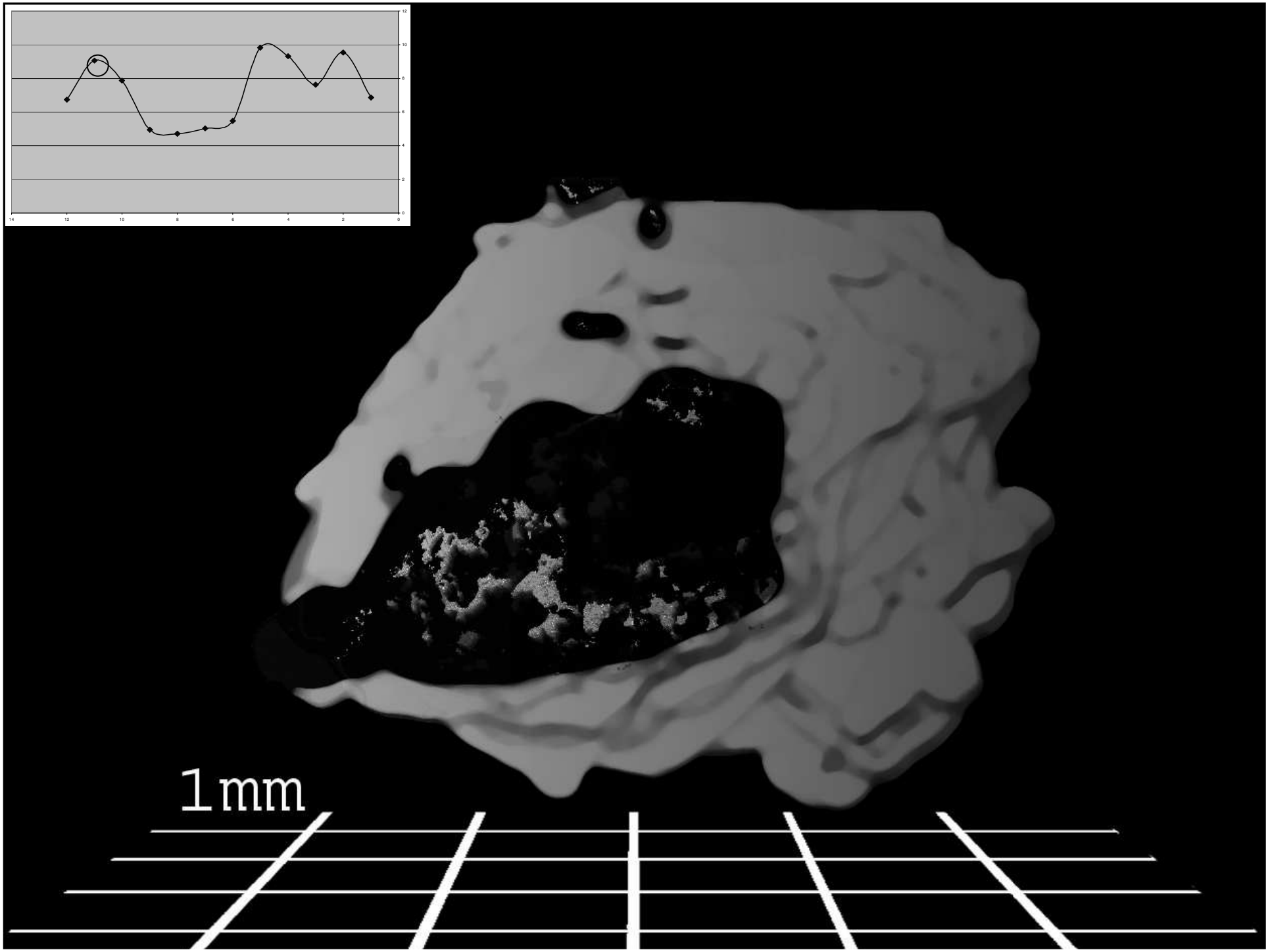
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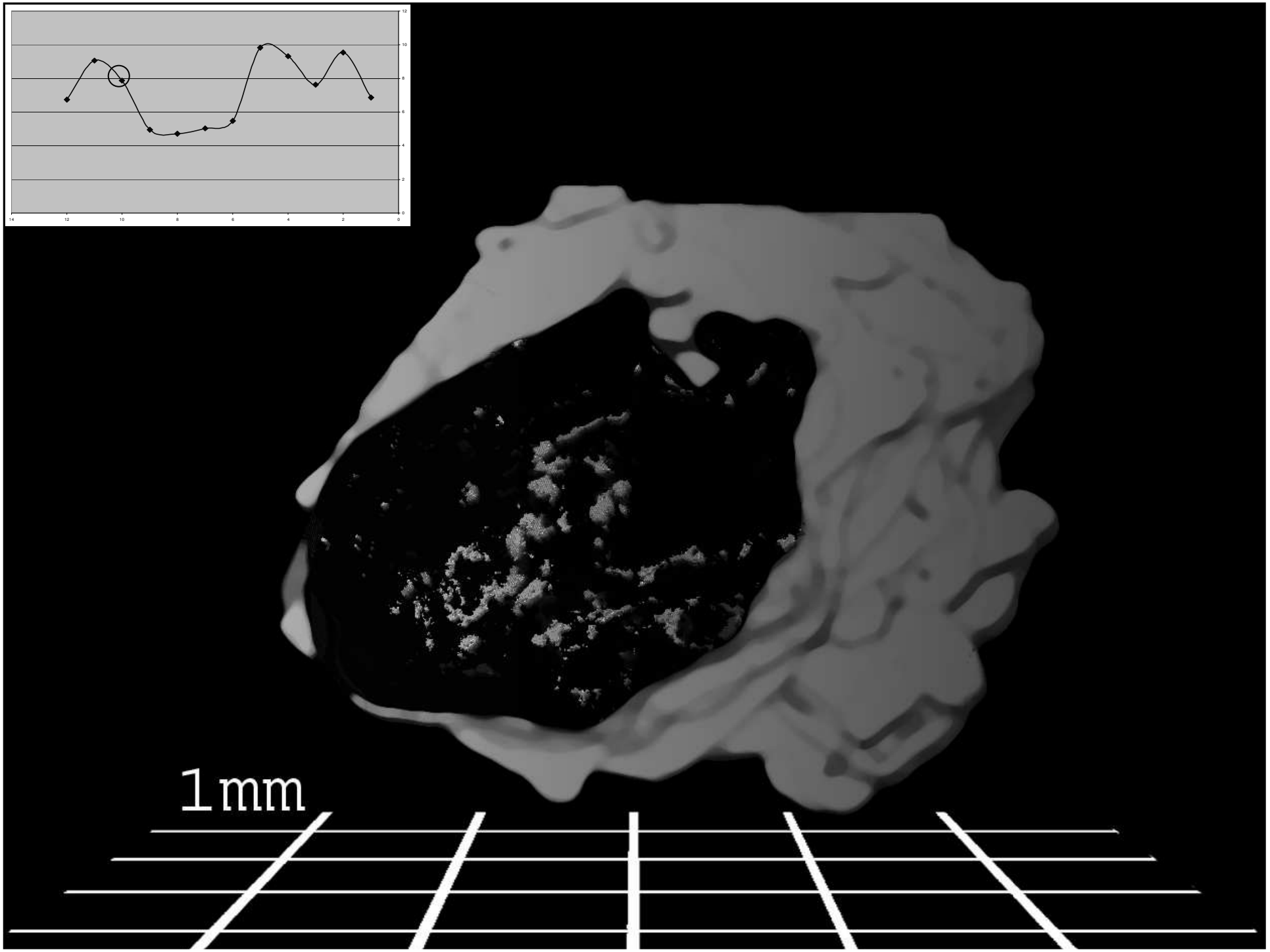
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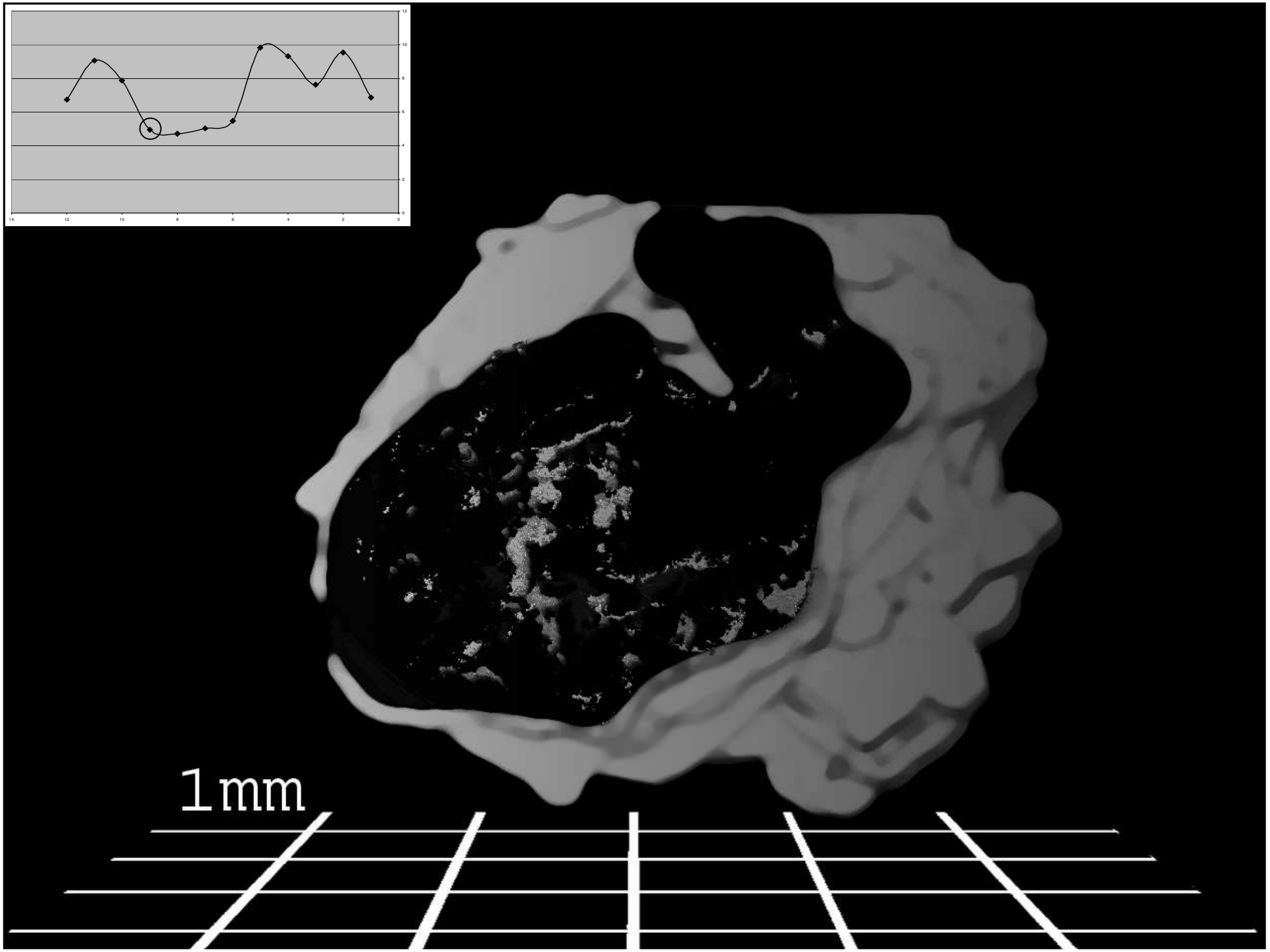
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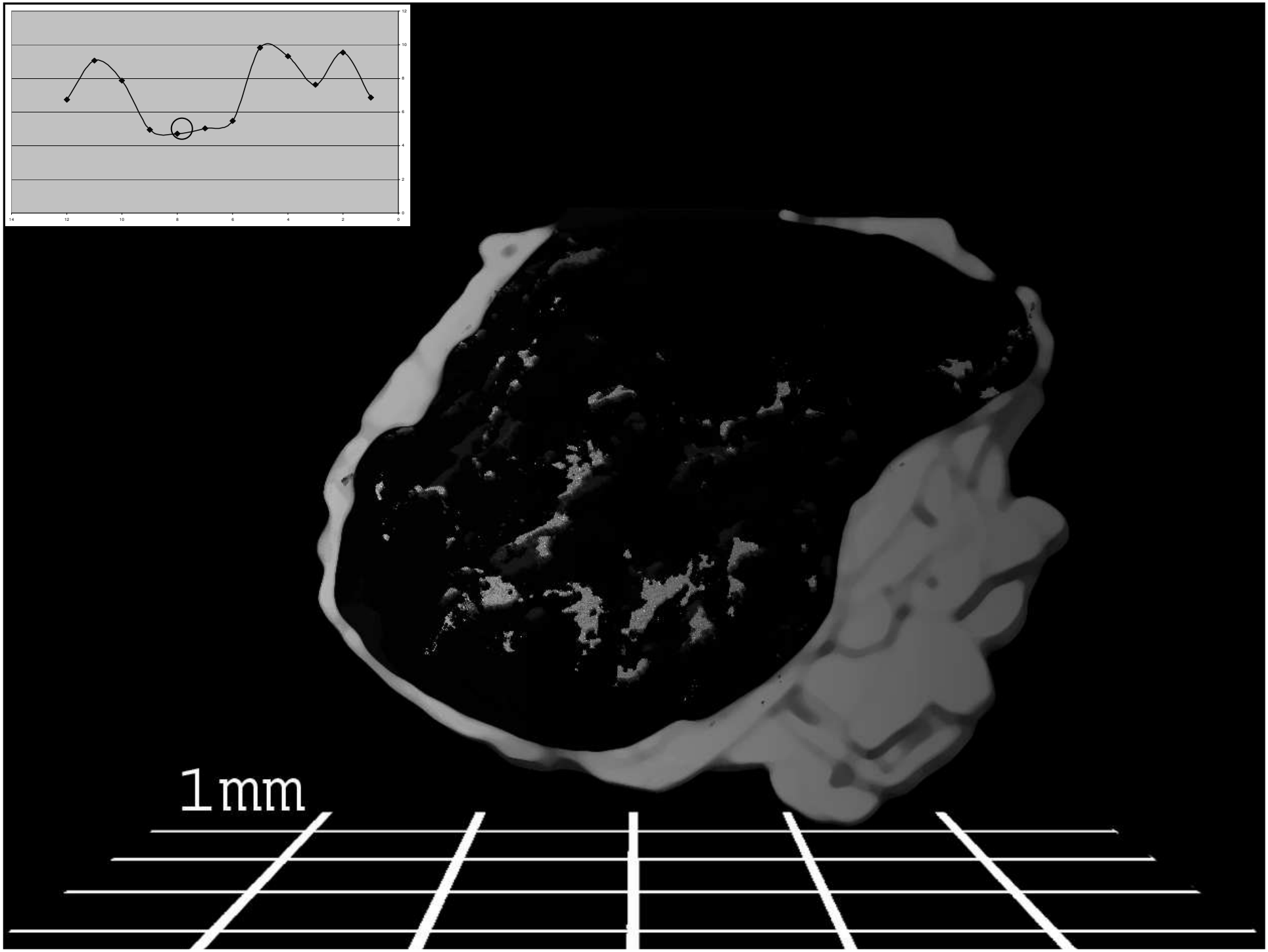
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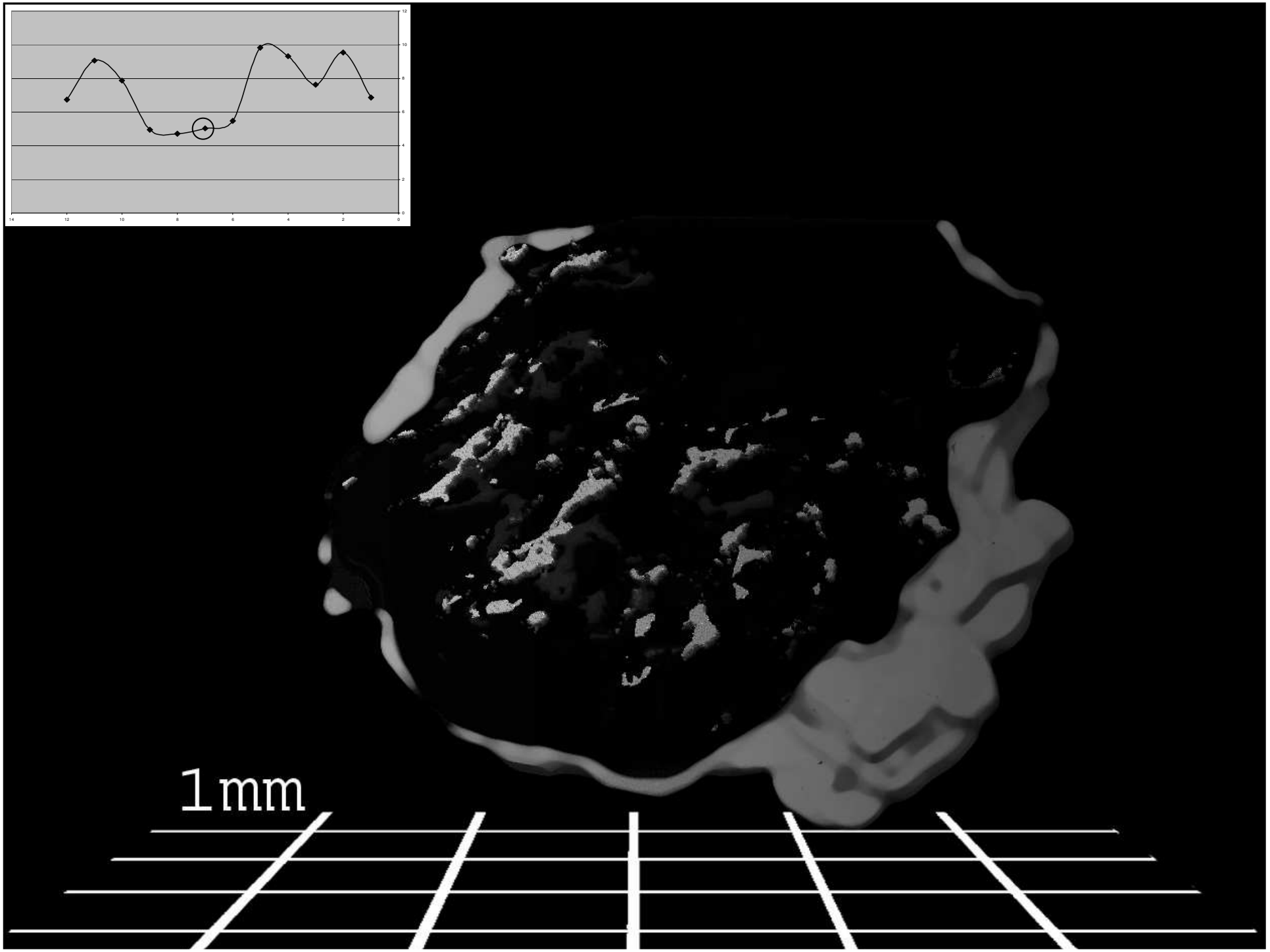
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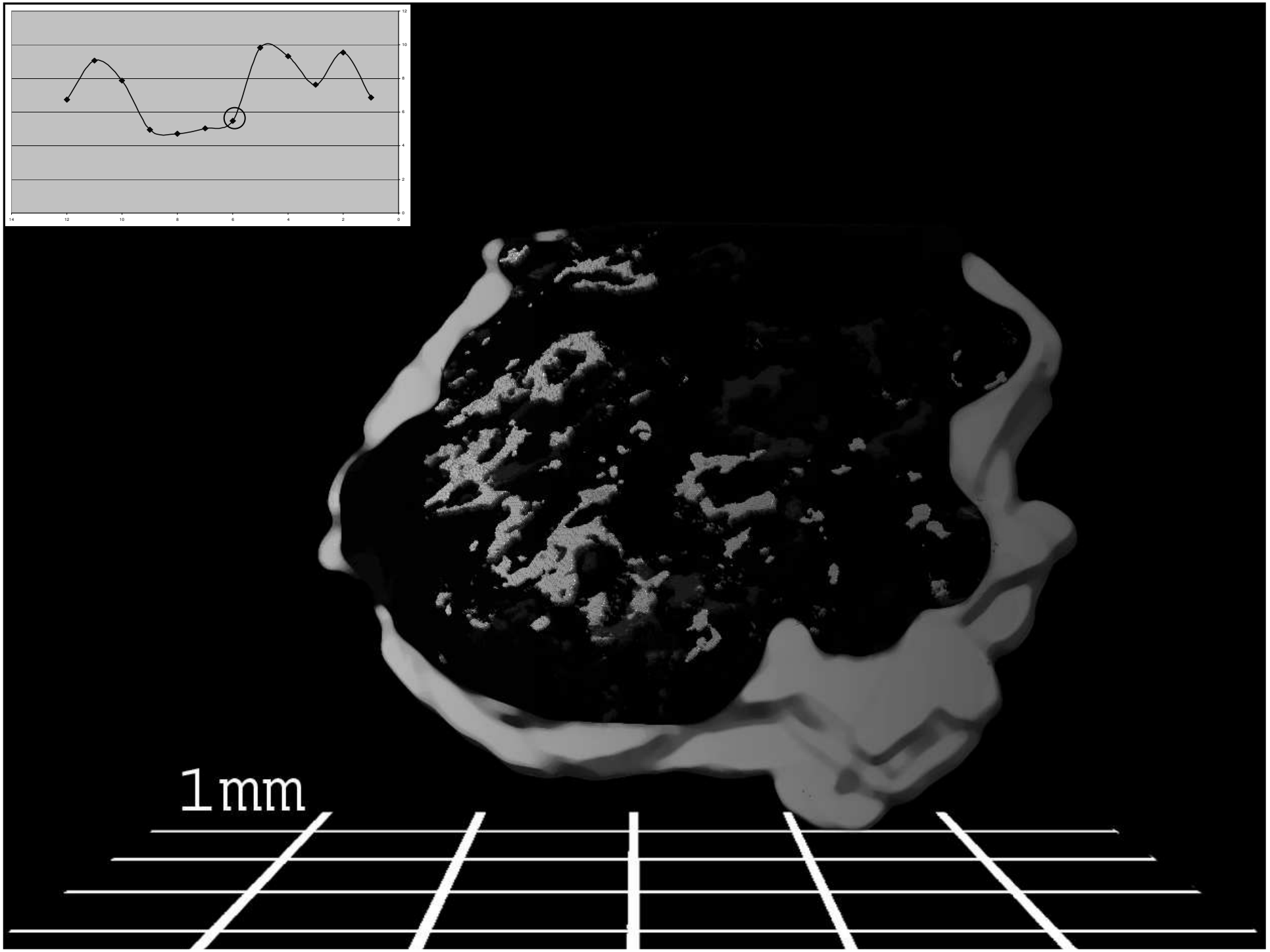
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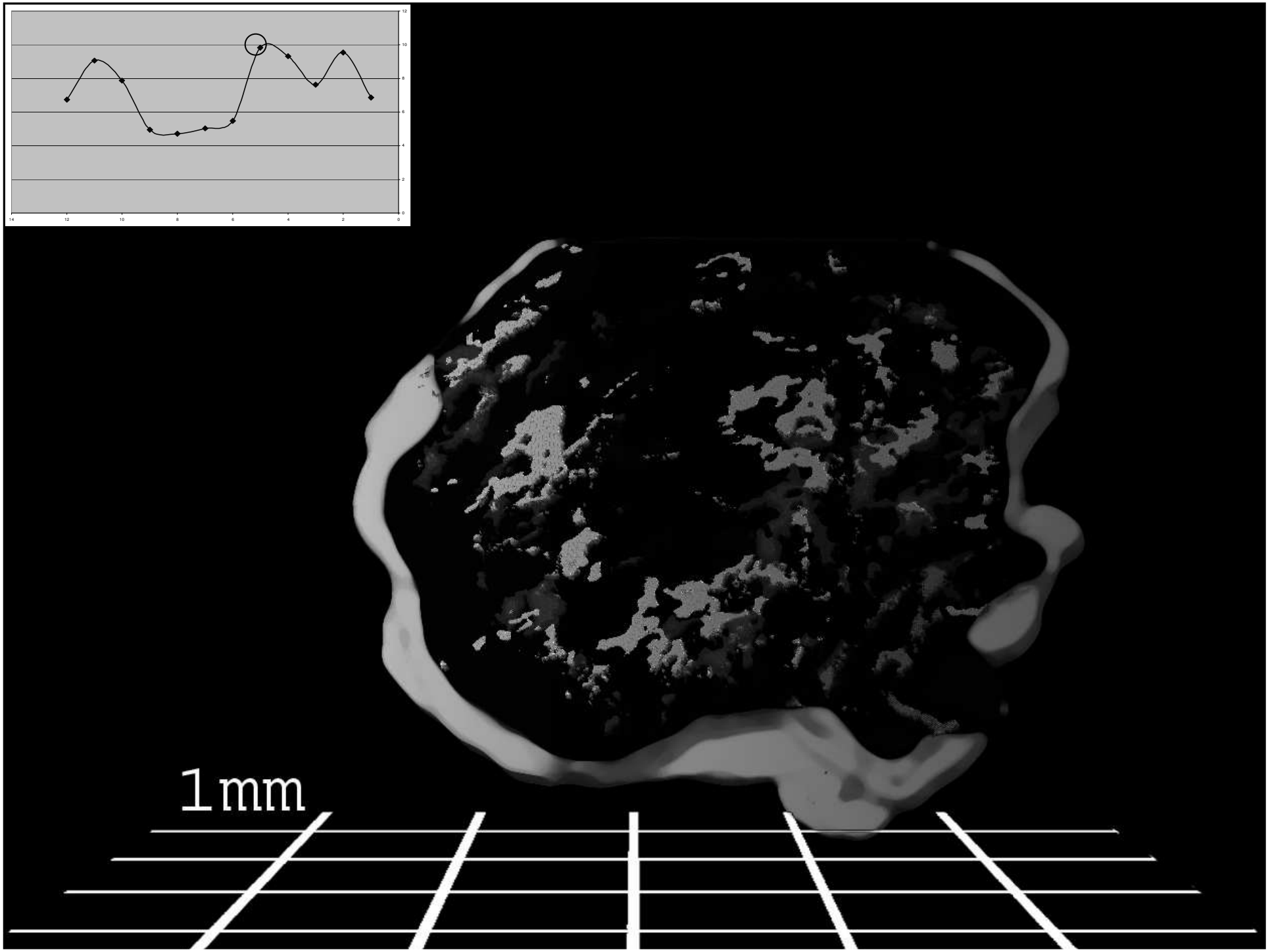
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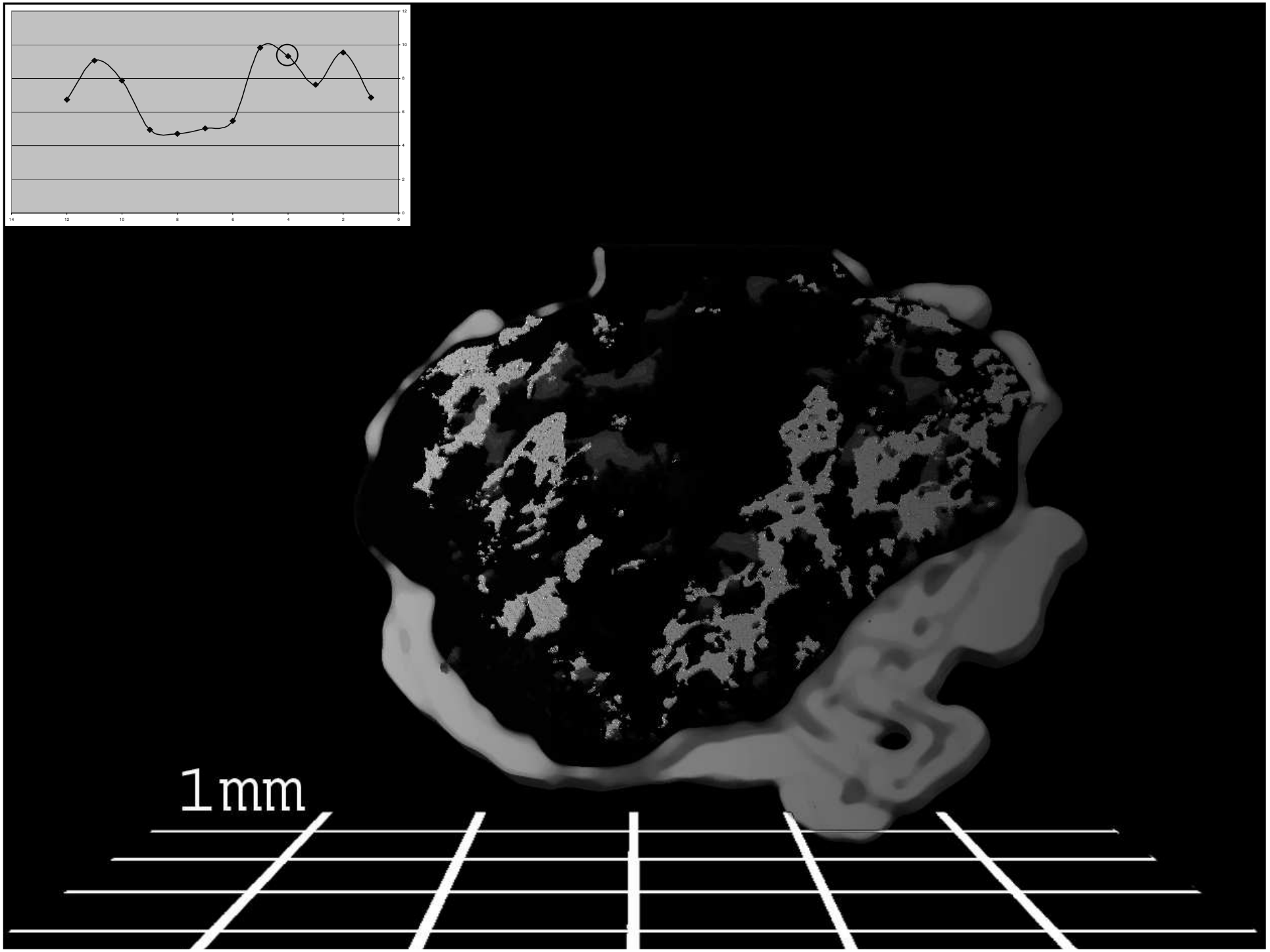
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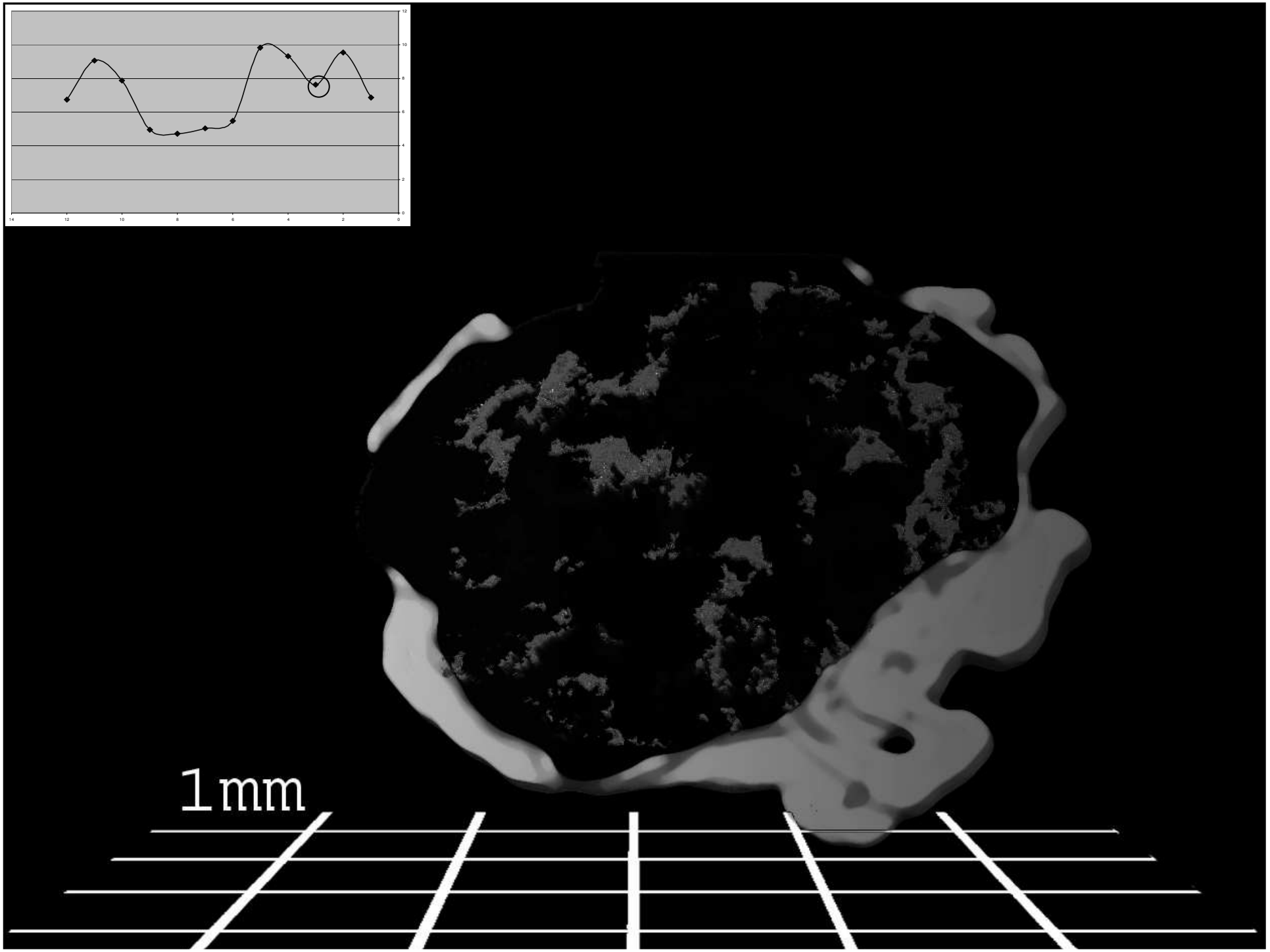
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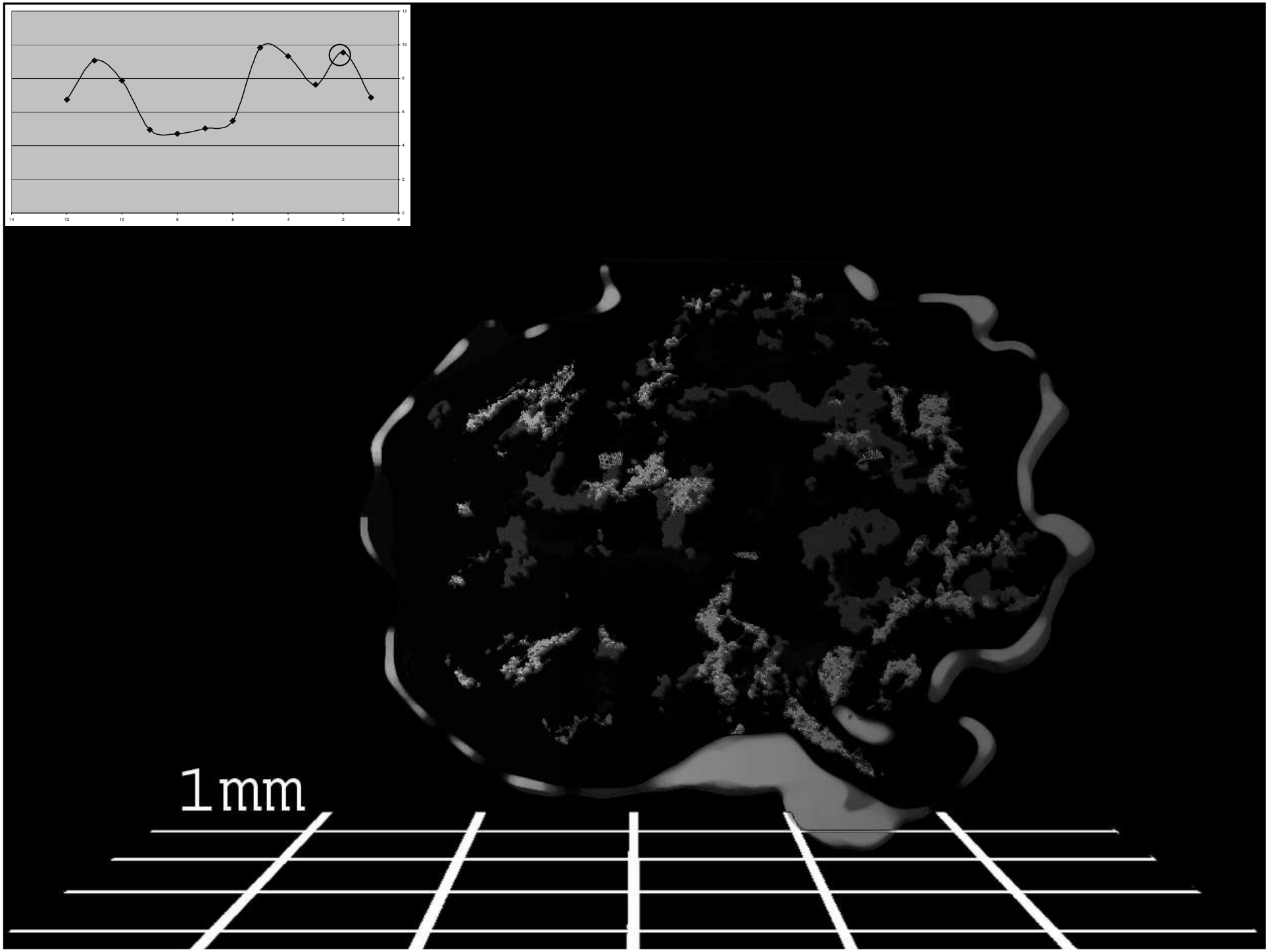
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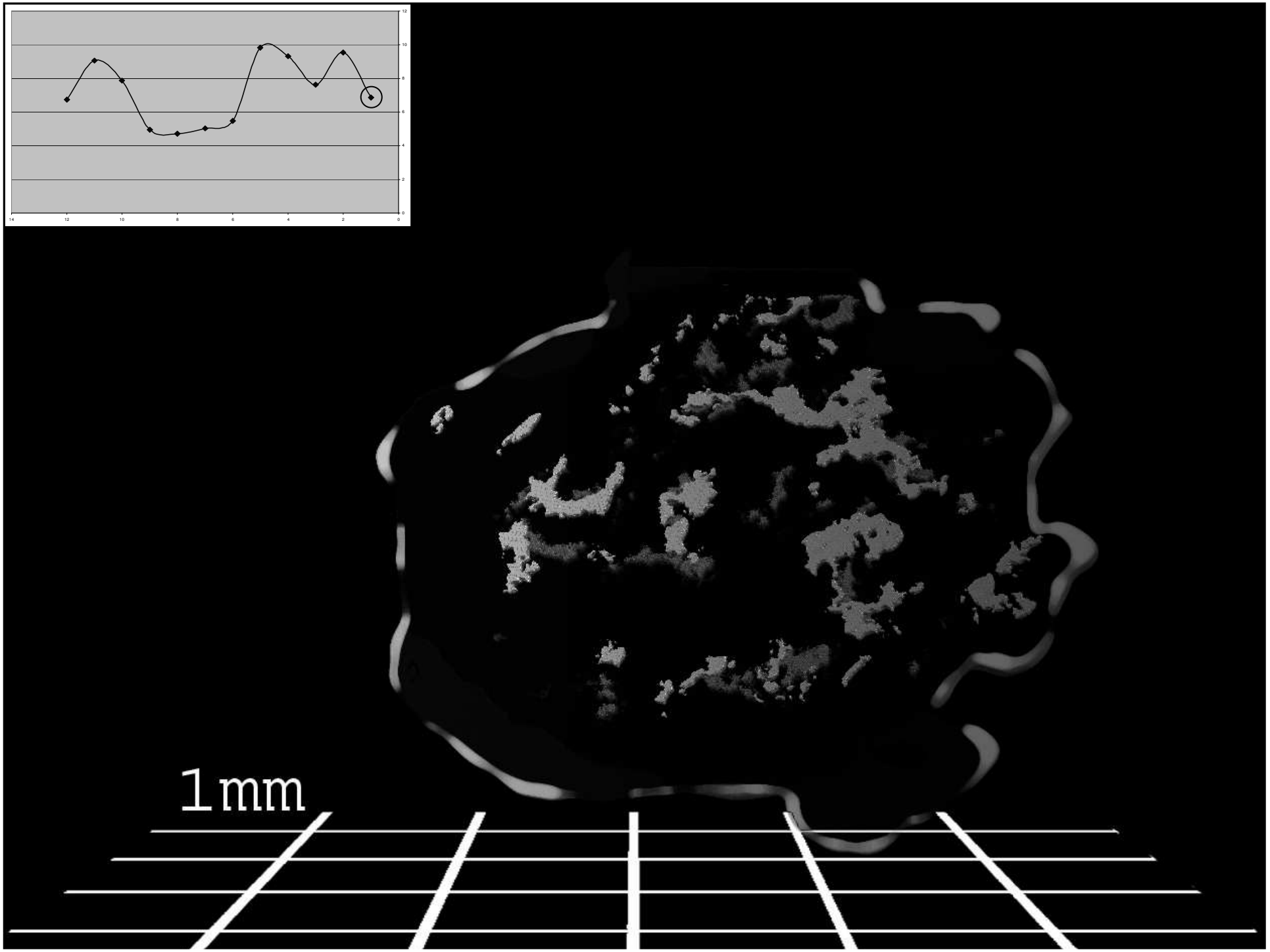
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What needs to be measured?

$$\textit{Sampling error} = \frac{d^3 f g c \beta}{\text{Mass of sample}}$$

- *d* – size of the largest staining unit
- f - shape factor (irregular *f*=0.25)
- *g* – size distribution factor (wide size distribution *g*=0.25)
- c – constitutional factor ~ density */ percentage*
- *β* – liberation factor which is =1 in our application since we do not homogenize tissue

What needs to be determined for each marker/tumor type combination?

- *d – size of the largest units, distributional heterogeneity*

-important for substances with sharp demarcation and homogenous distribution within positive areas, “ON-OFF” scenario

-foci, clusters….?

-needs work to establish reproducible methods

- *c – constitutional factor ~ density / percentage of distribution*

-important for constitutionally heterogeneous substances which are present in all cells at a different degree

-foci designation, density measurement?

-needs work to establish reproducible methods

How much is enough?

$$\text{Mass of sample} = \frac{\textit{Sampling error}}{0.06 \times d^3 c}$$

When d and c are known for a specific substance/tumor type combination, we can set a desired error and calculate needed size of a tissue sample

Thanks!

- Statistics

  -**Melania Pintile**

- David Hedley lab

  -**David Hedley**

  -Andrew Morrison

- Dick Hill lab

  -Dick Hill

  -Naz Chaudary

- Histology

  - Jing Jung
  - James Ho