

Project Goal

I wanted to get my hands dirty with an industry standard toolbox for 2-photon image analysis and work with real data. Through this, I hope to show my proficiency with image analysis and python programming along with my willingness to approach problems head-on. I followed the official [CalmAn documentation](#) to perform this analysis, but had to adapt the code to my conditions.

In [1]:

```
import imageio.v2 as imageio
from pathlib import Path
import matplotlib.pyplot as plt
import numpy as np
import cv2
from tqdm import tqdm
from bokeh.io import output_notebook
```

In [2]:

```
import caiman as cm
from caiman.motion_correction import MotionCorrect
from caiman.source_extraction.cnmf import cnmf, params
from caiman.utils.utils import download_demo
from caiman.utils.visualization import plot_contours, nb_view_patches, nb_pl
from visualization import nb_view_quilt
```

In [3]:

```
output_notebook()
```



Loading BokehJS ...

Dataset Description

This data was collected using a 2-photon microscope (B-scope; Thorlabs, USA). It shows GCaMP6f expression from the Retrosplenial cortex of 5 animals over 7 days. In this study, I will be working with data from the 7th day. The animal of interest was not indicated in the dataset's description. [Sun, Weilun \(2021\), "In vivo two-photon calcium imaging dataset_Day 7"](#), Mendeley Data, V1, doi: 10.17632/hjgtsmyyv5.1

In [4]:

```
num_animals = 5
num_frames = 1500
```

In [51]:

```
def build_image_dict(num_animals, num_frames):
    image_dict = {}
    for i in range(1, num_animals + 1):
        path_name = f"./Animal_{i}"
        input_path = Path(path_name)
        image_list = sorted(input_path.glob("*.tif"), key=lambda x: f"{int(s
        images = []
```

```

        for im_path in image_list[:num_frames]:
            images.append(imageio.imread(im_path))
        # Reshape into (Time x Height x Width).
        image_dict[f"Animal_{i}"] = np.dstack(images).reshape(-1, 256, 256)

    return image_dict

image_dict = build_image_dict(num_animals, num_frames)

```

```

In [52]: def write_movie(image_dict):
    for animal in tqdm(image_dict.keys()):
        print(f"Writing {animal}_movie.tif...")
        imageio.mimwrite(f'{animal}_movie.tif', image_dict[animal], format='')

write_movie(image_dict)

```

Computing Baseline Data

Max Projection

Looking at the maximum pixel values at each frame can generally indicate which neurons are activated at a given moment. However, before motion correction, these maximum intensity projections can be misleading due to motion artifacts. If the sample shifts between frames, the same neuron may appear in slightly different locations over time, causing blurring or spreading of high-intensity signals across multiple pixels. This results in a less distinct and noisier max projection image.

Local Correlations

Local correlations are a measure of how well pixel intensities in neighboring regions of the image correlate over time. Before motion correction, this measure can be distorted by motion artifacts because moving neurons may cause nearby pixels to show temporal patterns that are not genuinely related to the same underlying neuronal activity. For example, a neuron might shift in position from one frame to the next, making its signal appear to "spread out" or correlate with adjacent pixels that don't actually share the same temporal dynamics.

Expectations After Motion Correction

Using the NoRMCorre algorithm for motion correction, I expect to see:

- Max Projection
 - the neuron locations more stabilized across frames, leading to a sharper, more defined max projection, where neuronal structures are more accurately represented without being smeared by motion-induced displacement.
- Local Correlations

- reduced impact of false temporal correlations between pixels that are actually just displaced versions of the same neuron. Post-motion correction, pixels from neighboring regions should more accurately represent the same neural events over time, resulting in more reliable and precise local correlation maps.

```
In [53]: def get_baseline_movie_data(image_dict):
    max_projection_dict = {}
    correlation_image_dict = {}
    for animal in tqdm(image_dict.keys()):
        # Load data and reshape to (Time x Height x Width).
        movie_orig = imageio.imread(f"{animal}_movie.tif", memtest=False)
        movie_orig = np.dstack(movie_orig).reshape(-1, 256, 256)

        # Calculate maximum fluorescence.
        max_projection_dict[animal] = movie_orig.max(axis=0)

        # Calculate local correlation.
        correlation_image_dict[animal] = cm.local_correlations(movie_orig, s

        # Replace NaNs with zero.
        correlation_image_dict[animal][np.isnan(correlation_image_dict[animal])]

    return max_projection_dict, correlation_image_dict

max_projection_dict, correlation_image_dict = get_baseline_movie_data(image_
```

```
In [20]: def plot_baseline_movie_data(max_projection_dict, correlation_image_dict):
    fig, axes = plt.subplots(len(max_projection_dict), 2, figsize=(6, 3 * len(max_proje

    if len(max_projection_dict) == 1:
        axes = [axes]

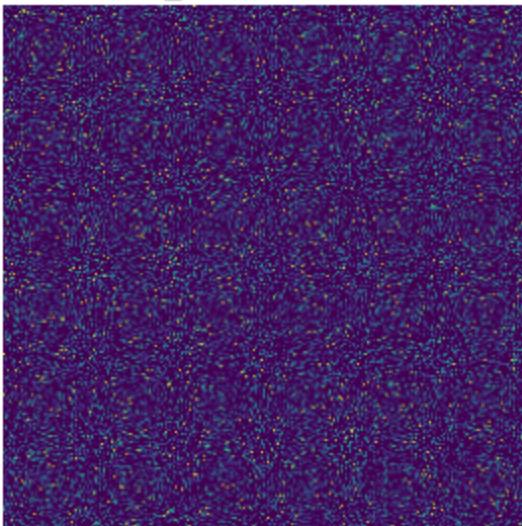
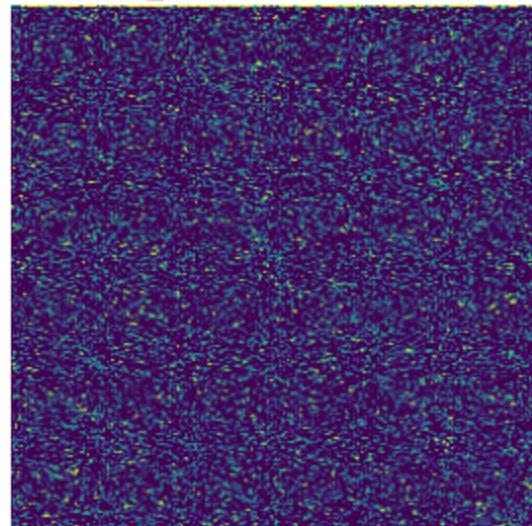
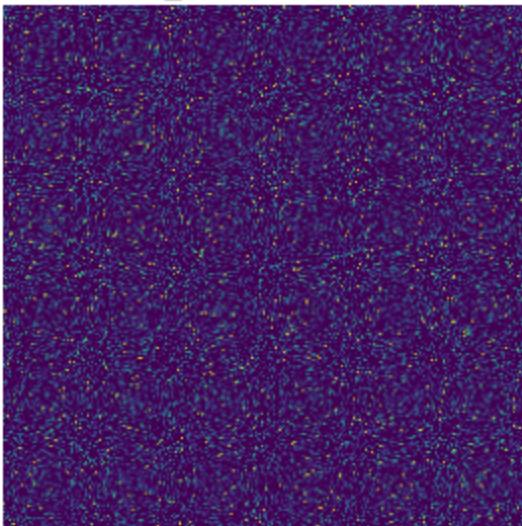
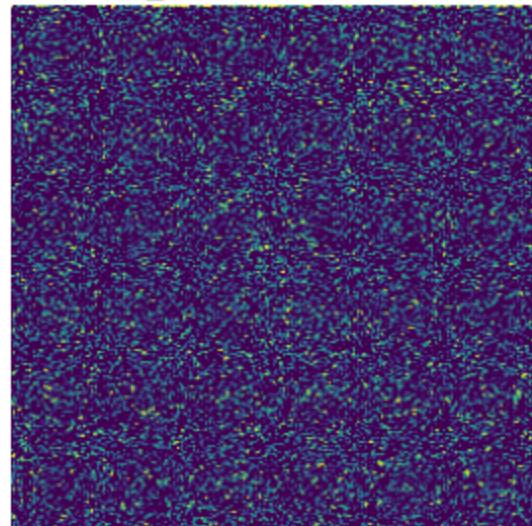
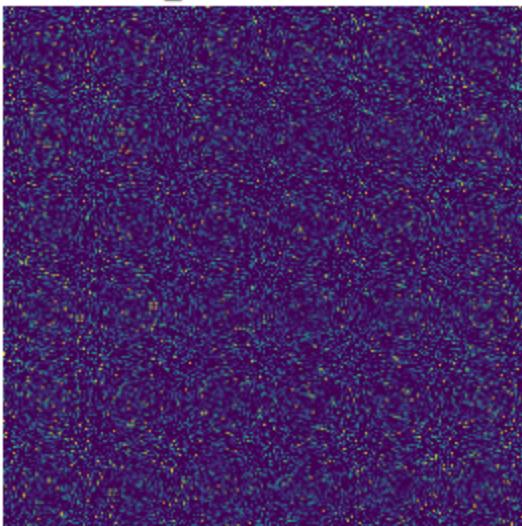
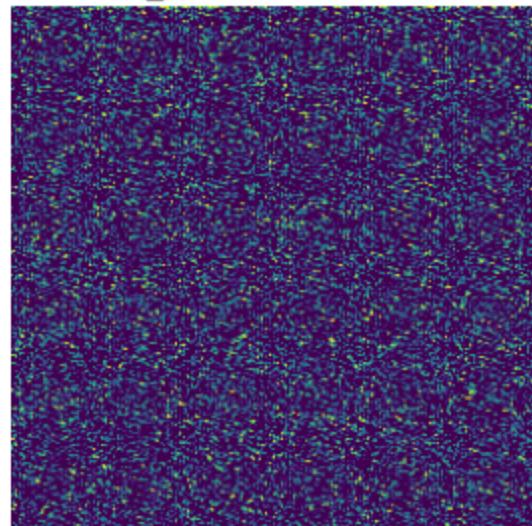
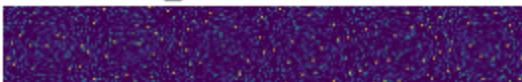
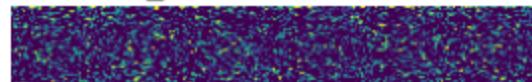
    for i, (animal, max_projection) in enumerate(max_projection_dict.items()):
        correlation_image = correlation_image_dict[animal]

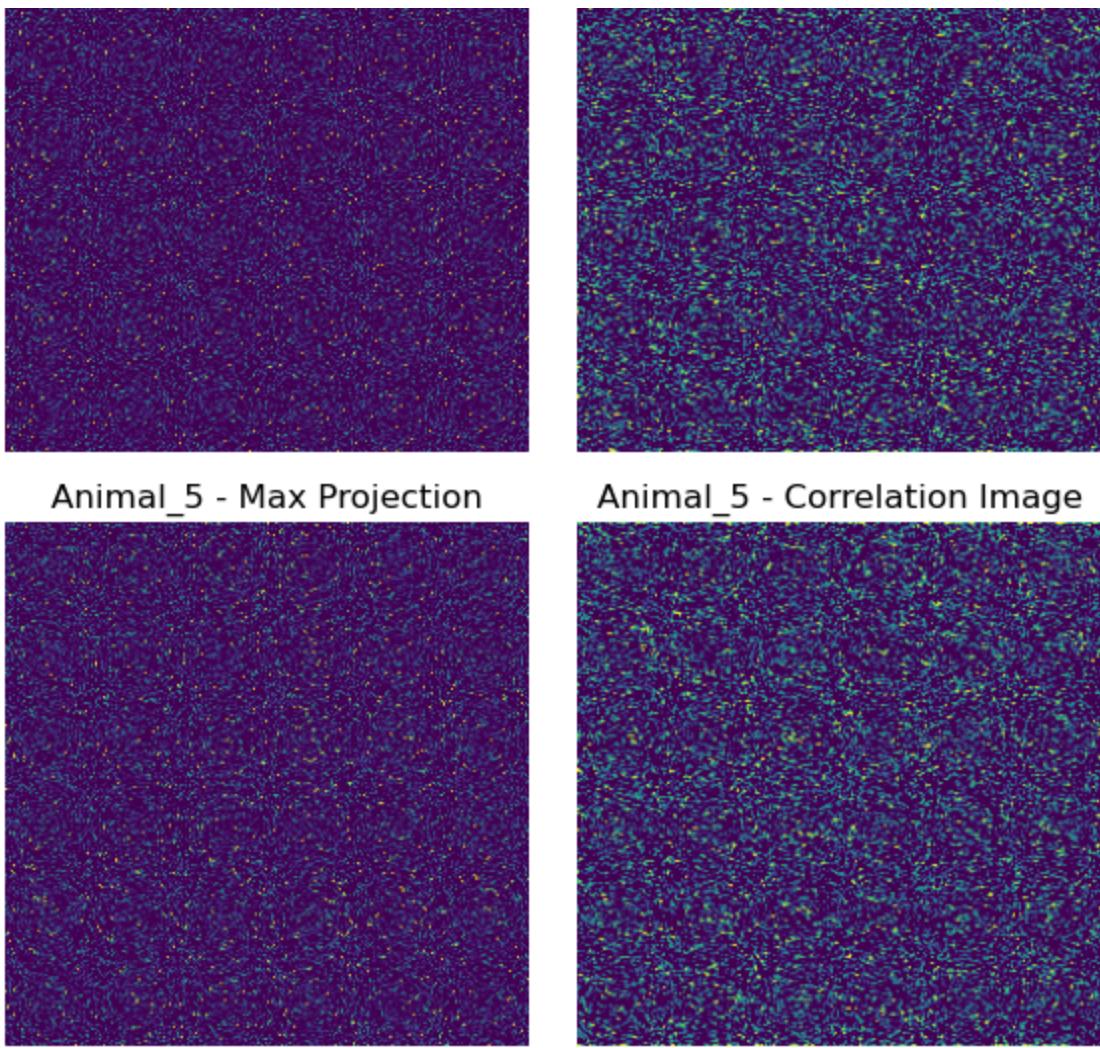
        # Plot Max Projection.
        ax_max = axes[i][0]
        ax_max.imshow(max_projection, cmap='viridis',
                      vmin=np.percentile(max_projection.ravel(), 50),
                      vmax=np.percentile(max_projection.ravel(), 99.5))
        ax_max.set_title(f"{animal} - Max Projection", fontsize=12)
        ax_max.axis("off")

        # Plot Correlation Image.
        ax_corr = axes[i][1]
        ax_corr.imshow(correlation_image, cmap='viridis',
                      vmin=np.percentile(correlation_image.ravel(), 50),
                      vmax=np.percentile(correlation_image.ravel(), 99.5))
        ax_corr.set_title(f"{animal} - Correlation Image", fontsize=12)
        ax_corr.axis("off")

    # Adjust layout for better visualization.
    plt.tight_layout()
    plt.show()
```

```
plot_baseline_movie_data(max_projection_dict, correlation_image_dict)
```

Animal_1 - Max Projection**Animal_1 - Correlation Image****Animal_2 - Max Projection****Animal_2 - Correlation Image****Animal_3 - Max Projection****Animal_3 - Correlation Image****Animal_4 - Max Projection****Animal_4 - Correlation Image**



Analysis Parameters

Dataset-dependent Parameters

- **fr:** Frame rate of imaging = 14.7 Hz
- **decay_time:** Decay time of GCaMP6f = 0.4 s
- **dxy:** Spatial resolution per pixel in um = 300/256 um/pixel

Motion Correction Parameters

- **strides:** Size of each region (patch) for piecewise rigid motion correction = (48, 48) pixels
- **overlaps:** Size of common region between patches: (24, 24) pixels
- **max_shifts:** Maximum shift-distance allowed for motion correction = (6, 6) pixels

- **max_deviation_rigid**: Maximum difference between patch shift and shift of whole image = 3 pixels
- **pw_rigid**: Enables algorithm to shift patches independently while maintaining a rigid transformation within each patch = True

CNMF parameters

- **p**: Order of model for linearly dependent deconvolution = 1
- **gnb**: Number of background components for the algorithm to account for (i.e. fluorescence from tissue that is not actively being imaged) = 2
- **merge_thr**: Two components are merged if they have a correlation greater than this threshold as they are likely to be part of the same neuron = 0.85
- **bas_nonneg**: Prevents model from fitting negative_values because they would be biologically unrealistic = True
- **rf**: Sets patch size = 15
- **stride_cnmf**: Set to approximate size of neuron. Controls overlap between patches = 10
- **K**: Approximate number of components per patch. Lower values of K correspond to lower false-negative readings at the cost of efficiency = 4
- **gSig**: Set to roughly half the approximate size of a neuron. Parameter for Gaussian Blur = [4, 4]
- **gSiz**: uses gSig to calculate kernel size for Gaussian Blur
- **method_init**: Focuses the algorithm on the regions of interest with higher fluorescence to optimize efficiency = 'greedy_roi'
- **ssub**: Lower values correspond to higher resolution at the cost of efficiency = 1
- **tsub**: Controls downsampling of temporal resolution = 1

Component Evaluation

- **min_SNR**: Minimum signal to noise ratio to accept a component = 2.0
- **rval_thr**: Threshold for spatial correlation between component and input data = 0.85
- **cnn_thr**: Threshold at the convolutional neural network accepts components = 0.99
- **cnn_lowert**: Lowest probability for a component to be accepted by the convolutional neural network. Lowers chances of false-positive = 0.1

```
In [21]: # general dataset-dependent parameters
fr = 15                                # imaging rate in frames per second
decay_time = 0.4                          # length of a typical transient in seconds
dxy = (300/256, 300/256)                 # spatial resolution in x and y in (um)

# motion correction parameters
strides = (48,48)                         # start a new patch for pw-rigid motion correction
overlaps = (24, 24)                        # overlap between patches (width of patch = strides[0])
max_shifts = (6, 6)                         # maximum allowed rigid shifts (in pixels)
max_deviation_rigid = 3                   # maximum shifts deviation allowed for patch width
pw_rigid = True                            # flag for performing non-rigid motion correction

# CNMF parameters for source extraction and deconvolution
p = 1                                     # order of the autoregressive system (set p=2 it is slow)
gnb = 2                                    # number of global background components (set to 1 if no background)
merge_thr = 0.85                           # merging threshold, max correlation allowed
bas_nonneg = True                           # enforce nonnegativity constraint on calcium traces
rf = 15                                    # half-size of the patches in pixels (patch width)
stride_cnmf = 10                            # amount of overlap between the patches in pixels
K = 4                                      # number of components per patch
gSig = np.array([4, 4])                     # expected half-width of neurons in pixels (Gaussian)
gSiz = 2*gSig + 1                          # Gaussian kernel width and height
method_init = 'greedy_roi'                  # initialization method (if analyzing dendritic trees)
ssub = 1                                    # spatial subsampling during initialization
tsub = 1                                    # temporal subsampling during initialization

# parameters for component evaluation
min_SNR = 2.0                             # signal to noise ratio for accepting a component
rval_thr = 0.85                            # space correlation threshold for accepting a component
cnn_thr = 0.99                             # threshold for CNN based classifier
cnn_lowest = 0.1                            # neurons with cnn probability lower than this value are not accepted
```

```
In [54]: def build_parameter_dict(num_animals):
    # Assuming animals are labeled as 1 - num_animals.
    animal_ids = range(1, num_animals + 1)

    parameters_dict = {}
    for animal_id in animal_ids:
        animal = f'Animal_{animal_id}'
        movie_path = f'{animal}_movie.tif'

        parameter_dict = {
            'fnames': movie_path,
            'fr': fr,
            'dxy': dxy,
            'decay_time': decay_time,
            'strides': strides,
            'overlaps': overlaps,
            'max_shifts': max_shifts,
            'max_deviation_rigid': max_deviation_rigid,
            'pw_rigid': pw_rigid,
            'p': p,
            'nb': gnb,
            'rf': rf,
            'K': K,
```

```

        'gSig': gSig,
        'gSiz': gSiz,
        'stride': stride_cnmf,
        'method_init': method_init,
        'rolling_sum': True,
        'only_init': True,
        'ssub': ssub,
        'tsub': tsub,
        'merge_thr': merge_thr,
        'bas_nonneg': bas_nonneg,
        'min_SNR': min_SNR,
        'rval_thr': rval_thr,
        'use_cnn': False,
        'min_cnn_thr': cnn_thr,
        'cnn_lowest': cnn_lowest,
    }

parameters = params.CNMFParams(params_dict=parameter_dict)
parameters_dict[animal] = parameters

return parameters_dict

parameters_dict = build_parameter_dict(num_animals=num_animals)

```

Motion Correction

Why is Motion Correction Necessary?

Motion correction helps eliminate the effects of slight movement that may cause artifacts in the image in order to maintain spatial consistency. This allows us to improve the signal to noise ratio in the data and accurately extract components in CNMF. Although measures like head-fixing and anesthetization are generally used to minimize movement and in turn artifacts, motion correction is still necessary to more closely represent accurate calcium expression.

Why NoRMCorre?

Although there are computational benefits to rigid motion correction, NoRMCorre accounts for complex motion types that are more characteristic of living organisms as opposed to the more linear expectation of rigid motion correction, thus improving the alignment.

In [55]:

```

%%time
def build_motion_correct_dict(parameters_dict):
    motion_correct_dict = {}
    for parameter in tqdm(parameters_dict.keys()):
        mot_correct = MotionCorrect(parameters_dict[parameter].data['fnames'
                                                               **parameters_dict[parameter].motion])

        # Perform the motion correction and save memory mapped file.

```

```

mot_correct.motion_correct(save_movie=True)
motion_correct_dict[parameter] = mot_correct

return motion_correct_dict

motion_correct_dict = build_motion_correct_dict(parameters_dict)

```

```

In [67]: def load_motion_corrected_movies(motion_correct_dict):
    movie_motion_correct_dict = {}
    for movie in tqdm(motion_correct_dict.keys()):
        # Load motion corrected movie.
        movie_motion_corrected = cm.load(motion_correct_dict[movie].mmap_file)

        # Set lower bound to 0 to handle negative outliers from background subtraction.
        # movie_motion_corrected = np.clip(movie_motion_corrected, a_min=0,
        movie_motion_correct_dict[movie] = movie_motion_corrected

    return movie_motion_correct_dict

movie_motion_correct_dict = load_motion_corrected_movies(motion_correct_dict)

```

Motion Correction Comparison Study

See analysis below

```

In [68]: # Calculate maximum fluorescence and local correlations for motion corrected
max_projection_mc_dict, correlation_image_mc_dict = get_baseline_movie_data()

```

```

In [66]: def compare_baseline_data(max_projection_dict, correlation_image_dict, \
                                max_projection_mc_dict, correlation_image_mc_dict):
    fig, axes = plt.subplots(num_animals, 4, figsize=(16, 4 * num_animals))
    axes = np.atleast_2d(axes)

    for i, animal in enumerate(max_projection_dict.keys()):
        # Get data before motion correction.
        max_orig = max_projection_dict[animal]
        corr_orig = correlation_image_dict[animal]

        # Get motion corrected data.
        max_corr = max_projection_mc_dict[animal]
        corr_corr = correlation_image_mc_dict[animal]

        # Plot Original Max Projection
        ax = axes[i, 0]
        ax.imshow(max_orig, cmap='viridis',
                  vmin=np.percentile(max_orig.ravel(), 50),
                  vmax=np.percentile(max_orig.ravel(), 99.5))
        ax.set_title(f'{animal} - Orig Max', fontsize=12)
        ax.axis("off")

        # Plot Corrected Max Projection
        ax = axes[i, 1]
        ax.imshow(max_corr, cmap='viridis',

```

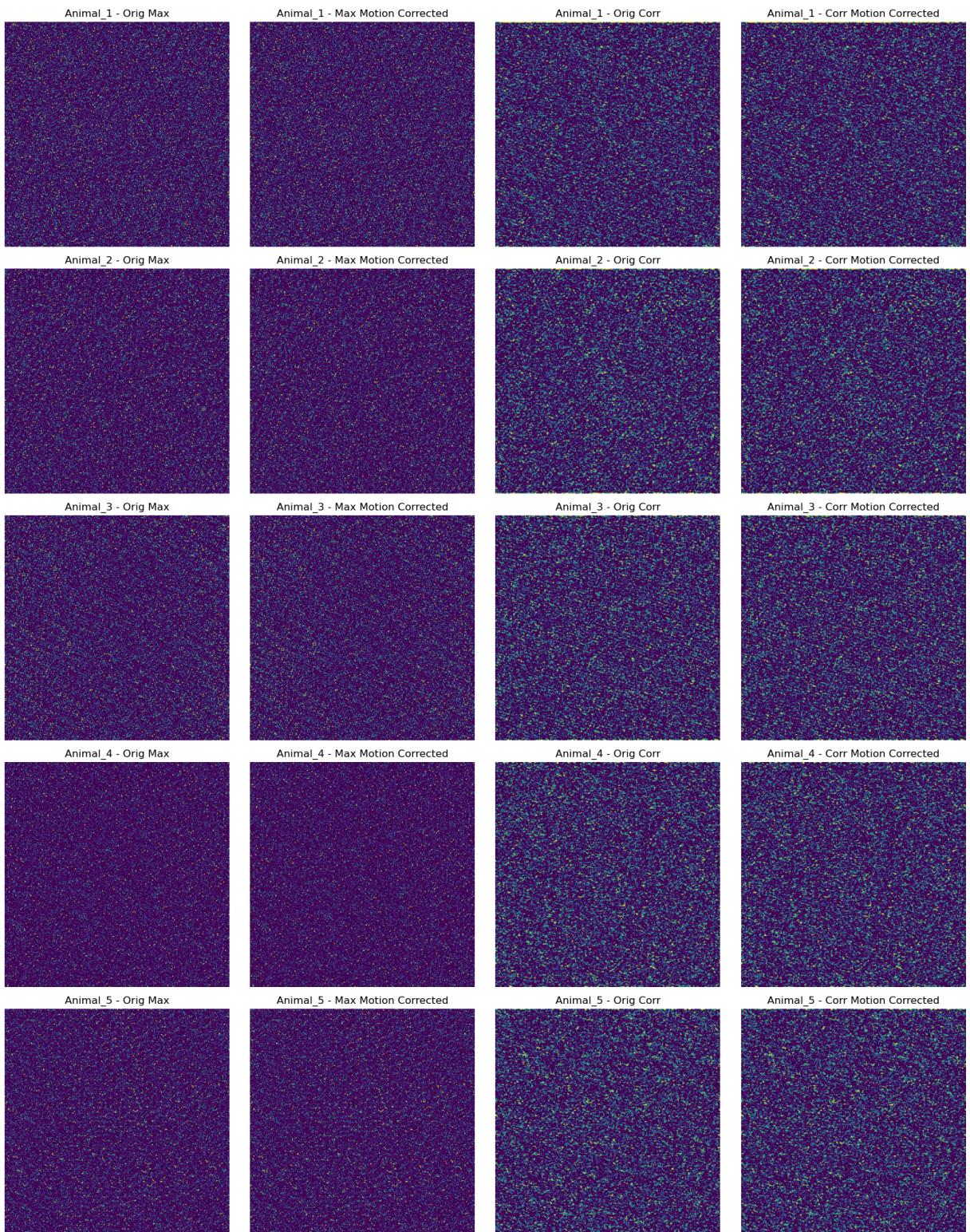
```
vmin=np.percentile(max_corr.ravel(), 50),
vmax=np.percentile(max_corr.ravel(), 99.5))
ax.set_title(f"{animal} - Max Motion Corrected", fontsize=12)
ax.axis("off")

# Plot Original Correlation Image
ax = axes[i, 2]
ax.imshow(corr_orig, cmap='viridis',
          vmin=np.percentile(corr_orig.ravel(), 50),
          vmax=np.percentile(corr_orig.ravel(), 99.5))
ax.set_title(f"{animal} - Orig Corr", fontsize=12)
ax.axis("off")

# Plot Corrected Correlation Image
ax = axes[i, 3]
ax.imshow(corr_corr, cmap='viridis',
          vmin=np.percentile(corr_corr.ravel(), 50),
          vmax=np.percentile(corr_corr.ravel(), 99.5))
ax.set_title(f"{animal} - Corr Motion Corrected", fontsize=12)
ax.axis("off")

plt.tight_layout()
plt.show()

compare_baseline_data(max_projection_dict, correlation_image_dict, \
                      max_projection_mc_dict, correlation_image_mc_dict)
```



Motion Correction Comparison Study Analysis

We would usually expect that after motion correction, the image becomes significantly more granular, meaning that fluorescence signals are more localized and well-defined. This suggests that neural activity is now more accurately captured, with individual neurons and their fluctuations more clearly separated from background noise. The increased fluorescence intensity across the image indicates that signal integration is no

longer disrupted by motion artifacts, leading to a more faithful representation of neural activity. However, in these images, it appears that there were not many artifacts due to movement from the imaged animal.

Motion Correction is valuable because motion artifacts can obscure true neural signals, making it difficult to accurately track activity, quantify fluorescence changes, or extract meaningful neuronal dynamics. By enhancing spatial precision and preserving the integrity of fluorescence fluctuations, motion correction ensures that downstream analyses—such as source extraction, spike inference, and functional connectivity studies—are based on more reliable data.

Constrained Non-Negative Matrix Factorization (CNMF)

Constrained Non-Negative Matrix Factorization, or CNMF, is often used for 2-photon image analysis. It operates by decomposing the fluorescence signals into spatial and temporal components that correspond to individual neuronal activity.

Unlike PCA or ICA, CNMF handles the problem of overlapping neurons well by explicitly modeling the spatial overlap of neurons. By enforcing non-negativity, it further improves the robustness of the extracted signals, modeling true biological behavior. Additionally, it can build upon motion correction by identifying stable neural components, filtering out motion-induced changes in intensity.

What to expect from this data?

After performing CNMF on the 2-photon data, I expect to be able to visualize distinct spatial components mapping to neurons. The algorithm should account for overlapping signals and return the isolated neurons accordingly. There should be very little background fluorescence due to CNMF's explicit separation of signals into their Spatial, Temporal, and Background components. Lastly, I hope to be able to isolate certain spikes in the neurons and attribute those spikes to specific function.

```
In [58]: def build_cnmf_model_dict(parameters_dict):
    cnmf_model_dict = {}

    for animal in tqdm(parameters_dict):
        cnmf_model = cnmf.CNMF(1, params=parameters_dict[animal])
        cnmf_model_dict[animal] = cnmf_model

    return cnmf_model_dict

cnmf_model_dict = build_cnmf_model_dict(parameters_dict)
```

```
In [59]: def build_processed_images_dict(motion_correct_dict):
    processed_images_dict = {}
```

```

    for i, motion_corrected_movie in enumerate(tqdm(motion_correct_dict)):
        mc_memmapped_fname = cm.save_memmap(
            motion_correct_dict[motion_corrected_movie].mmap_file,
            base_name=f'memmap_{i}_',
            order='C',
            border_to_0=0
        )

        # Load the memmapped file.
        raw_calcium_traces, dims, num_frames = cm.load_memmap(mc_memmapped_f

        # Reshape into Time x Height x Width.
        images = np.reshape(raw_calcium_traces.T, [num_frames] + list(dims),

        processed_images_dict[motion_corrected_movie] = images

    return processed_images_dict

processed_images_dict = build_processed_images_dict(motion_correct_dict)

```

In [60]:

```

%%time
def build_cnmf_dict(cnmf_model_dict, processed_images_dict, cnmf_refit_dict=
    cnmf_fit_dict = {}
    for animal in tqdm(processed_images_dict.keys()):
        # Perform refinement on initial fit if already computed.
        if cnmf_refit_dict is not None:
            cnmf_fit = cnmf_refit_dict[animal].refit(processed_images_dict[an
        else:
            cnmf_fit = cnmf_model_dict[animal].fit(processed_images_dict[ani

        cnmf_fit_dict[animal] = cnmf_fit

    return cnmf_fit_dict

cnmf_fit_dict = build_cnmf_dict(cnmf_model_dict, processed_images_dict)

```

CNMF Initial Fit Visualization

The initial segmentation does not seem to have yielded the results I was looking for. There is significant overlap between neurons, and the signal to noise ratio seems to be relatively high, as seen in the plots below. The distribution of neurons must be more discrete in order for the signal separation to yield any significant results.

In [40]:

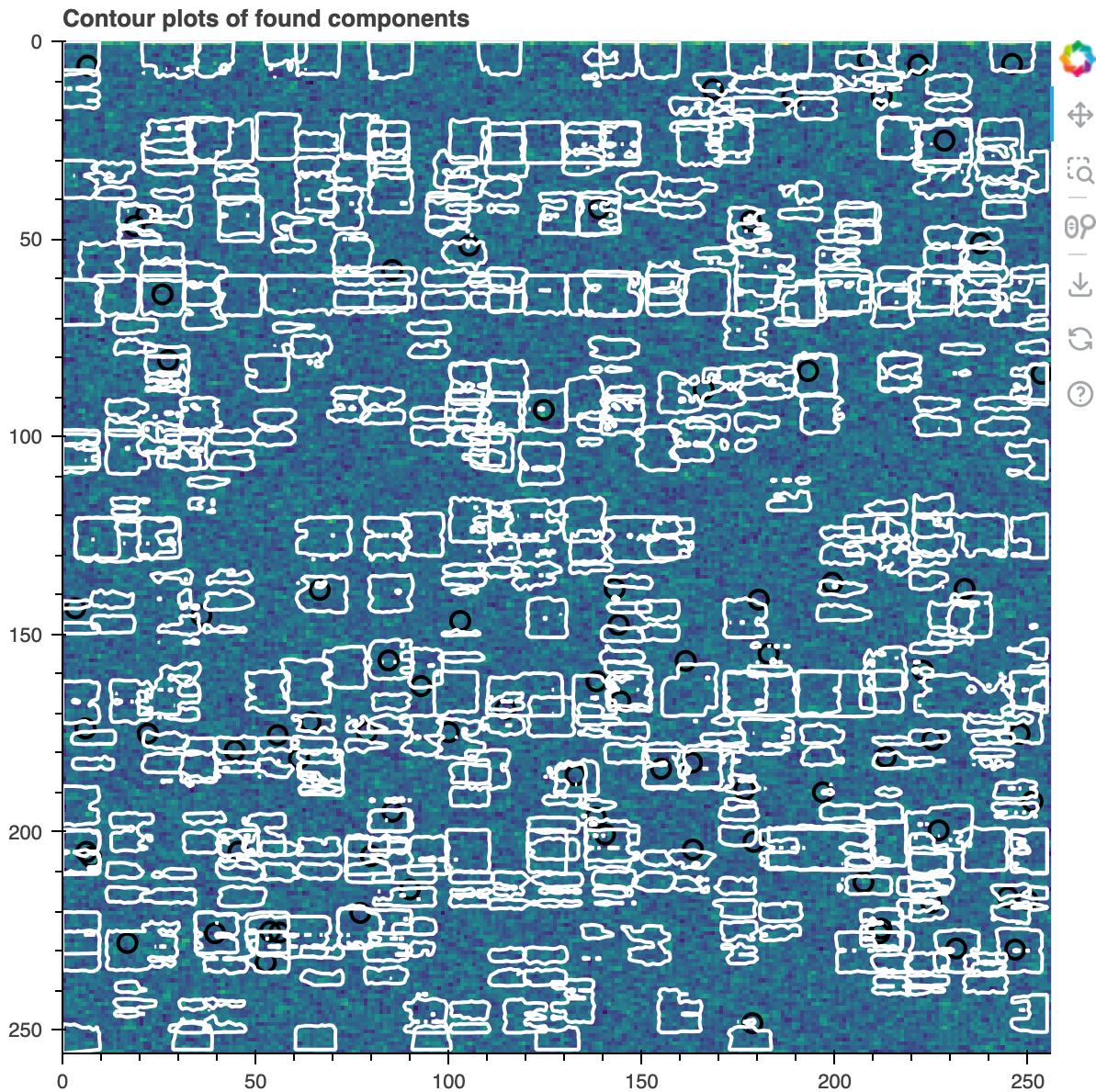
```

def plot_cnmf_contours(cnmf_fit_dict, correlation_image_mc_dict, evaluated=False):
    for animal in tqdm(cnmf_fit_dict.keys()):
        cnmf_fit = cnmf_fit_dict[animal]
        correlation_image = correlation_image_mc_dict[animal]
        if evaluated:
            cnmf_fit.estimates.plot_contours_nb(img=correlation_image, \
                idx=cnmf_fit.estimates.idx_c
        else:
            cnmf_fit.estimates.plot_contours_nb(img=correlation_image)

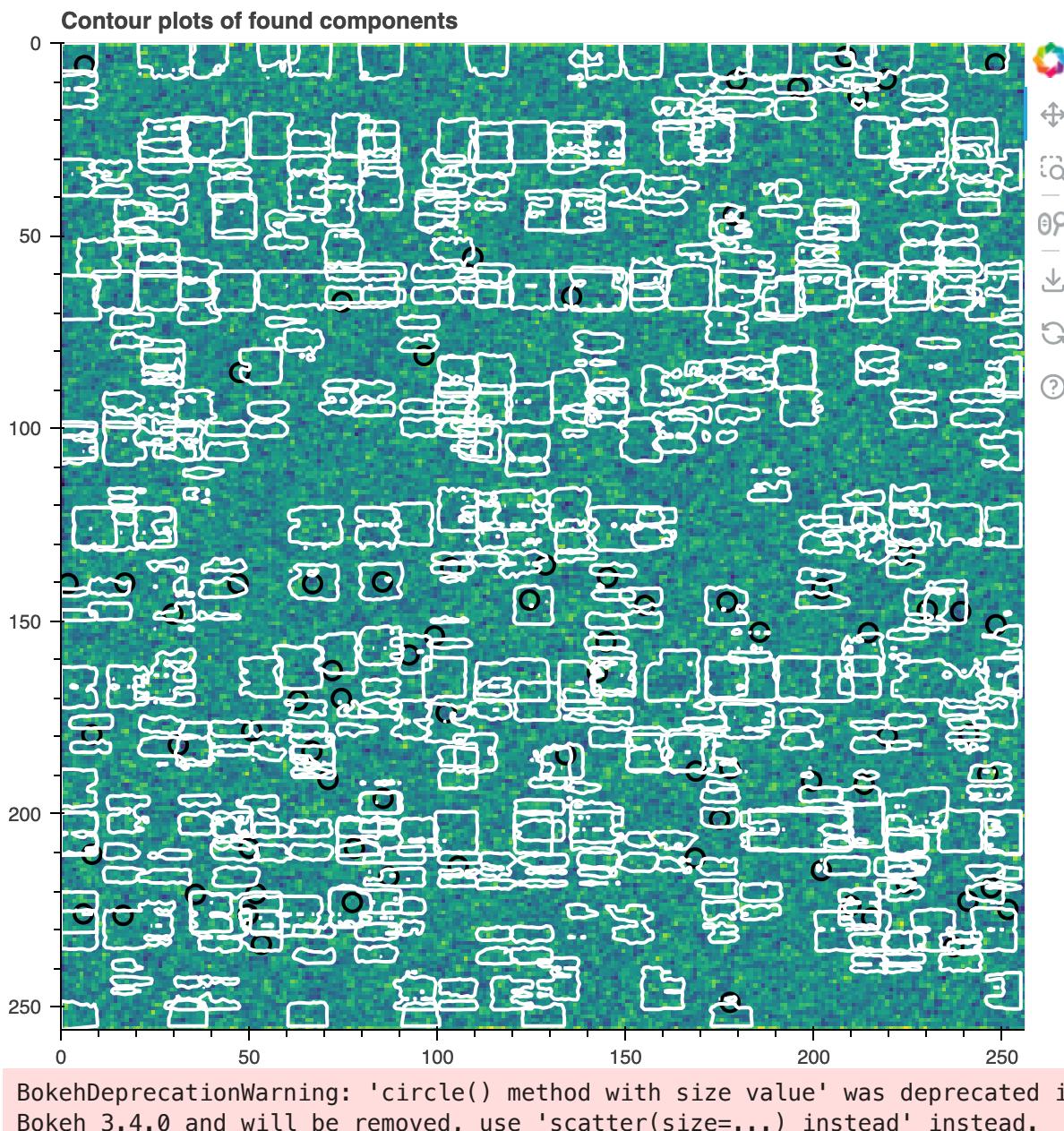
```

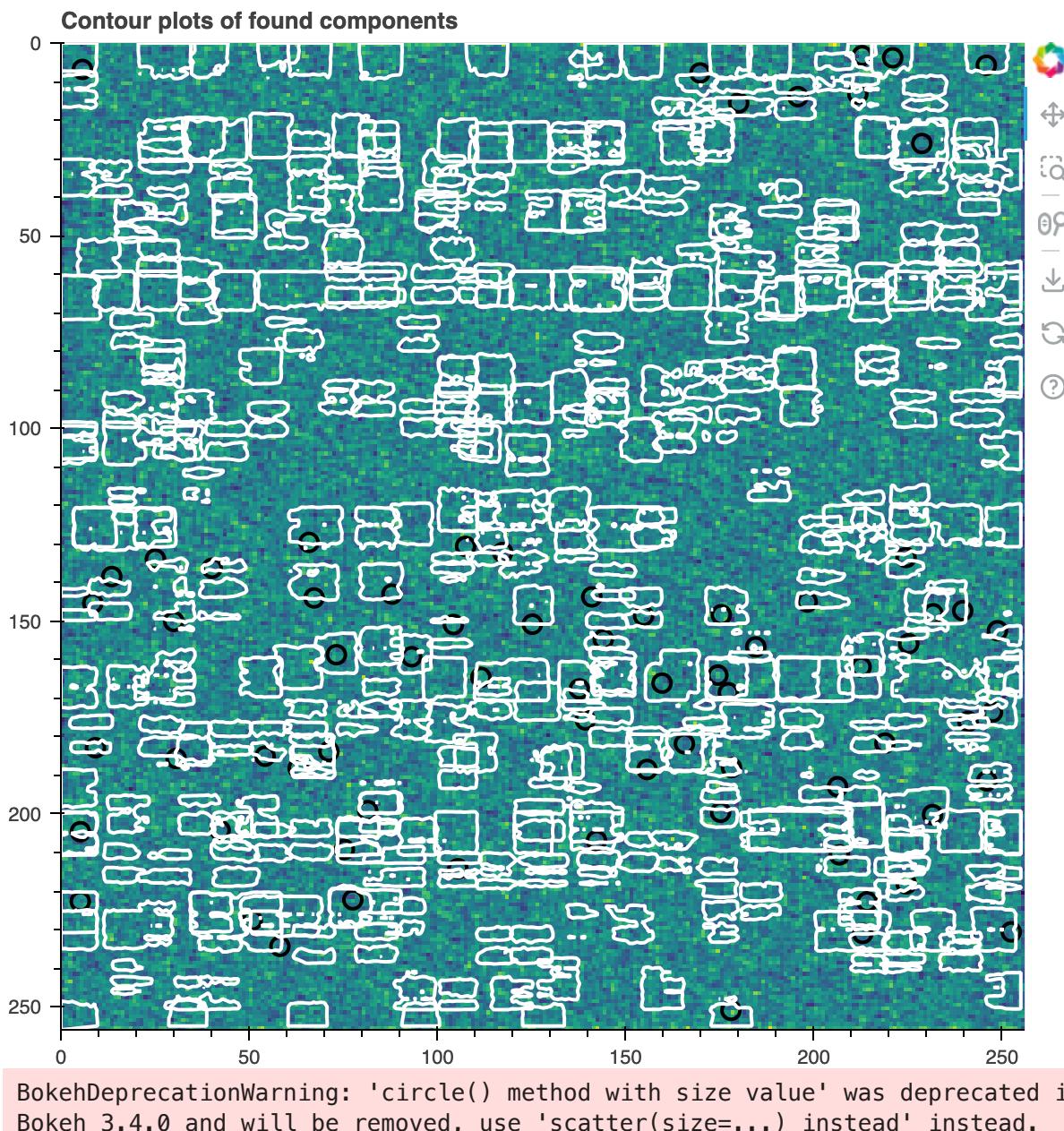
```
plot_cnmf_contours(cnmf_fit_dict, correlation_image_mc_dict)
```

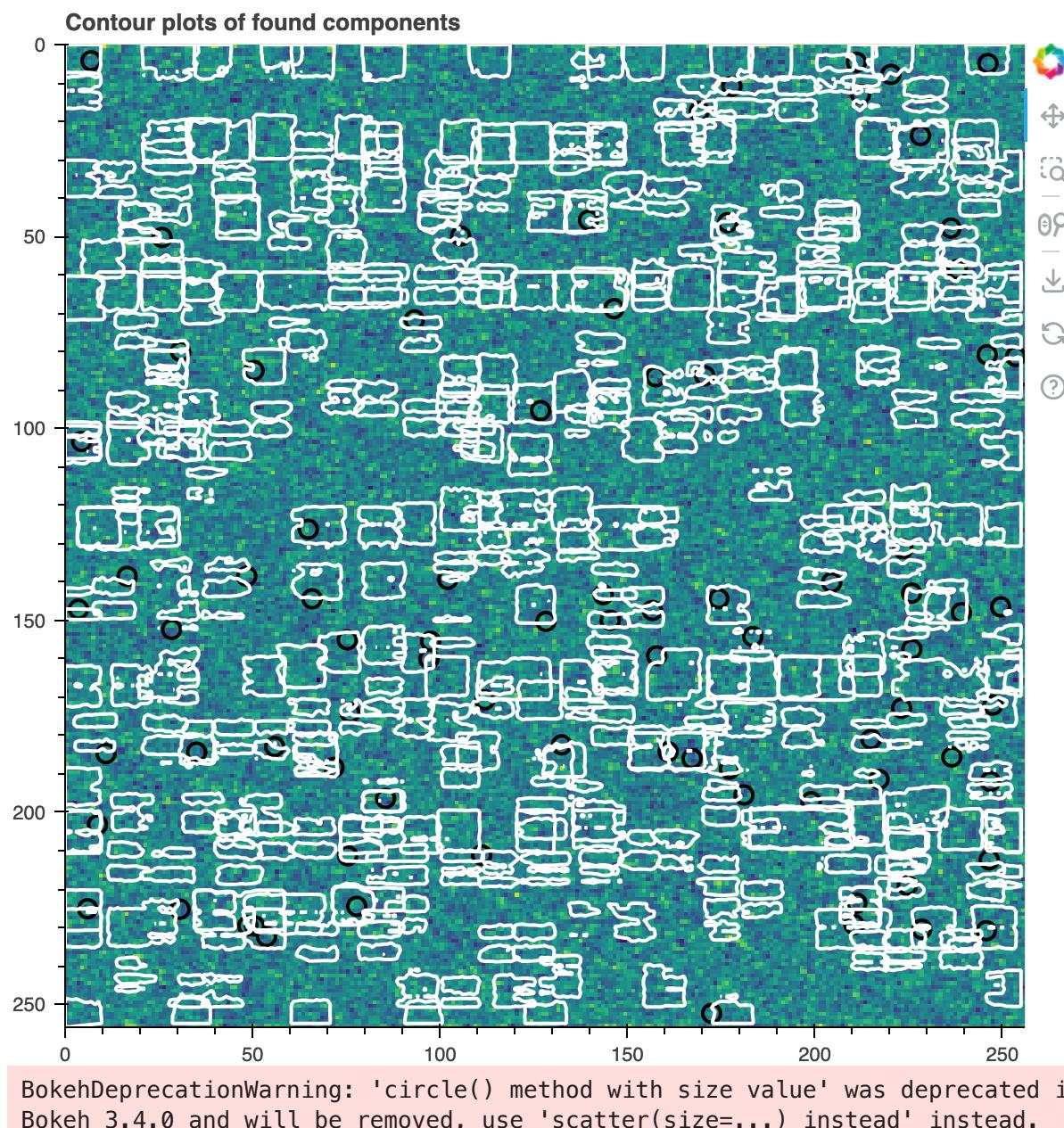
BokehDeprecationWarning: 'circle()' method with size value' was deprecated in Bokeh 3.4.0 and will be removed, use 'scatter(size=...)' instead' instead.

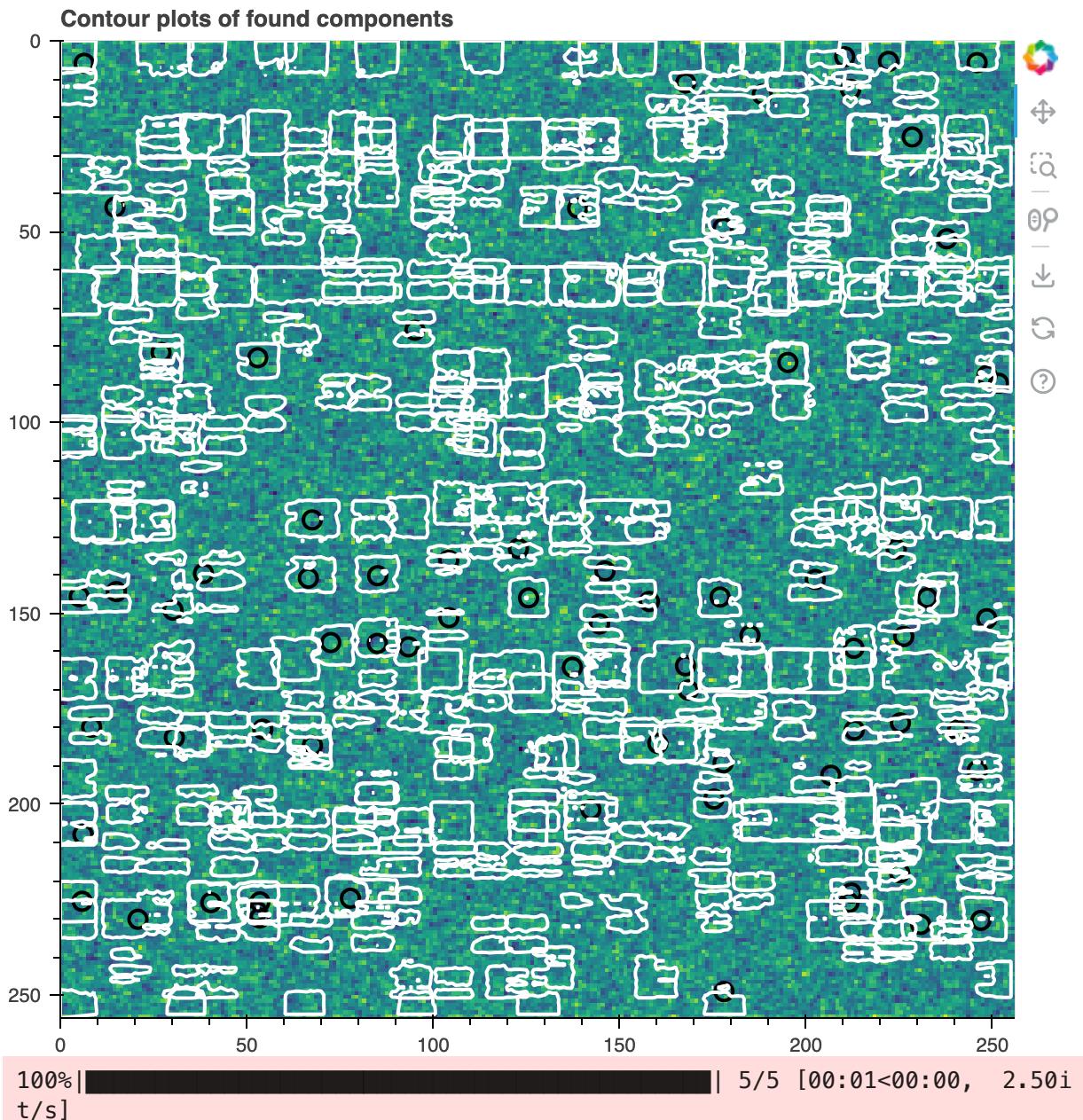


BokehDeprecationWarning: 'circle()' method with size value' was deprecated in Bokeh 3.4.0 and will be removed, use 'scatter(size=...)' instead' instead.









CNMF Re-Fit

To improve the accuracy of neural signal extraction, I performed a CNMF re-fit after the initial decomposition. The first pass can sometimes lead to overlapping components, missed low-amplitude signals, or insufficient separation of neural and background activity. Re-fitting allows for a more precise refinement of spatial components and calcium traces, enhancing signal quality and reducing background noise. This step ensures a more reliable representation of neural activity, improving the robustness of downstream analyses.

```
In [61]: %time
cnmf_refit_dict = build_cnmf_dict(cnmf_model_dict, processed_images_dict, cr
```

```
In [42]: plot_cnmf_contours(cnmf_refit_dict, correlation_image_mc_dict)
```

BokehDeprecationWarning: 'circle()' method with size value' was deprecated in Bokeh 3.4.0 and will be removed, use 'scatter(size=...)' instead' instead.

