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Tomogram Reconstruction and Gold Fiducial Removal with WarpTools, Etomo, and Fidder V.4

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We use this protocol and it's working

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Abstract

While gold nanoparticle fiducials greatly improve tilt-series alignment in cryo-electron tomography data, they can cause artifacts during later image processing steps. We present a workflow for high-quality fiducial removal using fidder, an open-source python package. This protocol demonstrates how to flexibly incorporate fidder into tomogram reconstruction using Warp and Etomo.

Overview

1 Gold nanoparticle fiducials in cryoET can greatly improve tilt-series alignment and tomogram quality. They can also introduce detrimental artifacts in downstream processing steps such as denoising or membrane segmentation. While there are several methods to remove gold beads from micrographs, these methods are not always high quality or easily integrated into existing workflows.

Here, we describe a workflow for tomogram reconstruction with WarpTools and Etomo, incorporating a step for gold fiducial removal with Fidder. The alignments are high quality because of the ability to use gold fiducials, but with no visible gold artifacts remaining in the final tomograms.

Install Software

2 Make sure you have these packages installed.

- **WarpTools**
- **Fidder**
- **IMOD**

To enable parallel processing with Etomo (recommended),

- Create the file /usr/local/lmodCalib/cpu.adoc **per these instructions**.

cpu.adoc 64B

- 'number' is the number of logical cpu cores on your machine. 'gpu.device' is the list of GPUs on your machine (note the list starts at 1, unlike CUDA device nomenclature).

WarpTools Preprocessing

3 We will be using WarpTools for the bulk of our processing. This section follows **this WarpTools tutorial**. Please refer to it for details on any specific command.

Values shown are data-specific (i.e. exposure, pixel size, paths), so please be careful when copying these commands. These are just example values!

3.1 **Create Settings Files**

frameseries

```
WarpTools create_settings --folder_data /where/your/movies/are --
folder_processing frameseries --output frameseries.settings --
extension "*.tif" --angpix 0.8125 --bin 1 --gain_path
/where/your/gain/is --exposure 3
```

tiltseries

- **You may need to create the /tomostar directory before this command.**

```
WarpTools create_settings --output tiltseries.settings --
folder_processing tiltseries --folder_data tomostar --extension
"*.tomostar" --angpix 0.8125 --bin 1 --gain_path
/where/your/gain/is --exposure 3 --tomo_dimensions 9600x12000x2500
```

If your data was collected at super-resolution like ours was, --bin 1 (2x bin) is very important!

3.2 Motion Correction and CTF Estimation

Motion correct and CTF estimate your data and output whole frame averages as well as even and odd frame-split micrographs for later denoising and/or missing-wedge correction.

```
WarpTools fs_motion_and_ctf --settings frameseries.settings --
m_grid 8x6x10 --c_grid 2x2x1 --c_range_max 7 --c_defocus_max 8 --
c_use_sum --out_averages --out_average_halves
```

Verify you have motion corrected micrographs and even/odd framesplit micrographs in /frameseries/average.

3.3 Import tiltseries metadata

You may need to edit your .mdoc files to reflect where your motion corrected micrographs are on your machine. If so, try this script.

```
#!/usr/bin/env python3

import os

mdoc_folder = '/where/your/mdocs/are'
new_subframe_path = 'where/your/micrographs/are'

def update_subframe_path(mdoc_path, new_path):
    with open(mdoc_path, 'r') as f:
        lines = f.readlines()

    for i, line in enumerate(lines):
        if line.startswith('SubFramePath'):
            existing_path = line.split('=')
[-1].strip().replace('\\', '/')
            _, existing_filename = os.path.split(existing_path)

            new_subframe_path = f'SubFramePath =
{os.path.join(new_path, existing_filename)}\n'
            lines[i] = new_subframe_path

    with open(mdoc_path, 'w') as f:
        f.writelines(lines)

def batch_update_paths(folder_path, new_path):
    for filename in os.listdir(folder_path):
        if filename.endswith('.mdoc'):
            mdoc_path = os.path.join(folder_path, filename)
            update_subframe_path(mdoc_path, new_path)
            print(f'SubFramePath updated for {filename}')

batch_update_paths(mdoc_folder, new_subframe_path)
```

Once you are confident that your .mdocs know where your averages are,

```
WarpTools ts_import --mdocs /where/your/mdocs/are --frameseries
frameseries --tilt_exposure 3 --min_intensity 0.3 --dont_invert --
output tomostar
```

3.4 Make Stacks

```
WarpTools ts_stack --settings tiltseries.settings --angpix 6.5
```

Notice we chose to make our stacks at 6.5 Å (bin 4 from physical pixel size) to save space and time. Whatever size you choose, remember it, as it is the resolution you will use for alignment and reconstruction later.

Verify you have ordered tiltseries .st and .rawtlt files in /tiltseries/tiltstack.

Etomo Tilt Series Alignment

4 We will use batch processing in etomo for aligning our newly created stacks. Components of this section follow **this warp tutorial.**

4.1 **Set up your etomo directory**

- Create the directory 'etomo' in your /tiltseries directory.
- Move your .st stacks and .rawtlt files from /tiltseries/tiltstack to your new /etomo directory. For example,

```
mv */*.st ../etomo
mv */*.rawtlt ../etomo
```

4.2 **Etomo**

Open etomo in your /tilseries/etomo directory and choose the 'Batch Tomograms' button in the GUI.

Set up your etomo session thusly:

Batch Setup Tab

- Select 'Move all stacks to dataset directory under:' and enter the path to your /tiltseries/etomo directory

Stacks Tab

- Add the stacks made in the last Warp step. The stacks will be the .st files.

Dataset Values (Basic Tab)

- Set fiducial bead size in nm (my BSA-coated 5nm gold beads are closer to 8nm)
- 'Coarse aligned stack binning - single frame' should be *1* (no binning)
- 'Aligned stack binning' should be *1* (no binning)
- Select 'R-weighted back projection' and deselect 'SIRT-like filter'

- Etomo will complain if you do not provide a tomogram thickness, so choose a number. Since we are only aligning and not reconstructing anything in etomo, this number is arbitrary. I did 4000px.

Dataset Values (Advanced Tab)

- Set 'Tilt Axis Rotation' (mine was 85)
- 'Use existing .rawtlt file for A/only axis:' should be set to *Yes.* ***(IMPORTANT)***
- 'Type of magnification solution' should be *0: fixed*
- 'Type of tilt angle solution' should be *5: grouped*
- 'Type of rotation solution' should be *-1: solve one*

Run Tab

- With the .adoc created, select your desired *# CPUs* value (I chose 56).
- Select 'Parallel GPUs' and set your *# GPUs* value (I chose 2)
- Select 'Run multiple batch jobs in parallel' if you dare. I was able to run 7 jobs with a maximum of 2 GPUs per job.
- Under 'Subset of Steps to Run', check *Stop after* and select *Fine alignment.* ***(IMPORTANT)***
- Click *Run* and rock and roll

This will output two alignments files for each tiltseries - .xf and .tlt.

WarpTools Importing Alignments

5 Head back to warp to import the etomo alignments and get ready for reconstruction. Still following **this guide.**

5.1 **Import alignments**
Copy the .xf and .tlt files from their etomo subdirectories and move to their own folder at tiltseries/alignments.

For example,

```
mkdir alignments
mv /path/to/warp/tiltseries/etomo/*/.xf alignments
mv /path/to/warp/tiltseries/etomo/*/.tlt alignments
```

Then you will need to remove the files that contain "fid" in the filename (**careful!**)

```
rm *fid*
```

You should be left with /alignments containing a .xf and .tlt file for each tiltseries.

Then run

```
WarpTools ts_import_alignments --settings tiltseries.settings --
alignments tiltseries/alignments --alignment_angpix 6.5
```

Remember we made our stacks at 6.5Å in step 2.4 and did no binning in etomo, so the alignments were all calculated at 6.5Å.

5.2 **Check Handedness**

Back to **this tutorial**.

```
WarpTools ts_defocus_hand --settings tiltseries.settings --check
```

Follow the instructions in the output of this job to change your handedness if necessary.

5.3 **CTF Estimation**

```
WarpTools ts_ctf --settings tiltseries.settings --range_high 7 --
defocus_max 8
```

Fidder Gold Removal

6 **Fidder** - involves two steps; creating masks of fiducials and erasing them. We have provided an example python script to complete both steps on your full and half micrographs so long as they are within the warp file structure. There is also the option to use the command-line for fidder.

6.1 Enter the path to your frameseries directory, micrograph pixel size, and probability_threshold value. Note - do not save this as fidder.py or your computer will be very confused.

```
#!/usr/bin/env python3

import os
from multiprocessing import Pool
import mrcfile
import torch
from fidder.predict import predict_fiducial_mask
from fidder.erase import erase_masked_region

#### User Defined Parameters ####
parent_dir = '/data/garrels/csg067/warp_2/frameseries/'
angpix = 1.35
thresh = 0.5


def check_directories(parent_dir):
    for subdir in subdirs:
        dir_path = os.path.join(parent_dir, subdir)
        if os.path.exists(dir_path):
            print(dir_path + ' found.')
        if not os.path.exists(dir_path):
            os.makedirs(dir_path)
            print(dir_path + ' created.')

    os.makedirs(os.path.join(parent_dir, 'average_erased'),
exist_ok=True)
    os.makedirs(os.path.join(parent_dir, 'average',
'even_erased'), exist_ok=True)
    os.makedirs(os.path.join(parent_dir, 'average', 'odd_erased'),
exist_ok=True)

def make_mask(filename, parent_dir):
    """ Apply fidder's predict_fidcucial_mask function to a single
micrograph and save the resultant mask as a new mrc file.

    Args:
        filename (str) : The name of the micrograph to process.
        parent_dir (str) : The directory containing micrographs in
/average and frame-split halves in
            /average/even, and /average/odd subdirectories.
    """
    mic_path = os.path.join(parent_dir, 'average', filename)
    mask_path = os.path.join(parent_dir, 'average', 'mask',
filename)
```

```
    image = torch.tensor(mrcfile.read(mic_path))

    mask, probabilities = predict_fiducial_mask(
        image, pixel_spacing=angpix, probability_threshold=thresh
    )

    mask_uint8 = mask.to(torch.uint8)

    os.makedirs(os.path.dirname(mask_path), exist_ok=True)

    with mrcfile.new(mask_path, overwrite=True) as mrc:
        mrc.set_data(mask_uint8.numpy())

    print(mask_path + '/' + filename + ' mask made.')

def erase_gold(filename, parent_dir, subdir):
    """Apply fidder's erase_masked_region function to a single
frame and save the result as a new mrc file.

    Args:
        filename (str): The name of the micrograph to process.
        parent_dir (str) : The directory containing micrographs in
/average and frame-split halves in
            /average/even, and /average/odd subdirectories.
    """
    mask_path = os.path.join(parent_dir, 'average', 'mask',
filename)

    mic_path  = os.path.join(parent_dir, subdir, filename)

    if subdir == 'average':
        output_subdir = os.path.join(parent_dir, 'average_erased')
    else:
        output_subdir = os.path.join(parent_dir, subdir +
'_erased')

    os.makedirs(output_subdir, exist_ok=True)

    image = torch.tensor(mrcfile.read(mic_path))
    mask = torch.tensor(mrcfile.read(mask_path))

    erased_image = erase_masked_region(image=image, mask=mask)

    mrc_output_path = os.path.join(output_subdir, filename)
```

```
    with mrcfile.new(mrc_output_path, overwrite=True) as mrc:
        mrc.set_data(erased_image.numpy())

    print(output_subdir +  '/' + filename + ' completed')

def process_gold(subdir, parent_dir):
    filenames = [i for i in os.listdir(os.path.join(parent_dir,
subdir)) if i.endswith('.mrc')]
    with Pool(48) as p:
        p.starmap(erase_gold, [(filename, parent_dir, subdir) for
filename in filenames])
    print('################################## all gold erased for '
+ subdir + ' ##################################')


subdirs = ['average', 'average/even', 'average/odd']

if __name__ == "__main__":
    check_directories(parent_dir)

    filenames = [i for i in os.listdir(os.path.join(parent_dir,
'average')) if i.endswith('.mrc')]
    for filename in filenames:
        mask_path = os.path.join(parent_dir, 'average','mask',
filename)
        if not os.path.exists(mask_path):
            make_mask(filename, parent_dir)
        else:
            print(f'{filename} aleady exists')
    print('################################## mask processing
complete ##################################')

    for subdir in subdirs:
        process_gold(subdir, parent_dir)
```

Depending on your computational resources, you may want to change the Pool() variable. erase_gold runs well with Pool(48) on our 64-thread machine.

Note that this script might hang after mask creation is finished. If this is the case, kill and rerun the script. It will see that the masks are already created and carry on with erasing. Sorry, I'm not a programmer!

6.2 Rename your existing (gold-containing) micrograph directories so they are not recognized by warp (e.g. /averages becomes /averages_gold etc.)

Move your newly gold-erased micrographs to the directories you just renamed (e.g. /frameseries/average, /frameseries/average/even, frameseries/average/odd)

You should be left with the following directory structure:

```
#your fidder-erased micrographs
/frameseries/average
/framseries/average/even
/frameseries/average/odd

#your original gold-containing micrographs
/frameseries/average_gold
/frameseries/average_gold/even_gold
/frameseries/average_gold/odd_gold
```

WarpTools Tomogram Reconstruction

7 To reconstruct your beautiful gold-erased tomograms:

```
WarpTools ts_reconstruct --settings tiltseries.settings --
dont_invert --halfmap_frames --angpix 10
```

Protocol references

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