Supplies needed:
- Gloves
- Lab coat
- Histosette cassettes (we use cassettes as seen in Fisher catalog [cat. #15-182-500A])
- Sieve for washing roots
- Drying oven
- 3 large beakers (1000mL)
- Graduated cylinders
- Heating plate
- 2.5 % KOH
- 1% HCl (acidified water)
- 0.05 % Trypan blue stain solution
- De-stain solution (acidified glycerol; 500 ml Glycerin (= Glycerol) + 450 ml distilled H₂O + 50 ml 1% HCl)
- 3% Hydrogen peroxide

Instructions:
1. If roots are dried, allow to re-hydrate for 1-2 hours in water.

2. Wash roots with tap water on a sieve then transfer some of the roots into a Histosette cassette.
   *Wear gloves, safety glasses and a lab coat for remaining steps.*

3. Clear roots in Histosettes by soaking them in 2.5 % KOH overnight, or by soaking them for 20-30 minutes in the heated KOH solution at 90 °C.
   **NOTE:** Check for color change to light brown then remove from heat. Do not over-clear; cortex of roots will disintegrate. Different root types will require different clearing times and intensities. Fine herbaceous roots will require much less time (15-20 minutes). To minimize the time in KOH preheat the KOH before adding cassettes.

4. Pour off KOH in a waste bottle, and rinse Histosettes in DI water 2-3 times.

5. To bleach root pigments place Histosettes in a solution of alkaline H₂O₂ for 10-30 minutes (see below for recipe). This bleaching step is only necessary for pigmented roots. **Not necessary for herbaceous roots.**
   a. Mix a fresh solution of alkaline 3% H₂O₂ just prior to usage.
   b. Mix and perform bleaching under hood.
   c. Preheat solution on heating block until it has just begun to boil. Turn down heat to prevent excessive foaming and boiling over.
   d. Place Histosettes in solution. Stir occasionally. Check roots periodically. Remove when roots are white or yellow/white in color.
   e. Pour off H₂O₂ into a waste bottle, and rinse again in DI water 2-3 times.

6. Acidify roots for 10 minutes in 1% HCl at room temperature. This is necessary for trypan blue stain to bind to fungal structures.

7. Pour off HCl in a waste bottle, and remove Histosettes from HCl and place in beaker with 0.05 % trypan blue stain. Place the solution in drying oven at 90 °C for 30-60 minutes or on hot plate at 90 °C for 5-20 minutes or leave it overnight.
8. Pour off trypan blue solution in a waste bottle, and place Histosettes in de-stain solution for a few days.
**If roots are not bleaching in alkaline H₂O₂ after considerable time or the trypan blue stain did not take, they were probably not cleared enough. You can start over by placing cassettes in 10% KOH. However, be aware that KOH is very destructive to the roots and if left in too long, the cortex will be stripped.

9. For permanent mounting, refer to the web site below:
http://invam.caf.wvu.edu/methods/recipes.htm

References:
A modified procedure for staining roots to detect VA mycorrhizas

Quantification of vesicular-arbuscular mycorrhizae in plant roots.