Video Article

# Assessing Urinary Tract Junction Obstruction Defects by Methylene Blue Dye Injection

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## Abstract

Urinary tract junction obstruction defects are congenital anomalies inducing hydronephrosis and hydroureter. Murine urinary tract junction obstruction defects can be assessed by tracking methylene blue dye flow within the urinary system. Methylene blue dye is injected into the renal pelvis of perinatal embryonic kidneys and dye flow is monitored from the renal pelvis of the kidney through the ureter and into the bladder lumen after applying hydrostatic pressure. Dye accumulation will be evident in the bladder lumen of the normal perinatal urinary tract, but will be constrained between the renal pelvis and the end point of an abnormal ureter, if urinary tract obstructions occur. This method facilitates the confirmation of urinary tract junction obstructions and visualization of hydronephrosis and hydroureter. This manuscript describes a protocol for methylene blue dye injection into the renal pelvis to confirm urinary tract junction obstructions.

## Video Link

The video component of this article can be found at https://www.jove.com/video/56247/

## Introduction

The urinary system consists of a pair of kidneys and ureters and a common bladder and urethra. The main function of the urinary system is to maintain body homeostasis by managing the water and electrolyte balance of the blood. The kidneys filter the blood to control electrolyte concentrations and acid-base balance,and produce urine to excrete excess water and waste including solutes and metabolites. Urine is then transported through the ureter from the renal pelvis of the kidney to the bladderin a unidirectional manner where it is stored and ultimately eliminated via the urethra[1].

The ureters are straight tubes originating from the nephric duct. After budding from the nephric duct at embryonic day 10.5 (E10.5) in the mouse, the ureter stalk elongates and differentiates into a multilayered structure called urothelium which is impermeable between mouse E12.5 and E16.5. The mesenchymal cells surrounding the ureter stalk are also differentiated into three layers consisting of inner stromal cells, intermediate thick smooth muscle cells, and outer adventitial fibroblasts. Ureteral peristaltic waves initiating in the renal pelvis are propagated through the smooth muscle layer of the ureter wall to the bladder to transport urine[2,3], which is produced starting at E16.5 in the mouse[1].

Congenital anomalies of the kidney and urinary tract (CAKUT) are among the most frequent genetic diseases, present in around 1% of human fetuses[1,4], and composed of a variety of phenotypes including hydronephrosis and hydroureter. The abnormal accumulation of urine in the kidney and ureter results in hydronephrotic kidney and hydroureter formation. One cause of hydronephrosis and/or hydroureter formation is an obstruction of the urinary tract. Ureteropelvic junction obstruction (UPJO) is caused by aberrant urine flow due to a blockage between the proximal ureter and the renal pelvis, resulting in hydronephrosis and proximal hydroureter narrowing with angulation or persistent folding[5,6]. In addition, the ectopic insertion of the distal ureter into either the bladder wall or the reproductive tract is called ureterovesical junction obstruction (UVJO). UVJO can also induce hydronephrosis and hydromegaureter formation[7,8]. An additional ureterovesical junction (UVJ) defect is vesicoureteric reflux (VUR). VUR is characterized by retrograde urine flow from the bladder toward the kidney at UVJ. Compared to UVJO, perinatal embryos with VUR do not distinctly show a distended hydronephrotic kidney or severe hydroureter phenotype[9].

In the laboratory mouse, urine flow can be examined by injection of a dye, such as methylene blue, into the renal pelvis[9]. The injectedmethylene blue solution will trace the urine trajectory from the renal pelvis through the ureter and into the bladder. Hydronephrosis can be recognized by an expansion of the dye in the kidney. UPJO can be detected as a blockage of dye flow at the proximal ureter with distended renal pelvis[5]. Any dilation of the ureter indicated by a widened diameter demonstrates an example of hydroureter. Finally, dye accumulation at the bladder wall or at the site of the reproductive tract indicates UVJO with distended hydronephrotic kidney and dilated hydromegaureter[7,10]. To detect VUR, the dye solution is injected into the bladder and subsequent retrograde flow is monitored in the kidney[9].

Here, a protocol for methylene blue dye injection into the renal pelvis of a perinatal embryo is presented. This protocol allows the tracing of urine flow from the renal pelvis through the ureter and into the bladder and verifies potential urinary tract junction obstructions such as UPJO or UVJO.

 

## Protocol

Mice (*Wnt5a flox/flox* mice (Wnt5a$^{tm1.1Tpy}$) and *Dll1Cre* line, UVJO mouse model)[7] were managed according to NIH guidelines for the care and use of laboratory animals and studied under a protocol approved by the NCI-Frederick Animal Care and Use Committee.

# 1. Preparation of Methylene Blue Dye Solution

1. Measure 0.1 g methylene blue powder.
2. Dissolve the methylene blue in 10 mL normal saline or 1x phosphate buffered saline (PBS) completely by vortexing.
3. Filter the 10 mg/mL methylene blue solution with a syringe filter (membrane pore size 0.45µm) to eliminate clogging during injection.
4. Assemble a sterile scalp vein set (27GX3/4″) with a 3 mL disposable syringe and fill the syringe with 3 mL filtered methylene blue solution.
   NOTE: To prevent hydrostatic pressure and subsequent dye flow, place the needle tip above the syringe filled with methylene blue solution.

# 2. Dissection of Prenatal Embryos

1. Clean dissecting scissors and forceps with 70% ethanol.
2. To collect perinatal embryos at E18.5 or E19.5, euthanize pregnant mice first using $CO_2$ inhalation and then perform cervical dislocation according to NIH guidelines.
   NOTE: The pregnant female mouse usually gives birth starting at E18.5, however, larger kidneys are easier to manipulate. Therefore, kidneys at E19.5 closer to birth are easier to perform this analysis on. However, pups with bilateral urinary tract obstructions die after birth. Depending on the characteristics of experimental mice, an appropriate collection day/time should be empirically determined.
3. Spray 70% ethanol on the ventral abdominal surface and then open the abdominal cavity ventrally using dissecting scissors and forceps.
4. Lift the entire uterus and separate it from the body by cutting with dissecting scissors at the tips of the uterine horns.
5. Rinse the entire uterus with 1x PBS in a petri dish.
6. Cut the uterus segmentally with dissecting scissors and remove placental decidua with dissecting forceps to expose the embryos in yolk sacs.
7. Remove the yolk sac first and then remove the amniotic membrane with dissecting forceps to liberate the perinatal embryos.
8. Decapitate an embryo with dissecting scissors. Wipe off excess blood with sterile gauze pads. Pin the embryo down with its ventral surface up on a dissecting microscope equipped with a digital camera for imaging. Collect its tail for genotyping if necessary.
   NOTE: Perform injections one embryo at a time.
9. Carefully open the abdominal cavity of an embryo with forceps by tearing the skin. Then, carefully remove excess organs and tissues such as liver, stomach, and intestine with forceps by cutting them or pulling them out to expose the kidneys, the ureters, and the bladder that are located dorsally (**Figure 1A**). Absorb excess blood from the dissected embryo with sterile gauze pads if necessary.
   NOTE: Excess blood interferes with identification of the renal pelvis for the dye injection.

# 3. Injection of Methylene Blue Dye into the Renal Pelvis and Monitoring Dye Flow

1. Remove bubbles in the needle and tubing by expelling methylene blue solution from the needle tip by hydrostatic pressure. Lift the syringe containing the dye solution above the level of the needle tip to start flow, and then lower the syringe to stop flow.
2. Insert the needle into the renal pelvis near the proximal ureter, taking care not to disturb it once placed. Perform dye injection into a kidney to determine its urinary tract obstruction.
   NOTE: Perform dye injection into any abnormal kidney[7] first by following the same way to determine its urinary tract obstruction.
3. Lift the syringe up about 20 cm to provide hydrostatic pressure and let 15 µL - 60 µL of the dye solution flow. The flow rate will be about 3 µL/s if the syringe is raised to this height above the embryo.
4. Monitor the blue color of the dye starting first in the renal pelvis, then in the length of the ureter, and finally in the bladder lumen.
   NOTE: It takes about 5 s to see a weak dye color emerge within the bladder lumen if the ureter is properly inserted into the bladder. Allow the dye to accumulate at the blocked site about 15 s if no dye color appears in the bladder lumen.
5. Place the syringe down to stop dye flow and remove the needle from the kidney.
6. Take images of the kidney, the ureter, and the bladder traced with dye solution using the camera and imaging program connected to a stereomicroscope.
7. Make a record of hydronephrosis of the kidney, hydroureter, and the final position of the dye solution in a lab notebook.
8. Perform injection of the contralateral kidney as described in sections 3.1) to 3.5) and allow dye flow about 20 s in total.
   NOTE: The bladder lumen will be filled with dye solution, indicated by a strong blue color, if the ureter is properly inserted into the bladder lumen. After assessment of urinary tract junction obstruction, the ureters and the bladder can be fixed for sectioning.

## Representative Results

The kidneys and lower urinary system are located dorsal to most other internal organs such as the liver and intestine. After removing these other internal organs, a pair of kidneys and ureters and a single bladder are visible (**Figure 1A**). Upon successful dissection, the dye injected into the renal pelvis will flow from the kidney into the bladder via the ureter. The renal pelvis is the funnel-like dilated proximal part of the ureter in the kidney. Therefore, the dye injection point is near the visible proximal ureter connected with the kidney as indicated with black arrows (**Figure 1B**-**1C**). After injection with methylene blue dye solution into one kidney, the dye flows from the renal pelvis through the ureter and into the bladder resulting in a visible blue color in the ureter and bladder lumen, if urinary tract obstructions do not exist (**Figure 1B**). The dye solution usually disappears in a normal ureter quickly even before imaging, and the dye color in the ureter is weak because the normal ureter is a thin tube. However, dye flow in the ureter can be observed with a dissecting microscope. A strong blue color in the bladder lumen appears as the result of a longer flow of about 20 s in total after injection of both kidneys with the dye solution (**Figure 1C**). Dye accumulation in the bladder after the contralateral injection indicates that the second urinary system does not have any urinary tract junction obstruction.

It is reasonable to inject an obviously abnormal kidney first to confirm any urinary tract junction obstructions. Abnormal hydronephrotic kidneys can be recognized by their bigger size or thin transparent surface with a broadened renal pelvis. An example distended hydronephrotic kidney and an abnormally dilated hydroureter are depicted in **Figure 2A**. A dilated hydroureter usually results in a much stronger visualization because its expanded volume retains much more dye solution than a normal, thin ureter. If the kidneys and/or the ureters are damaged during dissection, the examination of urinary tract junction obstructions is impossible because the dye will leak out aberrantly at the damaged site. For example, the abnormal hydroureter in **Figure 2A** was damaged in its middle and, therefore, the final position of the dye solution is unclear and UVJO can't be verified despite the absence of the dye in the bladder lumen (**Figure 2A**). However, this perinatal urinary system with dilated hydroureter and distended hydronephrotic kidney may have UVJO as the ureter is widened along its length and the kidney is distinctly distended. The contralateral urinary system appears normal as it has a thin ureter and the dye subsequently enters the bladder lumen (**Figure 2B**).

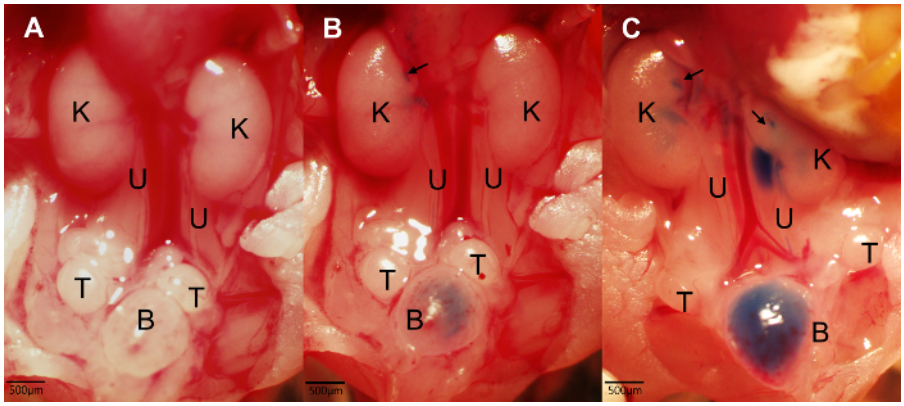


**Figure 1. Examples of methylene blue dye injection into a normal urinary system. (A)** A typical image of the urinary system from a perinatal embryo (E19.5) after removing excess tissue. Two kidneys, two ureters, and a bladder are seen with reproductive organs, here testes **(B)** An example of dye injection into a normal kidney. An injection point into the renal pelvis of the kidney (black arrow) can be seen. A weak dye color is visible in the ureter and the bladder lumen after allowing the dye to flow for 5 s. **(C)** Dye accumulation in the bladder lumen after injection of both normal kidneys. The color becomes strong from the accumulation of the dye solution in the bladder lumen after allowing the dye to flow for 20 s. The renal pelvis is also visible with the weak dye color in the kidney. B: bladder, K: kidney, T: testis, U: ureter. Please click here to view a larger version of this figure.

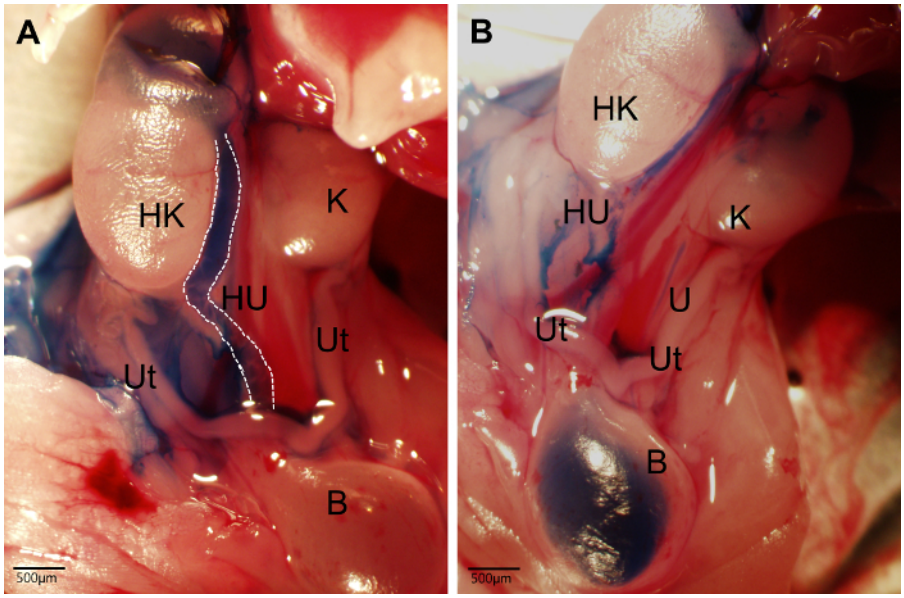


**Figure 2. Examples of methylene blue dye injection into a urinary system with unilateral hydroureter. (A)** An example of dye injection into a urinary system with a hydroureter and a hydronephrotic kidney of a neonatal female pup with dye flow of 15 s. The hydronephrotic kidney is clearly bigger than the contralateral kidney. The corresponding ureter is dilated and the dye color is strongly recognizable but the hydroureter is clearly damaged as observed by leaking dye solution in the body cavity. **(B)** Dye injection into the contralateral kidney of a urinary system with unilateral hydroureter with dye flow of 20 s. Injected dye solution into the normal kidney accumulates in the bladder lumen confirming that the abnormality is unilateral. B: bladder, HK: hydronephrotic kidney, HU: hydroureter K: kidney, U: ureter, Ut: uterine horn. Please click here to view a larger version of this figure.

## Discussion

Mouse kidneys are functional beginning at E16.5 and a dye injection test is theoretically possible from this time point. However, the kidney is too small to be injected with the dye solution and phenotypes such as hydronephrosis and hydroureter are not clearly observed since these phenotypes are a secondary effect of urine built up due to abnormal urine transport. These phenotypes, from the obstructions such as UPJO or UVJO, are evident at E18.5 by distended kidneys and ureters. Larger kidneys of embryos at E19.5 or neonatal pups are easier to manipulate than those of embryos at E18.5 or younger. However, pups with bilateral urinary tract obstructions die after birth. Therefore, perform experiments to validate CAKUT phenotypes by appropriately choosing perinatal embryos or pups depending on experimental mouse characteristics.

Dissect carefully to maintain an intact urinary system prior to performing dye injection to examine potential defects. This is essentially important for the abnormal hydronephrotic kidneys and ureters, which are very distended and easily damaged during the removal of extra tissue to expose the kidneys and urinary tracts. Damaging these organs during dissection will prevent a full determination of real defects. For example, **Figure 2** shows distinct dilation of a ureter, but the dye leakage fails to show the terminal point of the distal hydroureter.

Always be sure to remove air bubbles from the tubing and the needle prior to injection, as these bubbles will block proper dye flow. After successful dissection, the critical step to examine the urinary tract obstruction is insertion of the needle tip into the renal pelvis. The insertion of the needle into the upper half of the kidney, near the proximal ureter, is helpful to direct the needle tip into the renal pelvis. The needle tip should point toward the proximal ureter. Since the renal blood vessels are located near the proximal ureter, the dye may flow along the renal blood vessels unless the needle tip points toward the proximal ureter. In this case, the needle tip should be adjusted in the renal pelvis toward the proximal ureter. Further, the needle may pass through the kidney if inserted too deep, causing the dye to leak out of the kidney and into the body cavity. Careful insertion of the needle tip into the renal pelvis and monitoring the needle tip within the renal pelvis can help avoid this failure. Sometimes, the needle will become blocked by kidney tissue during injection if it is not inserted into the renal pelvis. In this case, remove the tissue from the needle, reset dye flow by restarting hydrostatic pressure and redirect the needle tip toward the renal pelvis. In addition, phenotypes associated with CAKUT may be unilateral or bilateral. Therefore, ensure that the injection is performed separately for left and right urinary systems. Optimally, perform the injection first with an obviously abnormal kidney to verify whether UVJO is unilateral.

Image immediately after the injection test since the dye passes from the renal pelvis through the ureter quickly. It is hard to see the color during imaging of a normal ureter because a normal ureter is a thin tube, though dye flow can be monitored under the dissecting microscope. As an alternate to methylene blue, 1% fast green in 1x PBS can also be used and will appear as a dark green color[9]. In addition to dye injection, histological sections of the kidneys and ureters following H&E staining will confirm the defects within the kidneys and ureters. Also, imaging of sections with a ureter specific reporter is an additional way to confirm UVJO[7]. However, these sections should be intact and serial sectioning is necessary to avoid false positives. Compared with traditional section-based analyses of urinary systems, this dye injection protocol is an instant way to observe the integrity of the kidney and patent ureter and to visualize obstructions such as UPJO or UVJO.

This protocol provides a simple and straightforward method toquickly visualize urinary tract structure in late stage mouse development, which allows the analysis of urinary tract development. The method described here can be used to assess mutations that cause developmental urinary tract defects, as a means of identifying changes in urinary tract maturation or ureter development.

## Disclosures

The author has nothing to disclose.

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