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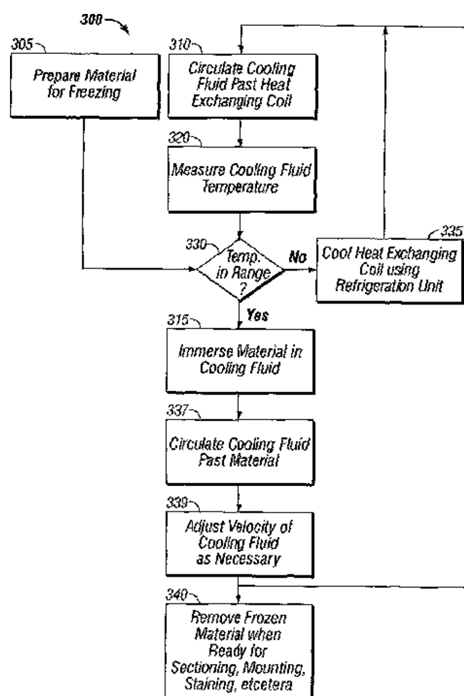
(54)【発明の名称】組織学的検査及び病理学的検査用の組織の試料を作成する方法及び装置

(57)【要約】
生きている生物材料を冷却液のタンクに材料を浸漬し、材料を冷凍するために実質的に一定の所定速度で材料を過ぎて冷却液を循環させることによって低温保存する。材料は、冷凍前に、処理なしで冷却液に直接浸漬するか又は化学的処理する。本発明の方法は、細胞構造内で氷結晶の形成を回避するのに十分迅速に生体材料を冷凍し、試料についてその解剖学的構造を維持し、冷凍後の生化学活性を残す。冷却液の温度は、応力破壊の発生及び熱変動による細胞膜におけるアーティファクトの形成を最小にするのに十分暖かい温度である－２０℃から－３０℃の間であるのが好ましい。本発明の方法を用いて冷凍した細胞は、病理的及び組織的手法で用いる他の低温保存によって冷凍された細胞より、かなり細胞及び細胞間のダメージが少なかった。本発明は材料がガラス化するように生体材料を冷凍することができるので、細胞内の生化学活性は冷凍後にも消失せず、本発明の種々の実施形態は、研究及び患者のケアの分野において、低温組織学及び免疫組織化学の新しい手法に対する生体材料を準備するための装置に用いてもよい。



【特許請求の範囲】
【請求項１】
生化学活性組織試料を冷凍する段階であって、該冷凍には、冷却液に組織試料を浸漬することと、組織試料がガラス化するように組織試料を冷凍するために実質的に一定の所定の速度及び温度で組織試料を過ぎて循環させることとを含み、ここで、組織試料はその解剖学的構造を維持し、解凍後にも生化学活性を残しているところの段階と；
組織試料を解凍する段階と；
解凍された試料を検査する段階と；を備えた方法。
【請求項２】
さらに、組織試料を断片化する段階を備えた請求項１に記載の方法。
【請求項３】
解凍された試料を検査する段階が組織学的検査を含む請求項１に記載の方法。
【請求項４】
解凍された試料を検査する段階が超構造検査を含む請求項１に記載の方法。
【請求項５】
検査する段階が免疫組織化学検査を含む請求項１に記載の方法。
【請求項６】
免疫組織化学には、蛍光標識化抗体染色が含まれる請求項５に記載の方法。
【請求項７】
組織試料の約５５％以上は細胞解剖学的構造にダメージはなく、解凍後に生化学活性を残している請求項１に記載の方法。
【請求項８】
組織試料の約４５％以上は細胞解剖学的構造にダメージはなく、解凍後に生化学活性を残している請求項１に記載の方法。
【請求項９】
組織試料の約８５％以上はその解剖学的構造を維持し、解凍後にダメージのないままである請求項１に記載の方法。
【請求項１０】
冷却液が約－２０℃から－３０℃の間の温度で維持されている請求項１に記載の方法。
【請求項１１】
組織試料を過ぎる冷却液の流速は、約２４インチ幅でかつ約４８インチ深さよりは大きくない面積を冷却液１フィート当たり及び１分当たり約３５リットル通過する速さである請求項１に記載の方法。
【請求項１２】
冷却液は、冷却液に浸漬するモータ／インペラアセンブリによって循環する請求項１に記載の方法。
【請求項１３】
さらに、冷却液に浸されたマルチパス熱交換コイルを過ぎて冷却液を循環させる段階を備え、ここで、冷却液が組織試料から除去されるときに、熱交換コイルは冷却液から少なくとも同じ量の熱を除去することができる請求項１に記載の方法。
【請求項１４】
冷却液に生化学活性組織試料を浸漬する段階と；
組織試料がガラス化し、組織試料がその解剖学的構造を維持し、組織試料が解凍後に生化学活性を残するように、実質的に一定の所定の速度及び温度で組織試料を過ぎて冷却液を循環させることによって、約－３０℃より高い温度まで直接組織試料を冷凍する段階と；を備えた検査のために組織試料を準備するのに用いる方法。
【請求項１５】
さらに、組織試料を断片化する段階を備えた請求項１４に記載の方法。
【請求項１６】
さらに、組織試料を解凍する段階を備えた請求項１４に記載の方法。

【請求項１７】
検査には、組織学的検査が含まれる請求項１４に記載の方法。
【請求項１８】
検査には、超構造検査が含まれる請求項１４に記載の方法。
【請求項１９】
検査には、免疫組織化学検査の使用が含まれる請求項１４に記載の方法。
【請求項２０】
免疫組織化学には、蛍光標識化抗体染色が含まれる請求項１９に記載の方法。
【請求項２１】
組織試料の約４０％以上はその細胞解剖学的構造を維持し、解凍後に生化学活性を残している請求項１４に記載の方法。
【請求項２２】
組織試料の約８０％以上はその細胞解剖学的構造を維持し、解凍後に生化学活性を残している請求項１４に記載の方法。
【請求項２３】
組織試料の約８５％以上はその解剖学的構造を維持し、解凍後にダメージのないままである請求項１４に記載の方法。
【請求項２４】
冷却液が約－２０℃から－３０℃の間の温度で維持されている請求項１４に記載の方法。
【請求項２５】
組織試料を過ぎる冷却液の流速は、約２４インチ幅でかつ約４８インチ深さよりは大きくない面積を冷却液１フィート当たり及び１分当たり約３５リットル通過する速さである請求項１４に記載の方法。
【請求項２６】
冷却液は、冷却液に浸漬したモータ／インペラアセンブリによって循環する請求項１４に記載の方法。
【請求項２７】
さらに、冷却液に浸されたマルチパス熱交換コイルを過ぎて冷却液を循環させる段階を備え、ここで、冷却液が組織試料から除去されるときに、熱交換コイルは冷却液から少なくとも同じ量の熱を除去することができる請求項１４に記載の方法。
【請求項２８】
冷却液に浸漬するために生化学活性組織試料を受容するように構成された冷却液溜まりと；
前記冷却液を循環させるように構成された一又は二以上の冷却液サーキュレータと；
前記冷却液から熱を除去する熱交換コイルと；
前記熱交換コイルから熱を除去するように構成された冷蔵ユニットと；を備え、
前記冷却液溜まりと、前記一又は二以上のサーキュレータと、前記冷蔵ユニットとが協力して、組織試料がガラス化し、組織試料がその解剖学的構造を維持し、組織試料が解凍後に生化学活性を残するように、実質的に一定の所定の速度及び温度で組織試料を過ぎて冷却液を循環させることによって、約－３０℃以上高い温度で組織試料を冷凍する検査用の組織試料を準備するのに用いる装置。
【請求項２９】
検査には、組織学的検査が含まれる請求項２８に記載の装置。
【請求項３０】
検査には、超構造検査が含まれる請求項２８に記載の装置。
【請求項３１】
検査には、免疫組織化学検査の使用が含まれる請求項２８に記載の装置。
【請求項３２】
免疫組織化学には、蛍光標識化抗体染色が含まれる請求項３１に記載の装置。
【請求項３３】

組織試料の約４０％以上はその細胞解剖学的構造を維持し、解凍後にも生化学活性を残している請求項２８に記載の装置。
【請求項３４】
組織試料の約８０％以上はその細胞解剖学的構造を維持し、解凍後にも生化学活性を残している２８に記載の装置。
【請求項３５】
組織試料の約８５％以上はその解剖学的構造を維持し、解凍後にもダメージのないままである請求項２８に記載の装置。
【請求項３６】
冷却液が約－２０℃から－３０℃の間の温度で維持されている請求項２８に記載の装置。 10
【請求項３７】
組織試料を過ぎる冷却液の流速は、約２４インチ幅でかつ約４８インチ深さよりは大きくない面積を冷却液１フィート当たり及び１分当たり約３５リットル通過する速さである請求項２８に記載の装置。
【請求項３８】
冷却液は、冷却液に浸漬したモータ／インペラアセンブリによって循環する請求項２８に記載の装置。
【請求項３９】
さらに、冷却液に浸されたマルチパス熱交換コイルを過ぎて冷却液を循環させる段階を備え、ここで、冷却液が組織試料から除去されるときに、熱交換コイルは冷却液から少なくとも同じ量の熱を除去することができる請求項２８に記載の装置。 20
【発明の詳細な説明】
【技術分野】
【０００１】
本発明は、低温保存に関し、特に、検査及び診断目的で保存する方法に関する。
本出願は、“組織学的検査及び病理学的検査用の組織の試料を作成する方法及び装置（Method And System For Preparing TIssues Samples For Histological And Pathological Examinations）”の発明の名称で、２００１年１月２日に出願した米国仮出願シリアル番号60/259,418を基礎として、合衆国法典第３５巻１１９条に基づいて優先権を主張するものである。 30
【背景技術】
【０００２】
組織のような生体材料は、組織学実験室において種々の処理が施されて、顕微鏡で観察するためにスライド上に標本（試料）として準備される。病理学者はスライドを慎重に調べ、病気の診断又は病気の過程において内科医の助けになるように所見を報告する。組織病理学は従来、一又は二以上の基礎的な方法によって準備（作成）された試料の検査に依存している。第１の組織学的方法では、試料は実験室において、組織を保存するための固定、組織から水分を除去するための脱水、パラフィンのような包埋剤での浸潤、包埋、スライド上に載置するための組織の断片化又は断片への切断、断片の載置、詳細を強調するための断片の染色等の重要な処理が施される。第２の方法、低温保存には通常、冷却液（冷却媒体）又は冷却環境でのスナップ冷凍、断片化、載置及び染色が含まれ、これによって、第１の方法の過程を大きく減少する。 40
【０００３】
第１の方法は非常に優れた可視化をもたらすが、通常最低でも１８時間から２４時間の長い処理時間を要する。従って、この方法では、例えば、手術中のような病理学的過程の迅速な診断を要する状況に適用することはできない。また、用いられる処理技術によって、組織の生物活動の全て又はその一部が破壊され得る。
【０００４】
第２の方法は、速度（３０分又は１時間）の点で有利であるが、低温作成を用いて準備した組織の標本はしばしば氷の結晶化によって細胞が損傷を受け、組織内の興味の対象であ 50

る分子の生物的機能の損失、及び、劣化した解剖学的構造として明らかとなる組織の完整性（integrity）の完全なる損失を引き起こし得る。多くの商業的な病理学実験室は、特別な環境を除いて、免疫組織化学のための冷凍組織の使用には向かない。というのも、保存された組織における氷の結晶の形成によって、試料内に多くの異常なアーティファクト（人工物）が発生して、診断の解釈が極めて困難になり、又は、場合によっては不可能にさえなるからである。

【０００５】

ポリクロナール抗体及びモノクローナル（単クローン）抗体の出現によって、従来の微視的な組織学及び病理学のいずれの焦点も、単純な主観的観察から直接対象染色処置へシフトしてきた。これらの最新の免疫組織化学（ＩＨＣ）技術は、病理学だけでは決定的でないときに、診断を決定づけるのに役立つ。しかしながら、ＩＨＣ技術は、適当な染色用の試料内の生物学的無処置のレセプターに依存する。従って、準備が完了した後に存在する活性な（アクティブな）生体材料の量を制限しない組織試料の作成方法を利用するのが望ましい。

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【発明の開示】

【課題を解決するための手段】

【０００６】

従って、必要なことは、現在使用できる方法に固有の問題の少なくとも一部を回避する、生存可能な単一の細胞、組織、器官、核酸、又は他の生物活性分子を低温保存する方法の改善である。従って、本発明は、冷却液に試料を浸漬し、かつ、冷却液を試料を通り過ぎて循環させることによって生化学活性組織試料を冷凍するための低温保存の方法を提供する。冷却液は、実質的に一定の所定の速度及び温度で組織試料を通過して循環することによって、組織試料はガラス化（ガラス状に）するが、その解剖学的構造を維持し、解凍後に生化学活性のままであるように組織試料を冷凍する。少なくとも一の実施形態では、冷却液は約－２０℃と約－３０℃との間の温度に維持し、組織試料を通過する冷却液の流速は、約２４インチ幅でかつ約４８インチ深さよりは大きくない領域を１フィートの冷却液及び１分当たり約３５リットル通過する速さである。また、本発明の少なくとも一の実施形態では、生物活性組織試料を冷却液に浸漬して、試料を直接約－３０℃より高い温度にまで冷凍する。本発明の他の実施形態では、冷却液に浸したマルチパス熱交換コイルを通過させて冷略液を循環することを備える。ここで、熱交換コイルは、冷却液が組織試料から除去されるときに、冷却液から少なくとも同じ量の熱を除去することができるものである。少なくとも一の実施形態は、上述の方法を実施する装置を提供する。

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【０００７】

本発明の少なくとも一の実施形態の目的は、生体材料の冷凍方法への応用であって、その方法において氷の結晶の形成及び応力破壊を回避し、冷凍後に細胞の生化学機能を維持するものである。

【発明の効果】

【０００８】

本発明の少なくとも一の実施形態の利点は、生体材料が冷凍中にガラス化するために、低温保存の回復率が非常に増大することである。

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【０００９】

本発明の少なくとも一の実施形態の他の利点は、生体材料が十分な高温においてガラス化して細胞膜内での応力破壊の発生を回避するために、低温保存の回復率が非常に増大することである。

【００１０】

本発明の少なくとも一の実施形態の他の利点は、低温保存の回復率が、現在利用されている方法に比べて、生体材料のかなり高い割合が解剖学的構造を維持し、解凍後に生化学活性が維持されているような値であることである。

【００１１】

本発明の少なくとも一の実施形態のさらなる利点は、低温保存の回復率が、生体材料試料

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を、伝統的な病理学的手法より短い時間で、断片化、処理、その後の組織学的、超構造及び免疫組織化学検査への応用に使用されるような値であることである。
【００１２】
本発明の少なくとも一の実施形態のさらなる利点は、一旦冷凍されると、現存の低温保存格納設備と機構とを用いて冷凍した生体材料を保存することができることである。
【発明を実施するための最良の形態】
【００１３】
方法、作用、関連する構成要素の機能、部分の組合せ及び製造における経済性と共に、本発明の他の目的、利点、特徴は、図面を参照して以下の詳細な説明と特許請求の範囲とから明らかになるだろう。図面は本明細書の一部を形成し、同符合の部材は同部材を示す。　10
【００１４】
好適な実施形態についての以下の詳細な説明は、この出願の一部を成すと共に本発明が実施される特定の好適な実施形態について図示する図面を参照して行う。それらの実施形態は、当業者が本発明を実施することができるのに十分詳細に説明されており、他の実施形態を利用してもよいし、本発明の精神又は範囲を逸脱することなく論理的、機械的、化学的及び電気的変更を行ってもよいことは理解されたい。当業者が本発明を実施するのに必要とされないほどの詳細さを回避するために、説明の中で当業者に公知の情報は省略している。従って、以下の詳細な説明は限定的な意味でとってはならないし、本発明の範囲は添付の特許請求の範囲によって画定される。
【００１５】　20
図１及び図２に、本発明の少なくとも一の実施形態による方法を実施するのに適した冷却装置について、冷却ユニット１００と称して示している。冷却ユニット１００は好適には、冷却液１４０を収容するタンク１１０を備えている。インペラ（羽根車）１３２、熱交換コイル１２０、ラック１５０を有するモータのようなサーキュレータ１３４であって、一の実施形態では、冷凍される生体材料を支持するトレイ１５５を備えたものが冷却液１４０に浸漬されている。生体材料は、限定するものではないが、生きている単一細胞、組織、器官、核酸及びその他の生物活性分子を含んでもよい。生体材料は特定の種類であることは要求されない。タンク１１０の外部には、熱交換コイル１２０に接続されて冷蔵ユニット１９０を備えている。
【００１６】　30
タンク１１０は、生体材料を冷却液１４０中で冷凍するために浸漬するのに要する寸法であればよく、12インチ×２４インチ×４８インチの倍数である。他のタンクの寸法もここに教示した内容に合致するものであれた使用できる。例えば、（図示しない）一の実施形態では、タンク１１０はちょうど冷却液１４０を保持する寸法であり、そのため、ガラス瓶、試験官、ビーカー、メスシリンダー等のような容器が、生体材料及び凍結防止剤を含む懸濁液の迅速冷凍のためのタンク１１０に載置することができる。他の実施形態では、タンク１１０は全器官及び／又は生物を迅速に冷凍するために完全に浸漬するのに十分な大きさである。タンク１１０は、冷凍される種々の寸法及び量の生体材料を効率的に収容する際の必要に応じて大きくしたり小さくしたりすることができることは利点である。生体材料は、タンク１１０に浸漬する前に凍結防止剤によって処理してもよい。　40
【００１７】
タンク１１０は冷却液１４０を収容できる。一の実施形態では、冷却液は食品グレードの溶質である。食品グレード品質の流体の好適な例は、プロピレングリコール、塩化ナトリウム溶液等にもとづくものである。他の実施形態では、冷却液はそれ自体が、ジメチルスルホキシド（ＤＭＳＯ）、エチレングリコール、プロピレングリコール、ポリエチレングリコール等の凍結防止剤である。場合によっては、凍結防止剤はそれ自体が食品グレード品質の流体である。他の実施形態では、他の流体及び好適には溶質を冷却液として用いてもよい。生体材料を収容するために種々の容器を使用してもよいが、本発明の実施形態によっては、迅速かつ有効な冷凍のために生体材料を冷却液に直接浸漬する。このような直接的な浸漬によって、組織、器官によっては低温保存を単純化する。　50

【００１８】
氷結晶の形成を回避しつつ生体材料を冷凍にするために、本発明の一の実施形態では、約２４インチ幅で４８インチ深さより大きくない領域に収容される冷却液の１フィート当たりかつ１分当たり３５リットルの比較的一定の流量で、冷凍する生体材料を通り過ぎて冷却液１４０を循環させる。必要な循環は、モータ１３０のような一又は二以上のサーキュレータ１３４によって行われる。本発明の少なくとも一の実施形態では、浸漬したモータ１３０はインペラ１３２を駆動して、冷却液１４０を冷凍される生体材料を過ぎて循環させる。（図示しない）種々のポンプを含む他のサーキュレータ１３４も、本発明の目的に合致するように使用することができる。本発明の少なくとも一の実施形態は、モータ１３０と少なくとも一のサーキュレータ１３４とを利用することによって冷却液が循環する面積及び体積を増加する。複数のサーキュレータ１３４を使用する実施形態では、冷却液循環の面積及び体積は、使用される付加された各サーキュレータに比例して増加する。例えば、好適な実施形態では、約２４インチ幅及び４８インチ深さより大きくない面積を通って循環する１フィートの冷却液に対して一の付加サーキュレータを用いる。

【００１９】
好適には、モータ１３０は、保存される生体材料を通って流れる一定の所定の冷却液の速度を維持するように制御でき、つまり、同時刻に、タンク１１０内の全ポイントで±0.5℃内の冷却液温度の分散で維持する。生体材料を通って循環する冷却液の実質的に一定の所定速度によって、一定の計測される熱の除去がなされ、冷凍中の生体材料のガラス化が可能となる。一の実施形態では、粘度、温度等の冷却液の特性を測定し、処理し、制御信号はモータ１３０に送られ、必要に応じてインペラ１３２の回転速度又はトルクを増大又は減少する。他の実施形態では、モータ１３０は流体の条件の全範囲にわたって所定の回転速度を維持するように構成されている。このような場合には、モータ１３０によって付与されたインペラ１３２のトルク又は回転速度は外部から制御されない。本発明の好適な実施形態を実施するのに、外部のではないポンプ、シャフト又はプーリが必要となる。モータ１３０又は他のサーキュレータ１３４を冷却液１４０に直接浸漬する。結果として、冷却液１４０はタンク１１０に配置された生体材料を冷凍するだけでなく、冷却液１４０はモータ１３０も冷却する。

【００２０】
熱交換コイル１２０は好適には“マルチパスコイル”であり、冷媒が通常一又は二以上の連続的なパス（経路）に制限されている従来の冷却コイルに対して、冷媒がマルチパス（すなわち、３又は４以上のパス）を通って流れることができる。また、コイルの寸法は、測定された量の冷却液１４０を含む断面積に直接関係する。例えば、好適な実施形態では、タンク１１０は１フィート長、２フィート深さ、４フィート幅であり、１フィート×２フィートの熱交換コイル１２０を用いる。タンク１１０の長さは２０フィートまで大きくなると、熱交換コイル１２０の長さも２０フィートまで大きくなる。結果として、熱交換コイル１２０は、同じ熱負荷を扱うのに要する従来のコイルの寸法の約５０％に作製できる。モータ１３０のようなサーキュレータ１３４は、冷凍される生体材料全体にわたって冷却された冷却液１４０を循環し、次いで、冷却液１４０に浸漬された熱交換コイル１２０へ暖かくなった冷却液を輸送する。少なくとも一の実施形態では、熱交換コイル１２０は、熱交換コイル１２０とシステムから熱を除去する冷蔵ユニット１９０に接続されている。

【００２１】
好適な実施形態では、冷蔵ユニット１９０は、熱交換コイル１２０の負荷条件に合致するように構成され、それによって熱は調和され効率的なやり方でシステムから除去され、材料が制御されて迅速に冷凍される。冷蔵ユニット１９０の効率は、効率的な供給又は熱交換コイル１２０及び冷蔵ユニット１９０で使用されるコンプレッサの効率的な出力による吸引圧を制御するための方法に直接関係する。この方法論は、冷媒温度と冷却液１４０の温度の間、及び、凝縮温度と周囲温度との間で維持するために、非常に近い許容度を要する。これらの温度の基準と熱交換コイル１２０の構成とによって、熱交換コイル１２０を

より効率的に供給することが可能となり、コンプレッサメーカーの標準規格として受け入れられているものよりコンプレッサから２５％程度越える大きなパフォーマンスを達成する調整されかつ厳しく制御された方法でコンプレッサを供給することができる。
【００２２】
図１で示した実施形態では、冷蔵ユニット１９０はは外部に離れて配備された冷蔵システムである。しかしながら、（図示しない）他の実施形態では、冷蔵ユニット１９０はタンク１１０の他のセクションに関連する。冷蔵ユニット１９０についての種々の構成が冷却液１００の所定の配置に対して大なり小なり適当であることは利点である。例えば、タンク１１０が極端に大きければ、独立した冷蔵ユニット１９０は望ましいが、携帯型の実施形態も集積型冷蔵ユニット１９０の恩恵を受ける。このような集積は、ここに記載したような構成を備えることによって達成される効率と、特に、小型化された熱交換コイルの使用とによって可能となる。
【００２３】
冷蔵ユニット１９０及び熱交換コイル１２０のために、好適な実施形態では、冷却液は、約±０．５℃以下の冷却液の温度のばらつきを伴って、−２０℃から−３０℃の間の温度まで冷却される。他の実施形態では、冷却液を、物質が冷凍される速度（率）を制御するために、−２０℃から−３０℃の間の温度範囲外の温度まで冷却する。他の実施形態では、冷却液の循環速度を制御して所望の冷凍速度を達成する。また、冷却液の体積を特定の冷凍速度を容易にするために変えてもよい。冷却液の循環速度と、冷却液の体積と、冷却液の温度との種々の組合せを用いて所望の冷凍速度を達成することができることは利点である。
【００２４】
図２において、図１で示した冷却装置の断面図について、比較的大きな量の生体材料を冷凍するのに適した冷却システム；比較的大きな量の生体材料を冷凍するのに適した冷却システム１００の実施形態について説明する。図１の符合と同じ図２の符合は、同一の構成を示している。タンク１１０は、ラック１５０が下がて入っていく冷却液１４０を収容している。ラック１５０はラック支持部２１０に可動に結合されており、それによって、ラック１５０は上昇し又は下降して、物質のタンク１１０への配置を容易にしている。
【００２５】
使用に際して、冷凍される生体材料をラック１５０のトレイ１５５に配置する。好適には、トレイ１５５は、ワイヤ、メッシュ等から成り、それによって、冷却液１４０はそれが載置される部材の上方、下方及び／又はその回りに自由に循環してよい。好適には、一旦冷却液を所望の温度に冷却すると、ラック支持部２１０は、冷却液１４０にトレイ１５５を浸漬するために、ラック１５０を下降してタンク１１０へ入れる。ラック１５０の下降は、手動で、又は、当業者に公知の種々のギヤ、チェーン及び／又はプーリ構成を用いて行う。サーキュレータ１３４は、迅速かつ制御された冷凍を行うために、トレイ１５５に載置された物質を過ぎて冷却液１４０を循環する。生体材料をタンク１１０に浸漬する他の構成を用いてもよいし、自動下降装置の使用が全環境における使用に対して必ずしも好適ではないことは利点である。
【００２６】
図２Ａに、多段らせん状パスコンベヤシステムを利用する本発明の実施形態を示す。図示したように、らせん状コンベヤ２００は、生体材料を冷却液１４０に浸漬するためにタンク１１０を内部にフィットさせる構成であってもよい。使用に際しては、一旦、冷却液を所望の温度まで冷却すると、冷凍される材料をコンベヤ１７０上でとられる入力フィード１６０に供給する。材料は、入力フィード１６０から下方らせん１７５上の冷却液１４０へ、上方らせん１７６上の冷却液１４０から出て、出力フィード１８０でらせんコンベヤから出る。すでに述べたように、冷却液１４０は一定の所定温度に維持され、冷凍される材料の迅速で安全な冷凍を保証する流速で循環するのが好ましい。材料が冷却液１４０に浸漬されている時間は、駆動ユニット２３０を調整することによって又は他の適当な手段によって変更することができる。理想的には、コンベヤベルト１７０の速度は、冷却液１

４０の温度と循環速度（流速）とを組み合わせて調整され、それによって、正確に所望量の熱が多段らせんパスコンベヤシステム２００上のタンク１１０を通って流れて、材料から除去される。

【００２７】

図３には、符合３００によって、本発明の一の実施形態による方法を示している。示した方法は、冷却液が熱交換コイルを通って循環するという段階３１０から始まる。熱交換コイルは上述のように冷却システムに作動可能に結合され、それを用いて、冷却液が熱交換コイルを通って循環するときに冷却液の温度が下げられる。段階３２０では、冷却液の温度を測定し、次いで、段階３３０に進んで、冷却液の温度が最適温度範囲か否かが決定される。この最適冷却液温度の範囲は異なる用途について異なっているが、多くの用途についての好適な最適温度範囲は－２０℃から－３０℃の間である。

【００２８】

冷却液が最適温度範囲内でないように決定されると、段階３３５が実施される。段階３３５では、熱交換コイルを冷蔵ユニット１９０によって冷却し、次に段階３１０に進み、冷却液は、冷却液の温度を下げるために熱交換コイルを通って循環する。好適には、段階３１０，３２０，３３０及び３３５を、冷却液が最適温度範囲に達するまで連続して実施する。

【００２９】

生体材料を冷凍するのに用いられる冷却液の温度は本発明の少なくとも一の実施形態では重要な要素である。従来のプロセスを用いてガラス化を達成するために、生体材料は通常、－１９６℃の温度の液体窒素でクエンチされる。このような非常に短い時間での温度の急峻な変化は細胞構造内の水を冷凍にし、それによって、氷結晶が形成する機会がなくなる。しかしながら、液体窒素でのクエンチングによる生体材料の冷凍によって、細胞膜における応力破壊が生じ、それによって、低温保存のために液体窒素でのクエンチングの有用性が制限される。本発明の好適な実施形態で用いられる温度は－２０℃から－３０℃の範囲であるので、温度変化による応力破壊が最小化され、ガラス化が細胞膜へのダメージが少なく行うことができる。

【００３０】

冷却液を適当な温度まで冷却している間、冷凍される材料は段階３０５での冷凍のために化学的処理を施してもよい。病理学のための使用される材料は通常、化学的処理は必要とされず、冷凍される材料を冷却液に入れることによって段階３０５を行うことはここでの教示と合致する。先述のように、生体材料は限定されないが、生きている単一の細胞、組織、器官、核酸、及び他の生物活性分子を含む。生体材料は、特定の種類でなくてもよい。生体材料を化学的に処理することには、細胞の成長又は死の間、細胞に保持されていた有害物質が除去されることによって細胞の生存可能性を大きくする添加剤（安定剤）によって生体材料を前処理することを含んでもよい。有効な安定剤にはこれらの化学物質及び化学化合物を含み、その多くは当業者には公知であり、酸素ラジカルのような高い反応性を有しかつ損傷を与える分子を隔離する。

【００３１】

生体材料を化学的に処理することには、順応段階（図示せず）を含んでもよい（図示せず）。前処理中またはその後、保存される生体材料は、培養温度から低下した温度まで順応されてもよいが、まだ冷凍のままである。これは、細胞の新陳代謝を遅延させること、及び、急な温度の遷移のショックを低減することによって低温保存のための生体材料の準備（処置）を支援するものである。しかしながら、順応段階は本発明の実施に必要というわけではないことに留意されたい。

【００３２】

好適な実施形態では、冷凍するために生体材料を化学的に処理することには、生体材料に凍結防止剤を装填することが含まれる。装填には通常、一又は二以上の凍結防止剤の溶液における生体材料を平衡させることも含まれる。装填中に利用される物質は装填剤と称してもよい。有効な装填剤は一又は二以上の脱水剤、透過及び非透過剤、浸透剤を含んでも

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よい。ＤＭＳＯやエチレングリコールのような透過剤、フルクトース、スクロース、グルコースのような透過及び非透過浸透剤の組合せのいずれも使用することができる。本発明の目的に合致する他の適当な凍結防止剤を使用してもよい点は利点である。

【００３３】

冷却液が適温に達した後、化学的に処理された生体材料を冷却液に浸漬する段階３１５を実施する。先述のように、生体材料を容器に配置してもよいし、冷却液の中に直接載置してもよい。次いで、浸漬したモータ／インペラアセンブリ又はポンプのようなサーキュレータを用いて、浸漬された生体材料を通って先述したような速度で冷却液を循環させる。冷却液は生体材料のそばを通るので、冷却液より高温で材料から熱が除去され、冷却液へ熱が移送され、熱を冷凍される生体材料から運びさられる。本発明の少なくとも一の実施形態では、冷凍される生体材料を通る冷却液の実質的に一定の循環は、準備された材料がガラス化されるように準備された生体材料を冷凍するために維持すべきである。

【００３４】

冷却液は冷凍される生体材料を通り過ぎて循環した後、段階３３９を実施する。段階３３９は冷却液の粘度、温度等の変化を説明するのに要するように冷却液の速度を調節する。冷却液の速度は、一又は二以上のサーキュレータによる力を調整することによって一定に保持されるのが好ましい。一旦生体材料が所望の冷凍状態に到達すると、段階３４０で示したようにそれが除去される。材料は、段階３４０において先述の手段によって冷却液から除去された後、蛍光標識化された抗体染色のような組織学的、超構造、免疫組織化学検査のために、断片化され、解凍されてもよい。

【００３５】

図３で示した段階を順に説明する。しかしながら、図示した方法は段階のうちのいくつか又は全てを連続的に実施してもよいし、異なる順で実施してもよい。例えば、本発明の少なくとも一の実施形態は単一の循環モータを用いて冷却液を循環する。このような実施形態では、冷却液は段階３１０におけるように熱交換コイルを過ぎて循環すると同時に、段階３３７で保存される生体材料を過ぎて循環する。また、本発明の一の実施形態では、冷却液の温度、粘度及び他の流体特性を連続して、かつ、システム内で多数の位置で測定する。

【００３６】

他の実施形態では、冷却液のいくつかの特性は直接は測定されない。すなわち、冷却液の特性の変化は循環モータの回転速度から間接的に決定される。モータはより遅い速度で回転すれば、モータを所望の回転速度に戻すようにさらなるパワーをモータに供給することができ、それによって、冷却液の特性の変化を補償する。少なくとも一の実施形態では、モータは回転を実質的に一定の速度に維持するように構成されている。この実質的に一定の回転速度によって、冷却液の循環が実質的に一定の流速となる。

【００３７】

本発明の一の実施形態のテストを実施したら、５ｍｌの水がフラスコで凍っていた。冷凍は、全体積の１％以下の増加であり、従来の冷凍の場合よりより少ない。他のテストでは、従来のフリーザー、及び、本発明の好適な方法による冷却システムにおいて、シート状に氷が形成した。冷凍後、氷は暗視野顕微鏡で調べた。期待されたように、従来の氷は結晶パターンを示しており、他方、本発明の原理を用いた方の凍った氷は明白な変位は示しておらず、氷の結晶が形成していないことが明らかとなった。

【００３８】

図４に、種々の低温保存法を比較した実験結果を示す。棒グラフ４００は、対照グループＡに対して４つの異なる低温保存法Ｂ、Ｃ、Ｄ及びＥを用いることによってダメージ（損傷）を受けた個々の細胞の数を比較している。対照グループＡでは低温保存は実施せず、方法Ａでは従来のフリーザーを用いて－２０℃の温度まで細胞を冷凍し、方法Ｃでは超低温のフリーザーを用いて－８０℃の温度まで細胞を冷凍し、方法Ｄでは液体窒素を用いて－１９６℃の温度まで細胞を冷凍し、方法Ｃでは本発明の好適な実施形態を用いて－２５℃の温度まで細胞を冷凍した。

【００３９】
棒グラフ４００で示した実験結果は、いかなる処理又は凍結防止剤なしで、本発明による方法と共に、先述の従来の方法によって凍らせた植物の細胞（種なしグレープ）を用いた。凍った植物の組織を解凍し、薄片に切断し、位相差顕微鏡を用いて調べた。実験で植物の組織を用いたのは、氷の結晶形成による組織の大きな乱れ又は冷凍に起因した水の膨張が組織の細胞壁構造を壊し、容易に観察ができるからである。図４で示した結果は、明らかに本発明の好適な実施形態による方法の優位性を示している。予想されたように、対照グループＡは細胞のダメージがなかった。方法Ｂ（－２０℃フリーザー）の場合は、細胞壁構造の約４５％にダメージを示しており；方法Ｃ（－８０℃フリーザー）の場合は、細胞壁構造の約５５％にダメージを示しており；方法Ｄ（液体窒素）の場合は、細胞壁構造の約５９％にダメージを示していた。しかしながら、本発明の好適な実施形態による方法では、細胞のダメージは約１２．５％しかなかった。
【００４０】
本発明の好適な実施形態による方法の優位性は、図５において、液体窒素方法及び本発明の好適な実施形態の冷凍方法の以下の冷凍－解凍サイクルの非凍結防止されたグレープの外観の組織位相差顕微鏡写真で示す。図５から、細胞壁のダメージを示す組織の変化した構造は、液体窒素を用いた方法で冷凍－解凍サイクルを行った組織より、本発明の好適な実施形態による冷凍－解凍方法の方がかなり小さいことがわかる。
【００４１】
図６に、標準の低温処理の手法を用いた冷凍後、及び、本発明の好適な実施形態による方法の適用後の心臓組織の外観の顕微鏡写真を示す。図６は、マウス及び犬の解剖用屍体から死後に収集した組織の試料において実施した異なる実験の結果を示すものである。組織の試料は５つの器官系から収集した：卵巣、心臓、肝臓、腎臓及び肺である。組織は、標準の冷凍手法を用い、さらに、以下に記載する本発明の好適な実施形態の方法による冷凍を用いて、従来の組織学、凍結切片、又は超構造検査について準備した。できた断片は経験ある臨床病理学者が評価した。予想されたように、決して冷凍しなかった試料は組織学的評価の際に優れた外観を示した。しかしながら、病理学者は、本発明の好適な実施形態による方法で冷凍した組織が少なくとも標準の低温処理手法を用いた組織と同様に保存されていたこと、及び、さらに複数の種類の組織、多くの注目すべき腎臓及び筋肉（心臓）が本発明による方法で冷凍したときに組織の完整性において顕著な改善を証明した報告している。図６は、標準の低温保存の手法は、本発明の実施形態による方法で処理された心臓の筋肉の試料と比較して、心臓の筋肉の試料内において“経本（アコーディオン）折り”６０５のような多くのアーティファクト（人工物）を有することを明らかにしている。
【００４２】
図７に、全く冷凍しなかった対照物と比較して、本発明の好適な実施形態による方法の適用後に見られる細胞のミトコンドリア７０５のおうな複雑な超構造を示した電子顕微鏡写真を示す。図７で示した電子顕微鏡写真は、本発明の好適な実施形態による方法によって冷凍した組織と全く冷凍されなかった対照組織との間の差はほとんどないことを明示している。また、液体窒素の標準的手法又は（図示していない）機械的な冷凍によって凍らせた組織の検査の際のダメージは、本発明の好適な実施形態による方法によって凍らせた組織のダメージよりかなり大きいということを示した。
【００４３】
先述のように、現在の技術を用いて作成した冷凍断片に関する主要な問題は、冷凍の際の特定の化学反応の損失である。この活動の損失は、抗体の染色に基づいた免疫組織化学の最新の手法に対して本質的に無用な試料を与えるものである。蛍光標識化抗体を用いて行った実験（５．１Ｈ１１、特定の筋肉に特定した人ＮＣＡＭ）は、蛍光のために前に染色した主要な豚に付随する細胞はこの抗体と共に、本発明の好適な実施形態の方法で準備したときの冷凍後、蛍光を発し続けることを証明した。しかしながら、液体窒素で冷凍された細胞は解凍後に蛍光を発し続けることができなかった。この実験の結果は、好適な方法によって、低温病理学及び免疫組織化学の最新手法を研究および患者の看護の分野に適用

できることを示している。
【００４４】
本発明は、材料がガラス化するように生体材料を冷凍できるので、細胞膜の応力破壊の発生を最小にし、細胞内の化学的活動を冷凍後に損失せず、本発明の種々の実施形態は、皮膚移植、角膜貯蔵、血管貯蔵、移植組織の冷凍及び不妊処置のような適当な化学的処置と共に他の医療分野においても、分子再生の病気（癌）の研究におけると共に応用可能である。
【００４５】
本発明の実施形態を変形例と共にここに詳細に示したが、本発明の教示を組み込んだ他の多くの変形の実施形態が当業者によって容易に構築されるだろう。従って、本発明はここに記載した特定の形態に限定されるものでなく、逆に、本発明の精神及び範囲内に道理にかなって含むことができるような変形、変更、均等物をカバーすることを意図したものである。
【図面の簡単な説明】
【００４６】
【図１】本発明の少なくとも一の実施形態による方法を実施する冷却装置の側面図である。
【図２】比較的大きな量の生体材料を冷凍するのに適した冷却装置の構成を示す図１で図示した冷却装置の断面図である。
【図２Ａ】本発明の一実施形態によるらせん状コンベヤを用いる構成の図１に示した冷却装置の断面図である。
【図３】本発明の少なくとも一の実施形態による装置の実施を示すフロー図である。
【図４】従来の冷凍方法と本発明の好適な実施形態による冷凍方法との実験比較の結果を示す棒グラフである。
【図５】液体窒素による方法と本発明の好適な実施形態による冷凍方法の冷凍－解凍サイクル後の非低温保存されていないグレープの組織の外観の顕微鏡写真であって、（ａ）対照物、（ｂ）新技術、（ｃ）液体窒素、の場合である。
【図６】標準の低温保存手法を用いた冷凍後と本発明の好適な実施形態による方法の適用後の心臓の組織の外観の顕微鏡写真であって、（ａ）標準凍結防止技術、（ｂ）新システム、の場合である。
【図７】本発明の好適な実施形態による方法の適用後に見られる細胞のミトコンドリアのような複雑な超構造を示す電子顕微鏡写真であって、（ａ）対照（非凍結）、（ｂ）新システム、の場合である。
【符号の説明】
【００４７】
１００　冷却ユニット
１１０　タンク
１２０　熱交換コイル
１３０　モータ
１３２　インペラ
１３４　サーキュレータ
１４０　冷却液
１５０　ラック
１５５　トレイ
１９０　冷蔵ユニット

【図３】

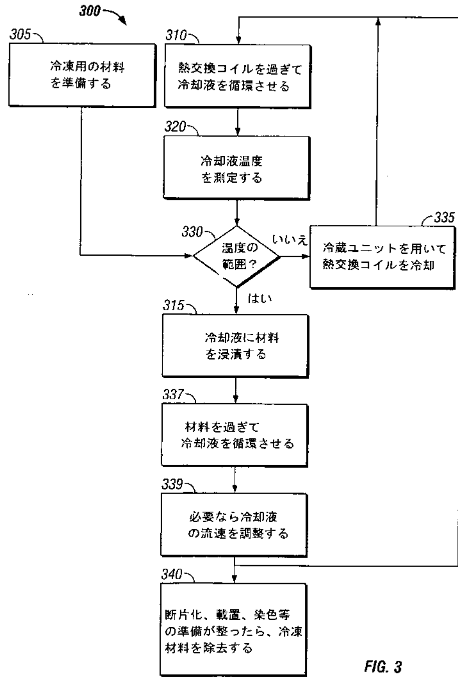


FIG. 3

【図４】

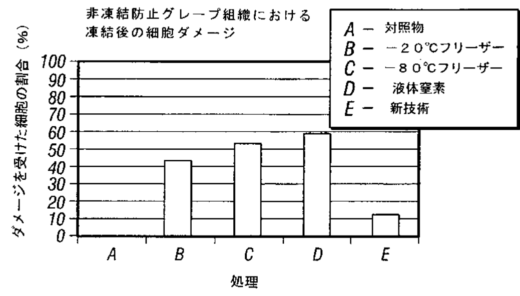
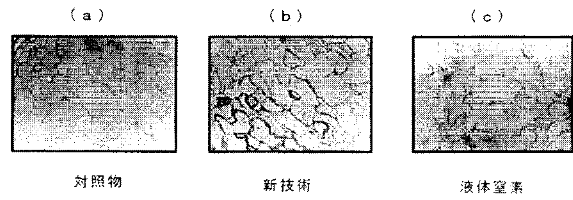


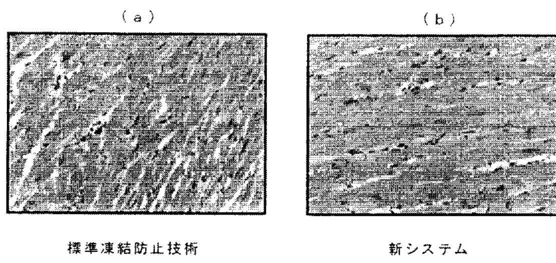
FIG. 4

【図５】



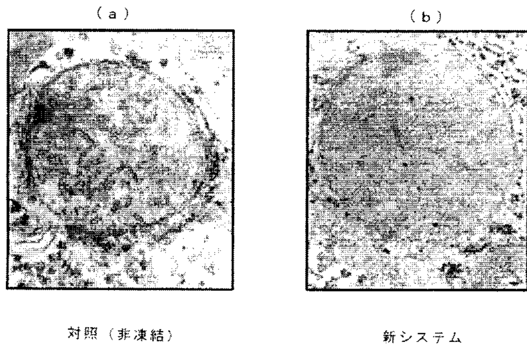
対照物　新技術　液体窒素

【図６】



標準凍結防止技術　新システム

【図７】



対照（非凍結）　新システム

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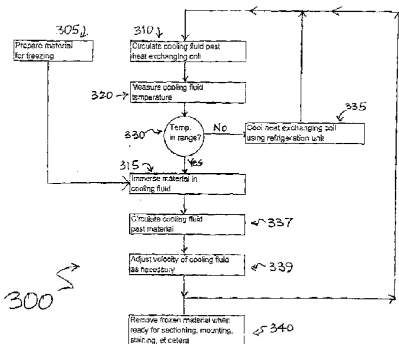
[Continued on next page]

(54) Title: METHOD AND SYSTEM FOR PREPARING TISSUE SAMPLES FOR HISTOLOGICAL AND PATHOLOGICAL EXAMINATION



(57) Abstract: Viable biological material is cryogenically preserved (cryopreservation) by immersing the material in a tank of cooling fluid, and circulating the cooling fluid past the material at a substantially constant predetermined velocity and temperature to freeze the material. The material may either be directly plunged into the cooling fluid without preparation, or chemically prepared prior to freezing. A method according to the present invention freezes the biologic material quickly enough to avoid the formation of ice crystals within cell structures (vitrification) and allows the samples to maintain anatomical structure and remain biochemically active after thaw. The temperature of the cooling fluid is preferably between -20 degrees centigrade and -30 degrees centigrade, which is warm enough to minimize the formation of stress fractures and other artefacts in cell membranes due to thermal changes. Cells frozen using a method according to the present invention have been shown to have a significantly less cellular and intercellular damage than cells frozen by other cryopreservation methods used for pathological and histological techniques. Because the present invention can freeze biological material such that the material is vitrified, biochemical activity within the cell is not lost after freezing and thus various embodiments of the present method may be employed in a system to prepare biological material for the newer techniques of cryopathology and immunohistochemistry in the areas of research and patient care.

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METHOD AND SYSTEM FOR PREPARING TISSUE SAMPLES FOR HISTOLOGICAL AND PATHOLOGICAL EXAMINATION

CROSS REFERENCE TO RELATED APPLICATION

This application claims benefit under 35 U.S.C.§ 119 of the following United
5 States provisional patent application Serial Number 60/259,418, entitled "Method And System For Preparing Tissue Samples For Histological And Pathological Examination", which was filed on January 2, 2001.

FIELD OF THE INVENTION

The present invention relates generally to cryogenic preservation and more
10 particularly to a method of preserving for examination and diagnostic purposes.

BACKGROUND OF THE INVENTION

Biological materials such as tissues are subjected to various treatments in an histology laboratory to prepare specimens on slides for viewing under a microscope. Pathologists carefully examine the slides and report their findings, which aids physicians
15 in the diagnosis of disease or disease processes. Histopathology has traditionally relied upon examination of samples prepared by one of two basic methods. In the first histological method, samples undergo significant processing in the laboratory, such as fixation to preserve tissues, dehydration to remove water from tissues, infiltration with embedding agents such as paraffin, embedment, sectioning or cutting sections of the
20 tissue for placement on a slide, mounting the sections, and staining the sections to enhance details. The second method , cryogenic preparation, significantly reduces the processing of the first method in that it generally involves snap freezing in a cold liquid or environment, sectioning, mounting, and staining.

While the first method yields significantly superior visualization, it requires an extended period of time for processing, generally a minimum of 18 to 24 hours. Thus this method cannot be applied in situations where a rapid diagnosis of a pathologic process is required, such as during a surgical procedure. Additionally, the processing techniques
5 employed may destroy all or part of the biological activity of the tissues.

The second method has the advantage of speed (30 minutes to 1 hour), however tissue specimens prepared using cryogenic preparation are often subject to cellular damage due to ice crystal formation, which can also cause the loss of biological function of molecules of interest within the tissues, and overall loss of tissue integrity manifested
10 as degraded anatomical structure. Many commercial pathology laboratories discourage the use of frozen tissue for immunohistochemistry in all but special circumstances, because ice crystal formation in stored tissue causes many abnormal artifacts within the sample which make diagnostic interpretation quite difficult, or even impossible in some cases.
15

With the advent of poly- and then monoclonal antibodies, the focus of both traditional microscopic histology and pathology has shifted from simple subjective observation, to direct objective staining procedures. These newer immunohistochemistry (IHC) techniques help in determining diagnosis when histopathology alone proves
20 inconclusive. However, IHC techniques are dependent on biologically intact receptors within the specimen for proper staining to occur. Therefore it is desirable to utilize a method of tissue specimen preparation that does not limit the amount of active biological material present after preparation is complete.

SUMMARY OF THE INVENTION

Therefore, what is needed is an improved way to cryogenically preserve viable single cells, tissues, organs, nucleic acids, or other biologically active molecules, that avoids at least some of the problems inherent in currently available methods. Accordingly, the present invention provides a method of cryopreservation for freezing a biochemically active tissue sample by immersing the sample in cooling fluid and circulating the cooling fluid past the material. The cooling fluid is circulated past the tissue sample at a substantially constant, predetermined velocity and temperature to freeze the tissue sample such that it is vitrified, yet the tissue sample maintains its anatomical structure and remains biochemically active after thaw. In at least one embodiment, the cooling fluid is maintained at a temperature of between about -20 degrees centigrade and -30 degrees centigrade, and the velocity of the cooling fluid past the tissue sample is about 35 liters per minute per foot of cooling fluid through an area not greater than about 24 inches wide and 48 inches deep. Additionally, at least one embodiment of the present invention immerses a biologically active tissue sample in cooling fluid to freeze the sample directly to a temperature higher than about -30 degrees centigrade. A further embodiment of the present invention provides for circulating the cooling fluid past a multi-path heat exchanging coil submersed in the cooling fluid, where the heat exchanging coil is capable of removing at least the same amount of heat from the cooling fluid as the cooling fluid removes from the tissue sample. At least one embodiment provides a system for implementing the above mentioned methods.

An object of at least one embodiment of the present invention is application of a method to freeze biological material wherein the formation of ice crystals and stress fractures is avoided, and cellular biochemical function is maintained after freezing.

An advantage of at least one embodiment of the present invention is that cryopreservation recovery rates are significantly increased, because biological material

is vitrified during freezing.

Another advantage of at least one embodiment of the present invention is that cryopreservation recovery rates are improved, because biological material is vitrified at a high enough temperature to avoid the formation of stress fractures within cell membranes.

Another advantage of at least one embodiment of the present invention is that cryopreservation recovery rates are such that a considerably higher percentage of the biological material maintains its anatomical structure and remains biochemically active after thaw as compared to currently available methods.

An additional advantage of at least one embodiment of the present invention is that cryopreservation recovery rates are such that the biological material samples lend themselves to the application of sectioning, processing and subsequent histological, ultrastructural, and immunohistochemistry examination in shorter periods of time than traditional pathology techniques, thus shortening time to results.

A further advantage of at least one embodiment of the present invention is that once frozen, existing cryopreservation storage facilities and mechanisms can be used to store the frozen biological materials.

BRIEF DESCRIPTION OF THE DRAWINGS

Other objects, advantages, features and characteristics of the present invention, as well as methods, operation and functions of related elements of structure, and the combination of parts and economies of manufacture, will become apparent upon 5 consideration of the following description and claims with reference to the accompanying drawings, all of which form a part of this specification, wherein like reference numerals designate corresponding parts in the various figures, and wherein:

FIG. 1 is a side view of a chilling apparatus for practicing a method according to at least one embodiment of the present invention;

10 FIG. 2 is a cross sectional view of the chilling apparatus illustrated in FIG. 1 indicating implementation of cooling systems suitable for freezing relatively large quantities of biological material;

Fig. 2A is a cross sectional view of the chilling apparatus shown in FIG. 1, configured for use with a spiral conveyor according to one embodiment of the present 15 invention;

FIG. 3 is a flow diagram illustrating a system implemented according to at least one embodiment of the present invention;

FIG. 4 is a bar chart showing the results of experimental comparisons between various prior art freezing methods and a freezing method according to a preferred 20 embodiment of the present invention;

FIG. 5 illustrates views, as seen through a microscope, of the morphological appearance of noncryoprotected grape tissue following freeze-thaw cycles of the method

of liquid nitrogen and the freezing method according to a preferred embodiment of the present invention;

FIG. 6 illustrates views, as seen through a microscope, of the morphological appearance of heart tissue after freezing using standard cryopreparative techniques, and after application of the method according to a preferred embodiment of the present invention; and

FIG. 7 is an electron microscope view illustrating the complex ultrastructural features such as cellular mitochondria that may be seen after application of the method according to a preferred embodiment of the present invention.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT OF THE INVENTION

In the following detailed description of the preferred embodiments, reference is made to the accompanying drawings which form a part hereof, and in which is shown by way of illustration specific preferred embodiments in which the invention may be practiced. These embodiments are described in sufficient detail to enable those skilled in the art to practice the invention, and it is to be understood that other embodiments may be utilized and that logical, mechanical, chemical and electrical changes may be made without departing from the spirit or scope of the invention. To avoid detail not necessary to enable those skilled in the art to practice the invention, the description may omit certain information known to those skilled in the art. The following detailed description is, therefore, not to be taken in a limiting sense, and the scope of the present invention is defined only by the appended claims.

Referring first to FIGS. 1 and 2, a chilling apparatus suitable for practicing a method according to at least one embodiment of the present invention is discussed, and designated generally as cooling unit 100. Cooling unit 100 preferably comprises tank 110 containing cooling fluid 140. Submersed in cooling fluid 140 are circulators 134 such as motors 130 having impellers 132, heat exchanging coil 120, and rack 150, which in one embodiment comprises trays 155 for supporting biological material to be frozen. Biological material may include, but is not limited to, viable single cells, tissues and organs, nucleic acids, and other biologically active molecules. Biological material is not required to be species-specific. External to tank 110, and coupled to heat exchanging coil 120, is refrigeration unit 190.

Tank 110 may be of any dimensions necessary to immerse biological material to be frozen in a volume of cooling fluid 140, in which dimensions are scaled multiples of 12 inches by 24 inches by 48 inches. Other tank sizes may be employed consistent with

the teachings set forth herein. For example, in one embodiment (not illustrated), tank 110 is sized to hold just enough cooling fluid 140, so containers such as vials, test tubes, beakers, graduated cylinders or the like, can be placed in tank 110 for rapid freezing of suspensions including biological materials and cryoprotectants. In other embodiments, tank 110 is large enough to completely immerse entire organs and or organisms for rapid freezing. It will be appreciated that tank 110 can be made larger or smaller as needed to efficiently accommodate various sizes and quantities of biological material to be frozen. The biological material may be treated with a cryoprotectant prior to being immersed in tank 110.

Tank 110 holds cooling fluid 140. In one embodiment, the cooling fluid is a food-grade solute. Good examples of food-grade quality fluids are those based on propylene glycol, sodium chloride solutions, or the like. In another embodiment, the cooling fluid is itself a cryoprotectant such as dimethylsulfoxide (DMSO), ethylene glycol, propylene glycol, polyethylene glycol or the like. Note that in some instances, the cryoprotectant is itself a food-grade quality fluid. In other embodiments, other fluids, and preferably solutes, are used as cooling fluids. While various containers may be used to hold the biological material, some embodiments of the present invention provide for the biological material to be directly immersed in the cooling fluid for rapid and effective freezing. Such direct immersion may simplify the cryopreservation of some tissues and organs.

In order to freeze biological material while avoiding the formation of ice crystals, one embodiment of the present invention circulates cooling fluid 140 past the biological material to be frozen, at a relatively constant rate of 35 liters per minute for every foot of cooling fluid contained in an area not more than about 24 inches wide by 48 inches deep. The necessary circulation is provided by one or more circulators 134, such as motors 130. In at least one embodiment of the present invention, submersed motors 130 drive impellers 132 to circulate cooling fluid 140 past biological material be to frozen. Other

circulators 134, including various pumps (not illustrated), can be employed consistent with the objects of the present invention. At least one embodiment of the present invention increases the area and volume through which cooling fluid is circulated by employing at least one circulator 134, in addition to motors 130. In embodiments using
5 multiple circulators 134, the area and volume of cooling fluid circulation are increased in direct proportion to each additional circulator employed. For example, in a preferred embodiment, one additional circulator is used for each foot of cooling fluid that is to be circulated through an area of not more than about 24 inches wide by 48 inches deep.

Preferably, motors 130 can be controlled to maintain a constant, predetermined
10 velocity of cooling fluid flow past the biological material to be preserved, while at the same time maintaining an even distribution of cooling fluid temperature within +/- 0.5 degrees centigrade at all points within tank 110. The substantially constant predetermined velocity of cooling fluid circulating past the biological material provides a constant, measured removal of heat, which allows for the vitrification of the biological
15 material during freezing. In one embodiment, cooling fluid properties such as viscosity, temperature, et cetera, are measured and processed, and control signals are sent to motors 130 to increase or decrease the rotational speed or torque of impellers 132 as needed. In other embodiments, motors 130 are constructed to maintain a given rotational velocity over a range of fluid conditions. In such a case, the torque or rotational speed of
20 impellers 132 imparted by motors 130 are not externally controlled. Of note is the fact that no external pumps, shafts, or pulleys are needed to implement a preferred embodiment of the present invention. Motors 130, or other circulators 134, are immersed directly in cooling fluid 140. As a result, cooling fluid 140 not only freezes biological material placed in tank 110, but cooling fluid 140 also provides cooling for motors 130.

25 Heat exchanging coil 120 is preferably a "multi-path coil," which allows refrigerant to travel through multiple paths (i.e., three or more paths), in contrast to conventional refrigeration coils in which refrigerant is generally restricted to one or two

continuous paths. In addition, the coil size is in direct relationship to the cross sectional area containing the measured amount of the cooling fluid 140. For example, in a preferred embodiment, tank 110 is one foot long, two feet deep, and four feet wide, and uses a heat exchanging coil 120 that is one foot by two feet. If the length of tank 110 is
5 increased to twenty feet, then the length of heat exchanging coil 120 is also increased to twenty feet. As a result, heat exchanging coil 120 can be made approximately fifty percent of the size of a conventional coil required to handle the same heat load. Circulators 134 such as motors 130, circulate chilled cooling fluid 140 over biological material to be frozen, and then transport warmer cooling fluid to heat exchanging coil
10 120, which is submersed in cooling fluid 140. In at least one embodiment, heat exchanging coil 120 is connected to refrigeration unit 190, which removes the heat from heat exchanging coil 120 and the system.

In a preferred embodiment, refrigeration unit 190 is designed to match the load requirement of heat exchanging coil 120, so that heat is removed from the system in a
15 balanced and efficient manner, resulting in the controlled, rapid freezing of a material. The efficiency of the refrigeration unit 190 is directly related to the method employed for controlling suction pressures by the efficient feeding or the heat exchange coil 120 and the efficient output of compressors used in refrigeration unit 190. This methodology requires very close tolerances to be maintained between the refrigerant and cooling fluid
20 140 temperatures, and between the condensing temperature and the ambient temperature. These temperature criteria, together with the design of the heat exchange coil 120, allow heat exchange coil 120 to be fed more efficiently, which in turn allows the compressor to be fed in a balanced and tightly controlled manner to achieve in excess of twenty five percent greater performance from the compressors than that which is accepted as the
25 compressor manufacturer's standard rating.

Note that in the embodiment illustrated in FIG. 1, refrigeration unit 190 is an external, remotely located refrigeration system. However, in another embodiment (not

illustrated), refrigeration unit 190 is incorporated into another section of tank 110. It will be appreciated that various configurations for refrigeration unit 190 may be more or less appropriate for certain configurations of cooling unit 100. For example, if tank 110 is extremely large, a separate refrigeration unit 190 may be desirable, while a portable 5 embodiment may benefit from an integrated refrigeration unit 190. Such an integration is only made possible by the efficiencies achieved by implementing the principles as set forth herein, and particularly the use of a reduced-size heat exchanging coil.

By virtue of refrigeration unit 190 and heat exchanging coil 120, in a preferred embodiment, the cooling fluid is cooled to a temperature of between -20 degrees 10 centigrade and -30 degrees centigrade, with a temperature differential throughout the cooling fluid of less than about +/- 0.5 degrees centigrade. In other embodiments, the cooling fluid is cooled to temperatures outside the -20 degrees centigrade to -30 degrees centigrade range in order to control the rate at which a substance is to be frozen. Other embodiments control the circulation rate of the cooling fluid to achieve desired freezing 15 rates. Alternatively, the volume of cooling fluid may be changed in order to facilitate a particular freezing rate. It will be appreciated that various combinations of cooling fluid circulation rate, cooling fluid volume, and cooling fluid temperature can be used to achieve desired freezing rates.

Referring now to FIG. 2, a cross sectional view of the chilling apparatus 20 illustrated in FIG. 1 indicating implementation of cooling systems suitable for freezing relatively large quantities of biological material; an embodiment of cooling system 100 suitable for freezing relatively large quantities of biological material is discussed. Reference numerals in FIG. 2 that are like, similar, or identical to reference numerals in FIG. 1 indicate like, similar, or identical features. Tank 110 contains cooling fluid 140, 25 into which rack 150 may be lowered. Rack 150 is movably coupled to rack support 210, such that rack 150 may be raised or lowered to facilitate the placement of substances into tank 110.

In use, biological material to be frozen is placed in trays 155 of rack 150. Preferably, trays 155 are constructed of wire, mesh, or otherwise, so that cooling fluid 140 may freely circulate over, under, and/or around items placed thereon. Preferably, once the cooling fluid is chilled to a desired temperature, rack support 210 lowers rack 150 into tank 110, in order to submerge trays 155 in cooling fluid 140. Lowering rack 150 may be accomplished manually or using various gear, chain, and/or pulley configurations known to those skilled in the art. Circulators 134 circulate cooling fluid 140 across substances placed in trays 155 to provide quick and controlled freezing. It will be appreciated that other arrangements for immersing biological material into tank 110 may be employed, and that use of an automatic lowering system is not necessarily preferred for use in all circumstances.

Referring now to FIG. 2A, an embodiment of the present invention employing a multi-tiered spiral path conveyor system is discussed. As illustrated, spiral conveyor 200 may be configured to fit inside tank 110 in order to submerge biological material into cooling fluid 140. In use, once the cooling fluid is chilled to a desired temperature, materials to be frozen are fed into an input feed 160 where they are taken onto conveyor belt 170. The material travels from input feed 160, into the cooling fluid 140 on downward spiral 175, out of cooling fluid 140 on upward spiral 176, and out of spiral conveyor at output feed 180. As noted earlier, the cooling fluid 140 is preferably kept at a constant predetermined temperature, and circulated at a rate that ensures rapid, safe freezing of material to be frozen. The time the material spends submerged in cooling fluid 140 can be varied by adjusting the drive unit, 230, or by other suitable means. Ideally, the speed of conveyor belt 170, in combination with the temperature and circulation rate of cooling fluid 140, will be adjusted so that exactly the desired amount of heat will be removed from materials as they travel through tank 110 on the multi-tiered spiral path conveyor system 200.

Referring now to FIG. 3, a method according to one embodiment of the present

invention is illustrated, and designated generally by reference numeral 300. The illustrated method begins at step 310, where cooling fluid is circulated past a heat exchange coil. The heat exchange coil is operably coupled to a refrigeration system as discussed above, and is used to reduce the temperature of the cooling fluid as the cooling fluid is circulated past the heat exchange coil. In step 320, the temperature of the cooling fluid is measured, and the method proceeds to step 330, where it is determined whether the temperature of the cooling fluid is within an optimal temperature range. This optimal cooling fluid temperature range may be different for different applications, however a preferred optimal temperature range for many applications is between -20 degrees centigrade and -30 degrees centigrade.

Should the cooling fluid temperature be determined not to be within an optimal, predetermined temperature range, step 335 is performed. In step 335, the heat exchanging coil is cooled by a refrigeration unit, and the method returns to step 310, in which the cooling fluid is circulated past the heat exchange coil in order to lower the temperature of the cooling fluid. Preferably, steps 310, 320, 330, and 335 are performed continually until the cooling fluid reaches the optimal temperature range.

The temperature of the cooling fluid used to freeze the biological material is an important element of at least one embodiment of the present invention. In order to achieve vitrification using conventional processes, biological material is generally quenched in liquid nitrogen, at a temperature of -196 degrees centigrade. Such a drastic change in temperature over a very short period of time freezes water within cell structures so quickly that ice crystals do not have a chance to form. However, freezing biological material by quenching in liquid nitrogen can cause stress fractures in cellular membranes, thereby limiting the usefulness of quenching in liquid nitrogen for cryopreservation. Since the temperatures used in a preferred embodiment of the present invention are between -20 degrees centigrade and -30 degrees centigrade, stress fractures due to temperature changes are minimized, and vitrification can be achieved with far less

damage to cellular membranes.

While the cooling fluid is being cooled to the proper temperature, biological material to be frozen may be chemically prepared for freezing in step 305. It will be appreciated that materials to be used for pathology do not normally require chemical preparation, and foregoing step 305 by plunging materials to be frozen directly into a cooling fluid is consistent with the teachings set forth herein. As noted earlier, biological material includes, but is not limited to, viable single cells, tissues and organs, nucleic acids, and other biologically active molecules. The biological material does not have to be species specific. Chemically preparing the biological material may include pretreatment of the biological material with agents (stabilizers) that increase cellular viability by removing harmful substances secreted by the cells during growth or cell death. Useful stabilizers include those chemicals and chemical compounds, many of which are known to those skilled in the art, which sequester highly reactive and damaging molecules such as oxygen radicals.

Chemically preparing biological material may also include an acclimation step (not illustrated). During or at some time after pretreatment, the biological material to be preserved may be acclimated to a temperature which is reduced from culturing temperatures, but still above freezing. This may help prepare the biological material for the cryopreservation process by retarding cellular metabolism and reducing the shock of rapid temperature transition. Note well, however, than an acclimation step is not required in order to practice the present invention.

In a preferred embodiment, chemically preparing biological material for freezing includes loading the biological material with a cryoprotectant. Loading generally involves the equilibration of biological material in a solution of one or more cryoprotectants. Substances utilized during loading may be referred to as loading agents. Useful loading agents may include one or more dehydrating agents, permeating and non-

permeating agents, and osmotic agents. Both permeating agents such as DMSO and ethylene glycol, and a combination of permeating and non-permeating osmotic agents such as fructose, sucrose or glucose, and sorbitol, mannitol, or glycerol can be used. It will be appreciated that other suitable cryoprotectants may be employed consistent with 5 the objects of the present invention.

After the cooling fluid reaches a proper temperature, step 315 is performed, in which the chemically prepared biological material is immersed in cooling fluid. As noted earlier, the biological material may be held in a container, or placed directly into the cooling fluid. The method then proceeds to step 337, in which a circulator, such as a 10 submersed motor/impeller assembly or pump, is used to circulate the cooling fluid at the velocity previously discussed, past the immersed biological material. As the cooling fluid passes by the biological material, heat is removed from the material, which is at a higher temperature than the temperature of the cooling fluid, and is transferred to be cooling fluid, which transports the heat away from the biological material to be frozen. 15 According to at least one embodiment of the present invention, a substantially constant circulation of cooling fluid past the biological material to be frozen should be maintained in order to freeze the prepared biological material such that the prepared material is vitrified.

After the cooling fluid is circulated past the biological material to be frozen, step 20 339 is performed. Step 339 adjusts the velocity of the cooling fluid as necessary to account for changes in the cooling fluid viscosity, temperature, and the like. Preferably, the velocity of the cooling fluid is held constant by adjusting the force provided by one or more circulators. Once the biological material has reached the desired frozen state, it is removed as shown in step 340. After the material is removed from the cooling fluid 25 in step 340 by means previously discussed, it may be sectioned and thawed for histological, ultrastructural, and immunohistochemistry examinations, such as fluorescent labeled antibody staining.

The steps illustrated in FIG. 3 are shown and discussed in a sequential order. However, the illustrated method is of a nature wherein some or all of the steps are continuously performed, and may be performed in a different order. For example, at least one embodiment of the present invention uses a single circulating motor to circulate the
5 cooling fluid. In such an embodiment, cooling fluid is circulated past a heat exchanging coil as in step 310, and past the biological material to be preserved in step 337 at the same time. In addition, one embodiment of the present invention measures cooling fluid temperatures, viscosities, and other fluid properties continually, and at multiple locations within the system.

10 In yet another embodiment, some properties of the cooling fluid are not directly measured. Rather, the change in cooling fluid properties is determined indirectly from the rotational speed of a circulation motor. If the motor is turning at a slower rate, then additional power can be supplied to the motor to return the motor to the desired rotational speed, thereby compensating for the change in cooling fluid properties. In at least one
15 embodiment, a motor is configured to maintain a substantially constant rate of rotation. This substantially constant rate of rotation will result in a substantially constant rate of cooling fluid circulation.

A test of one embodiment of the present invention was performed in which five milliliters (5 ml) of water was frozen in a graduated container. Upon freezing, there was
20 less than one percent increase in total volume, much less than expected with conventional freezing. In another test, ice was frozen in sheets in a conventional freezer, and in a cooling system according to a preferred method of the present invention. After freezing, the ice was examined under dark microscope. As expected, the conventional ice displayed a crystalline pattern, whereas the ice frozen according to the principles of
25 the present invention exhibited no light displacement, indicating little to no ice crystal formation.

Refer now to FIG. 4, in which experimental results comparing various cryopreservation methods are compared. Bar graph 400 compares the number of individual cells damaged by use of four different cryopreservation methods B, C, D, and E against a control group A. No cryopreservation was performed on control group A, method B used a conventional freezer to freeze cells to a temperature of -20 degrees centigrade, method C used an ultralow freezer to freeze cells to a temperature of -80 degrees centigrade, method D used liquid nitrogen to freeze cells to a temperature of -196 degrees centigrade, and method E used a preferred embodiment of the present invention to freeze cells to a temperature of -25 degrees centigrade.

The results of the experiments, shown in bar graph 400, used plant tissue (seedless grapes) which were frozen by the conventional methods previously discussed, as well as by the method as embodied by the present invention, without any form of preparation or cryoprotectant. The frozen plant tissue was then thawed and thin sections were cut and examined, unstained, using phase-contrast microscopy. Plant tissue was employed in the experiments because gross distortion of the tissue by ice crystal formation or water expansion caused by freezing would disrupt the tissue's cell wall structure and could be readily observed. The results, as illustrated in FIG. 4, clearly show the superiority of the method performed according to a preferred embodiment of the present invention. As expected, the control, A, exhibited no cellular damage. Method B, the -20 C freezer, exhibited damage in approximately 45% of the cellular wall structures; method C, the -80 C freezer, exhibited damage in approximately 55% of the cellular wall structures; method D, liquid nitrogen, exhibited damage in approximately 59% of the cellular wall structures. However, the method performed according to a preferred embodiment of the present invention exhibited only about 12.5% cellular damage.

The superiority of the method performed according to a preferred embodiment is also seen in FIG. 5, which illustrates views, as seen through a phase-contrast microscope,

of the morphological appearance of noncryoprotected grape tissue following freeze-thaw cycles of the method of liquid nitrogen and the freezing method according to a preferred embodiment of the present invention. Note in FIG. 5 the altered form and structure of the tissue indicating cellular wall damage is seen to be considerably less in the freeze-thaw method performed according to a preferred embodiment than that seen in the view of tissue freeze-thaw cycled with a method using liquid nitrogen.

Referring now to FIG. 6, views, as seen through a microscope, of the morphological appearance of heart tissue after freezing using standard cryopreparative techniques, and after application of the method according to a preferred embodiment of the present invention is discussed. FIG. 6 illustrates the results of a different experiment which was performed on tissue samples collected post-mortem from mice and canine cadavers. Tissue samples were collected from five organ systems: ovarian, heart, liver, kidney, and lung. Tissues were prepared for conventional histology, cryo-sectioning, or ultrastructural examination using standard freezing techniques, and also following freezing by the method performed according to a preferred embodiment of the present invention. The resulting sections were then evaluated by a trained clinical pathologist. As expected, samples that were never frozen exhibited superior morphology upon histological evaluation. However, the pathologist report states that tissue frozen according to the method of a preferred embodiment of the present invention was at least as well preserved as tissue using standard cryogenic technology, and further that several types of tissue, most notably kidney and muscle (heart) demonstrated marked improvement in tissue integrity when frozen according to the method embodied by the present invention. FIG. 6 clearly indicates that the standard cryopreparative technique has numerous artifacts, such as "accordion folds" 605 seen within the heart muscle sample, as compared to the heart muscle sample which underwent the method as embodied by the present invention.

Refer now to FIG. 7, in which an electron microscope view illustrates the

complex ultrastructural features, such as cellular mitochondria 705, seen after application of the method according to a preferred embodiment of the present invention as compared to a control which was never frozen. The electron microscope views illustrated in FIG. 7 clearly show little if any difference between the tissues frozen by the
5 method according to a preferred embodiment of the present invention and control tissue which had never been frozen. Additionally, tissues frozen by the standard techniques of liquid nitrogen or mechanical freezing (not illustrated) exhibited significantly more damage upon examination than those of tissues frozen by the method according to a preferred embodiment of the present invention.

10 As stated earlier, a major problem with frozen sections created using the current technology is the loss of specific chemical reactions upon freezing. Loss of this activity renders these samples essentially useless for the more modern techniques of immunohistochemistry based upon antibody stain. An experiment which was conducted using a fluorescent labeled antibody (5.1H11, a human NCAM that is muscle specific)
15 demonstrated that primary porcine satellite cells which were previously stained for fluorescence with this antibody continued to fluoresce after freezing when prepared according to the method of a preferred embodiment of the present invention. However, cells frozen in liquid nitrogen failed to fluoresce after thaw. The results of this experiment indicate that the method of a preferred embodiment will allow the newer
20 techniques of cryopathology and immunohistochemistry to be applied in the areas of research and patient care.

Because the present invention can freeze biological material such that the material is vitrified, the formation of stress fractures in cellular membranes is minimized, and chemical activity within the cell is not lost after freezing, various embodiments of the
25 present invention may find application in other medical fields with proper chemical preparation, such as skin grafts, cornea storage, circulatory vessel storage, freezing of transplant tissues, and infertility treatment, as well as in the investigation of molecular

regeneration disease (cancer).

Although an embodiment of the present invention has been shown and described in detail herein, along with certain variants thereof, many other varied embodiments that incorporate the teachings of the invention may be easily constructed by those skilled in
5 the art. Accordingly, the present invention is not intended to be limited to the specific form set forth herein, but on the contrary, it is intended to cover such alternatives, modifications, and equivalents, as can be reasonably included within the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1 1. A method comprising:
2 freezing a biochemically active tissue sample, wherein freezing includes:
3 immersing the tissue sample in cooling fluid;
4 circulating the cooling fluid past the tissue sample at a substantially
5 constant predetermined velocity and temperature to freeze the tissue
6 sample such that the tissue sample is vitrified; and wherein
7 the tissue sample maintains its anatomical structure and remains
8 biochemically active after thaw;
9 thawing the tissue sample; and
10 examining the thawed tissue sample.

1 2. The method as in Claim 1, further comprising sectioning the tissue sample.

1 3. The method as in Claim 1, wherein examining the thawed tissue sample
2 includes histological examination.

1 4. The method as in Claim 1, wherein examining the thawed tissue sample
2 includes ultrastructural examination.

1 5. The method as in Claim 1, wherein examining includes the use of
2 immunohistochemistry examination.

1 6. The method as in Claim 5, wherein immunohistochemistry includes
2 fluorescent labeled antibody staining.

1 7. The method as in Claim 1, wherein more than about 55 percent of the tissue
2 sample exhibits no damage to cellular anatomical structure and remains
3 biochemically active after thaw.

1 8. The method as in Claim 1, wherein more than about 45 percent of the tissue
2 sample exhibits no damage to cellular anatomical structure and remains
3 biochemically active after thaw.

1 9. The method as in Claim 1, wherein more than about 85 percent of the tissue
2 sample maintains its anatomical structure and remains undamaged after thaw.

1 10. The method as in Claim 1, wherein the cooling fluid is maintained at a
2 temperature of between about -20 degrees centigrade and about -30 degrees
3 centigrade.

1 11. The method as in Claim 1, wherein the velocity of the cooling fluid past the
2 tissue sample is about 35 liters per minute per foot of cooling fluid through an
3 area not greater than about 24 inches wide and 48 inches deep.

1 12. The method as in Claim 1, wherein, the cooling fluid is circulated by a
2 motor/impeller assembly immersed in the cooling fluid.

1 13. The method as in Claim 1, further comprising circulating the cooling fluid past
2 a multi-path heat exchanging coil submersed in the cooling fluid, and wherein
3 the heat exchanging coil is capable of removing at least the same amount of
4 heat from the cooling fluid, as the cooling fluid removes from the tissue
5 sample.

1 14. A method for use in preparing a tissue sample for examination, the method
2 comprising:
3 immersing a biologically active tissue sample in cooling fluid; and
4 freezing the tissue sample directly to a temperature higher than about -30
5 degrees centigrade by circulating the cooling fluid past the tissue sample at
6 a substantially constant predetermined velocity and temperature such that
7 the tissue sample is vitrified, the tissue sample maintains its anatomical
8 structure, and the tissue sample remains biochemically active after thaw.

1 15. The method as in Claim 14, further comprising sectioning the tissue sample.

1 16. The method as in Claim 14, further comprising thawing the tissue sample.

1 17. The method as in Claim 14, wherein examination includes histological
2 examination.

1 18. The method as in Claim 14, wherein examination includes ultrastructural
2 examination.

1 19. The method as in Claim 14, wherein examination includes the use of
2 immunohistochemistry examination.

1 20. The method as in Claim 19, wherein immunohistochemistry includes
2 fluorescent labeled antibody staining.

1 21. The method as in Claim 14, wherein more than about 40 percent of the tissue
2 sample maintains its anatomical structure and remains biochemically active
3 after thaw.

1 22. The method as in Claim 14, wherein more than about 80 percent of the tissue
2 sample maintains its anatomical structure and remains biochemically active
3 after thaw.

1 23. The method as in Claim 14, wherein more than about 85 percent of the tissue
2 sample maintains its anatomical structure and remains undamaged after thaw.

1 24. The method as in Claim 14, wherein the cooling fluid is maintained at a
2 temperature of between about -20 degrees centigrade and about -30 degrees
3 centigrade.

1 25. The method as in Claim 14, wherein the velocity of the cooling fluid past the
2 tissue sample is about 35 liters per minute per foot of cooling fluid through an
3 area not greater than about 24 inches wide and 48 inches deep.

1 26. The method as in Claim 14, wherein, the cooling fluid is circulated by a
2 motor/impeller assembly immersed in the cooling fluid.

1 27. The method as in Claim 14, further comprising circulating the cooling fluid
2 past a multi-path heat exchanging coil submersed in the cooling fluid, and
3 wherein the heat exchanging coil is capable of removing at least the same
4 amount of heat from the cooling fluid, as the cooling fluid removes from the
5 tissue sample.

1 28. A system for use in preparing a tissue sample for examination, the system
2 comprising:
3 a cooling fluid reservoir configured to receive a biochemically active tissue
4 sample for immersion in cooling fluid;
5 one or more cooling fluid circulators configured to circulate said cooling fluid;
6 a heat exchanging coil for removing heat from said cooling fluid;
7 a refrigeration unit configured to remove heat from said heat exchanging coil;
8 and wherein
9 said cooling fluid reservoir, said one or more circulators, and said refrigeration
10 unit cooperate to freeze the tissue sample directly to a temperature higher
11 than about -30 degrees centigrade by circulating the cooling fluid past the
12 tissue sample at a substantially constant predetermined velocity and
13 temperature such that the tissue sample is vitrified, the tissue sample
14 maintains its anatomical structure, and the tissue sample remains
15 biochemically active after thaw.

1 29. The system as in Claim 28, wherein examination includes histological
2 examination.

1 30. The system as in Claim 28, wherein examination includes ultrastructural
2 examination.

1 31. The system as in Claim 28, wherein examination includes the use of
2 immunohistochemistry examination.

1 32. The system as in Claim 31, wherein immunohistochemistry includes
2 fluorescent labeled antibody staining.

1 33. The system as in Claim 28, wherein more than about 40 percent of the tissue

2 sample maintains its anatomical structure and remains biochemically active
3 after thaw.

1 34. The system as in Claim 28, wherein more than about 80 percent of the tissue
2 sample maintains its anatomical structure and remains biochemically active
3 after thaw.

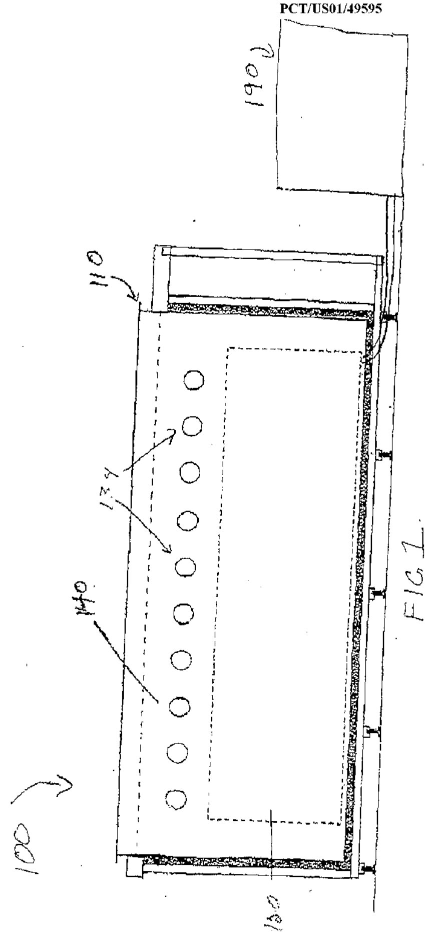
1 35. The system as in Claim 28, wherein more than about 85 percent of the tissue
2 sample maintains its anatomical structure and remains undamaged.

1 36. The system as in Claim 28, wherein the cooling fluid is maintained at a
2 temperature of between about -20 degrees centigrade and about -30 degrees
3 centigrade.

1 37. The system as in Claim 28, wherein the velocity of the cooling fluid past the
2 tissue sample is about 35 liters per minute per foot of cooling fluid through an
3 area not greater than about 24 inches wide and 48 inches deep.

1 38. The system as in Claim 28, wherein, the circulator is a motor/impeller
2 assembly immersed in the cooling fluid.

1 39. The system as in Claim 28, wherein the cooling fluid is circulated past a multi-
2 path heat exchanging coil submersed in the cooling fluid, and wherein the heat
3 exchanging coil is capable of removing at least the same amount of heat from
4 the cooling fluid, as the cooling fluid removes from the tissue sample.



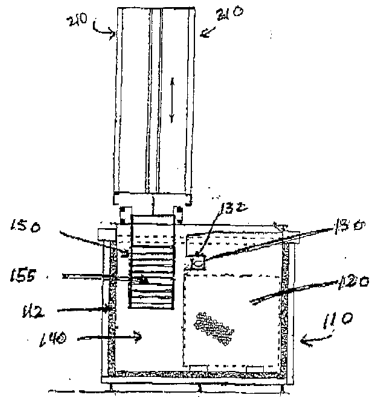


FIG. 2

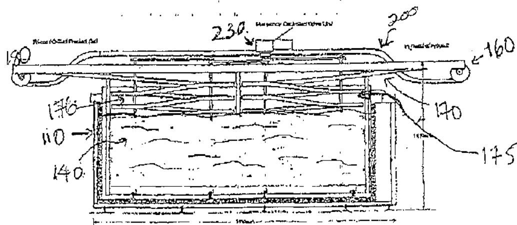


FIG. 2A

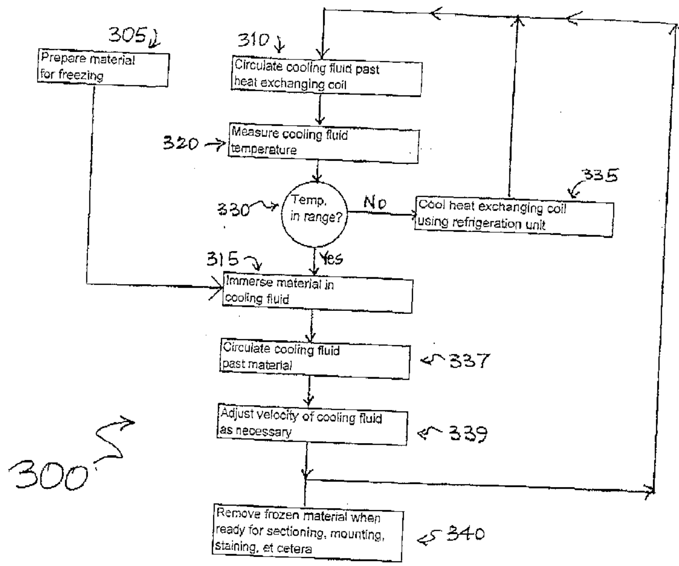


FIG. 3

FIG. 4. Cell Damage Following Freezing in Noncryoprotected Grape Tissue

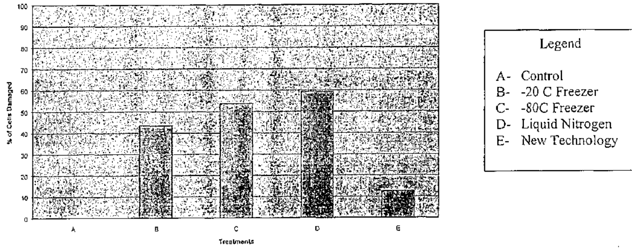
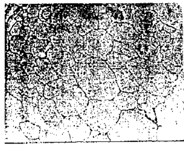


FIG. 5.

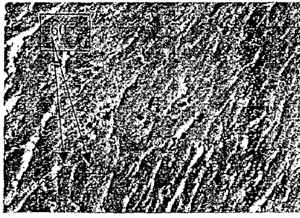


Control

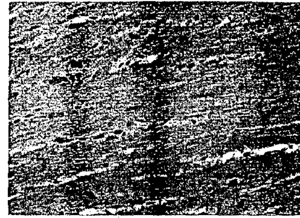
New Technology

Liquid Nitrogen

FIG. 6.

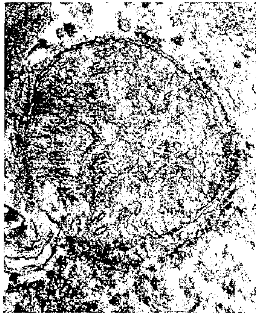


Standard Cryopreparative Technique

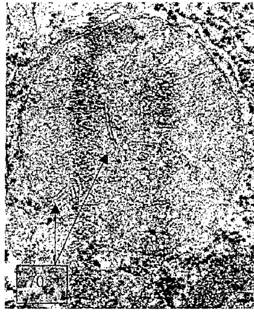


New System

FIG. 7.



Control, never frozen



New System

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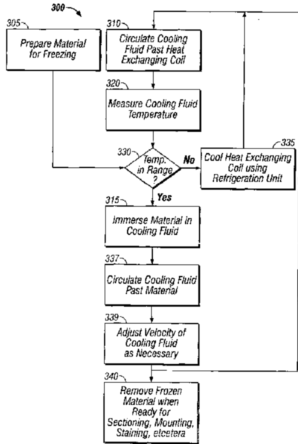
(54) Title: METHOD AND SYSTEM FOR PREPARING TISSUE SAMPLES FOR HISTOLOGICAL AND PATHOLOGICAL EXAMINATION



(57) **Abstract:** Viable biological material is cryogenically preserved (cryopreservation) by immersing the material in a tank of cooling fluid, and circulating the cooling fluid past the material at a substantially constant predetermined velocity and temperature to freeze the material. The material may either be directly plunged into the cooling fluid without preparation, or chemically prepared prior to freezing. A method according to the present invention freezes the biologic material quickly enough to avoid the formation of ice crystals within cell structures (vitrification) and allows the samples to maintain anatomical structure and remain biochemically active after thaw. The temperature of the cooling fluid is preferably between -20 degrees centigrade and -30 degrees centigrade, which is warm enough to minimize the formation of stress fractures and other artefacts in cell membranes due to thermal changes.

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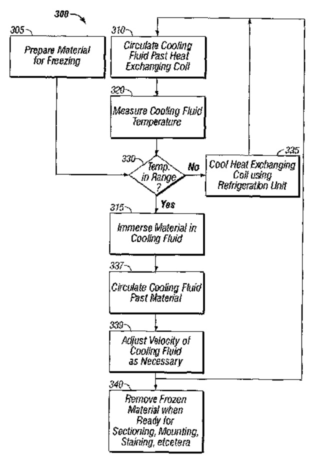

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METHOD AND SYSTEM FOR PREPARING TISSUE SAMPLES FOR HISTOLOGICAL AND PATHOLOGICAL EXAMINATION

CROSS REFERENCE TO RELATED APPLICATION

This application claims benefit under 35 U.S.C.§ 119 of the following United
5 States provisional patent application Serial Number 60/259,418, entitled "Method And System For Preparing Tissue Samples For Histological And Pathological Examination", which was filed on January 2, 2001.

FIELD OF THE INVENTION

The present invention relates generally to cryogenic preservation and more
10 particularly to a method of preserving for examination and diagnostic purposes.

BACKGROUND OF THE INVENTION

Biological materials such as tissues are subjected to various treatments in an histology laboratory to prepare specimens on slides for viewing under a microscope. Pathologists carefully examine the slides and report their findings, which aids physicians
15 in the diagnosis of disease or disease processes. Histopathology has traditionally relied upon examination of samples prepared by one of two basic methods. In the first histological method, samples undergo significant processing in the laboratory, such as fixation to preserve tissues, dehydration to remove water from tissues, infiltration with embedding agents such as paraffin, embedment, sectioning or cutting sections of the
20 tissue for placement on a slide, mounting the sections, and staining the sections to enhance details. The second method , cryogenic preparation, significantly reduces the processing of the first method in that it generally involves snap freezing in a cold liquid or environment, sectioning, mounting, and staining.

While the first method yields significantly superior visualization, it requires an extended period of time for processing, generally a minimum of 18 to 24 hours. Thus this method cannot be applied in situations where a rapid diagnosis of a pathologic process is required, such as during a surgical procedure. Additionally, the processing techniques employed may destroy all or part of the biological activity of the tissues.

The second method has the advantage of speed (30 minutes to 1 hour), however tissue specimens prepared using cryogenic preparation are often subject to cellular damage due to ice crystal formation, which can also cause the loss of biological function of molecules of interest within the tissues, and overall loss of tissue integrity manifested as degraded anatomical structure. Many commercial pathology laboratories discourage the use of frozen tissue for immunohistochemistry in all but special circumstances, because ice crystal formation in stored tissue causes many abnormal artifacts within the sample which make diagnostic interpretation quite difficult, or even impossible in some cases.

With the advent of poly- and then monoclonal antibodies, the focus of both traditional microscopic histology and pathology has shifted from simple subjective observation, to direct objective staining procedures. These newer immunohistochemistry (IHC) techniques help in determining diagnosis when histopathology alone proves inconclusive. However, IHC techniques are dependent on biologically intact receptors within the specimen for proper staining to occur. Therefore it is desirable to utilize a method of tissue specimen preparation that does not limit the amount of active biological material present after preparation is complete.

SUMMARY OF THE INVENTION

Therefore, what is needed is an improved way to cryogenically preserve viable single cells, tissues, organs, nucleic acids, or other biologically active molecules, that avoids at least some of the problems inherent in currently available methods. Accordingly, the present invention provides a method of cryopreservation for freezing a biochemically active tissue sample by immersing the sample in cooling fluid and circulating the cooling fluid past the material. The cooling fluid is circulated past the tissue sample at a substantially constant, predetermined velocity and temperature to freeze the tissue sample such that it is vitrified, yet the tissue sample maintains its anatomical structure and remains biochemically active after thaw. In at least one embodiment, the cooling fluid is maintained at a temperature of between about -20 degrees centigrade and -30 degrees centigrade, and the velocity of the cooling fluid past the tissue sample is about 35 liters per minute per foot of cooling fluid through an area not greater than about 24 inches wide and 48 inches deep. Additionally, at least one embodiment of the present invention immerses a biologically active tissue sample in cooling fluid to freeze the sample directly to a temperature higher than about -30 degrees centigrade. A further embodiment of the present invention provides for circulating the cooling fluid past a multi-path heat exchanging coil submersed in the cooling fluid, where the heat exchanging coil is capable of removing at least the same amount of heat from the cooling fluid as the cooling fluid removes from the tissue sample. At least one embodiment provides a system for implementing the above mentioned methods.

An object of at least one embodiment of the present invention is application of a method to freeze biological material wherein the formation of ice crystals and stress fractures is avoided, and cellular biochemical function is maintained after freezing.

An advantage of at least one embodiment of the present invention is that cryopreservation recovery rates are significantly increased, because biological material

is vitrified during freezing.

Another advantage of at least one embodiment of the present invention is that cryopreservation recovery rates are improved, because biological material is vitrified at a high enough temperature to avoid the formation of stress fractures within cell
5 membranes.

Another advantage of at least one embodiment of the present invention is that cryopreservation recovery rates are such that a considerably higher percentage of the biological material maintains its anatomical structure and remains biochemically active after thaw as compared to currently available methods.

10 An additional advantage of at least one embodiment of the present invention is that cryopreservation recovery rates are such that the biological material samples lend themselves to the application of sectioning, processing and subsequent histological, ultrastructural, and immunohistochemistry examination in shorter periods of time than traditional pathology techniques, thus shortening time to results.

15 A further advantage of at least one embodiment of the present invention is that once frozen, existing cryopreservation storage facilities and mechanisms can be used to store the frozen biological materials.

BRIEF DESCRIPTION OF THE DRAWINGS

Other objects, advantages, features and characteristics of the present invention, as well as methods, operation and functions of related elements of structure, and the combination of parts and economies of manufacture, will become apparent upon 5 consideration of the following description and claims with reference to the accompanying drawings, all of which form a part of this specification, wherein like reference numerals designate corresponding parts in the various figures, and wherein:

FIG. 1 is a side view of a chilling apparatus for practicing a method according to at least one embodiment of the present invention;

10 FIG. 2 is a cross sectional view of the chilling apparatus illustrated in FIG. 1 indicating implementation of cooling systems suitable for freezing relatively large quantities of biological material;

Fig. 2A is a cross sectional view of the chilling apparatus shown in FIG. 1, configured for use with a spiral conveyor according to one embodiment of the present 15 invention;

FIG. 3 is a flow diagram illustrating a system implemented according to at least one embodiment of the present invention;

FIG. 4 is a bar chart showing the results of experimental comparisons between various prior art freezing methods and a freezing method according to a preferred 20 embodiment of the present invention;

FIG. 5 illustrates views, as seen through a microscope, of the morphological appearance of noncryoprotected grape tissue following freeze-thaw cycles of the method

of liquid nitrogen and the freezing method according to a preferred embodiment of the present invention;

FIG. 6 illustrates views, as seen through a microscope, of the morphological appearance of heart tissue after freezing using standard cryopreparative techniques, and after application of the method according to a preferred embodiment of the present invention; and

FIG. 7 is an electron microscope view illustrating the complex ultrastructural features such as cellular mitochondria that may be seen after application of the method according to a preferred embodiment of the present invention.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT OF THE INVENTION

In the following detailed description of the preferred embodiments, reference is
5 made to the accompanying drawings which form a part hereof, and in which is shown by way of illustration specific preferred embodiments in which the invention may be practiced. These embodiments are described in sufficient detail to enable those skilled in the art to practice the invention, and it is to be understood that other embodiments may be utilized and that logical, mechanical, chemical and electrical changes may be made
10 without departing from the spirit or scope of the invention. To avoid detail not necessary to enable those skilled in the art to practice the invention, the description may omit certain information known to those skilled in the art. The following detailed description is, therefore, not to be taken in a limiting sense, and the scope of the present invention is defined only by the appended claims.

15 Referring first to FIGS. 1 and 2, a chilling apparatus suitable for practicing a method according to at least one embodiment of the present invention is discussed, and designated generally as cooling unit 100. Cooling unit 100 preferably comprises tank 110 containing cooling fluid 140. Submersed in cooling fluid 140 are circulators 134 such as motors 130 having impellers 132, heat exchanging coil 120, and rack 150, which in
20 one embodiment comprises trays 155 for supporting biological material to be frozen. Biological material may include, but is not limited to, viable single cells, tissues and organs, nucleic acids, and other biologically active molecules. Biological material is not required to be species-specific. External to tank 110, and coupled to heat exchanging coil 120, is refrigeration unit 190.

25 Tank 110 may be of any dimensions necessary to immerse biological material to be frozen in a volume of cooling fluid 140, in which dimensions are scaled multiples of 12 inches by 24 inches by 48 inches. Other tank sizes may be employed consistent with

the teachings set forth herein. For example, in one embodiment (not illustrated), tank 110 is sized to hold just enough cooling fluid 140, so containers such as vials, test tubes, beakers, graduated cylinders or the like, can be placed in tank 110 for rapid freezing of suspensions including biological materials and cryoprotectants. In other embodiments, tank 110 is large enough to completely immerse entire organs and or organisms for rapid freezing. It will be appreciated that tank 110 can be made larger or smaller as needed to efficiently accommodate various sizes and quantities of biological material to be frozen. The biological material may be treated with a cryoprotectant prior to being immersed in tank 110.

Tank 110 holds cooling fluid 140. In one embodiment, the cooling fluid is a food-grade solute. Good examples of food-grade quality fluids are those based on propylene glycol, sodium chloride solutions, or the like. In another embodiment, the cooling fluid is itself a cryoprotectant such as dimethylsulfoxide (DMSO), ethylene glycol, propylene glycol, polyethylene glycol or the like. Note that in some instances, the cryoprotectant is itself a food-grade quality fluid. In other embodiments, other fluids, and preferably solutes, are used as cooling fluids. While various containers may be used to hold the biological material, some embodiments of the present invention provide for the biological material to be directly immersed in the cooling fluid for rapid and effective freezing. Such direct immersion may simplify the cryopreservation of some tissues and organs.

In order to freeze biological material while avoiding the formation of ice crystals, one embodiment of the present invention circulates cooling fluid 140 past the biological material to be frozen, at a relatively constant rate of 35 liters per minute for every foot of cooling fluid contained in an area not more than about 24 inches wide by 48 inches deep. The necessary circulation is provided by one or more circulators 134, such as motors 130. In at least one embodiment of the present invention, submersed motors 130 drive impellers 132 to circulate cooling fluid 140 past biological material be to frozen. Other

circulators 134, including various pumps (not illustrated), can be employed consistent with the objects of the present invention. At least one embodiment of the present invention increases the area and volume through which cooling fluid is circulated by employing at least one circulator 134, in addition to motors 130. In embodiments using
5 multiple circulators 134, the area and volume of cooling fluid circulation are increased in direct proportion to each additional circulator employed. For example, in a preferred embodiment, one additional circulator is used for each foot of cooling fluid that is to be circulated through an area of not more than about 24 inches wide by 48 inches deep.

Preferably, motors 130 can be controlled to maintain a constant, predetermined
10 velocity of cooling fluid flow past the biological material to be preserved, while at the same time maintaining an even distribution of cooling fluid temperature within +/- 0.5 degrees centigrade at all points within tank 110. The substantially constant predetermined velocity of cooling fluid circulating past the biological material provides a constant, measured removal of heat, which allows for the vitrification of the biological
15 material during freezing. In one embodiment, cooling fluid properties such as viscosity, temperature, et cetera, are measured and processed, and control signals are sent to motors 130 to increase or decrease the rotational speed or torque of impellers 132 as needed. In other embodiments, motors 130 are constructed to maintain a given rotational velocity over a range of fluid conditions. In such a case, the torque or rotational speed of
20 impellers 132 imparted by motors 130 are not externally controlled. Of note is the fact that no external pumps, shafts, or pulleys are needed to implement a preferred embodiment of the present invention. Motors 130, or other circulators 134, are immersed directly in cooling fluid 140. As a result, cooling fluid 140 not only freezes biological material placed in tank 110, but cooling fluid 140 also provides cooling for motors 130.

25 Heat exchanging coil 120 is preferably a "multi-path coil," which allows refrigerant to travel through multiple paths (i.e., three or more paths), in contrast to conventional refrigeration coils in which refrigerant is generally restricted to one or two

continuous paths. In addition, the coil size is in direct relationship to the cross sectional area containing the measured amount of the cooling fluid 140. For example, in a preferred embodiment, tank 110 is one foot long, two feet deep, and four feet wide, and uses a heat exchanging coil 120 that is one foot by two feet. If the length of tank 110 is
5 increased to twenty feet, then the length of heat exchanging coil 120 is also increased to twenty feet. As a result, heat exchanging coil 120 can be made approximately fifty percent of the size of a conventional coil required to handle the same heat load. Circulators 134 such as motors 130, circulate chilled cooling fluid 140 over biological material to be frozen, and then transport warmer cooling fluid to heat exchanging coil
10 120, which is submersed in cooling fluid 140. In at least one embodiment, heat exchanging coil 120 is connected to refrigeration unit 190, which removes the heat from heat exchanging coil 120 and the system.

In a preferred embodiment, refrigeration unit 190 is designed to match the load requirement of heat exchanging coil 120, so that heat is removed from the system in a
15 balanced and efficient manner, resulting in the controlled, rapid freezing of a material. The efficiency of the refrigeration unit 190 is directly related to the method employed for controlling suction pressures by the efficient feeding or the heat exchange coil 120 and the efficient output of compressors used in refrigeration unit 190. This methodology requires very close tolerances to be maintained between the refrigerant and cooling fluid
20 140 temperatures, and between the condensing temperature and the ambient temperature. These temperature criteria, together with the design of the heat exchange coil 120, allow heat exchange coil 120 to be fed more efficiently, which in turn allows the compressor to be fed in a balanced and tightly controlled manner to achieve in excess of twenty five percent greater performance from the compressors than that which is accepted as the
25 compressor manufacturer's standard rating.

Note that in the embodiment illustrated in FIG. 1, refrigeration unit 190 is an external, remotely located refrigeration system. However, in another embodiment (not

illustrated), refrigeration unit 190 is incorporated into another section of tank 110. It will be appreciated that various configurations for refrigeration unit 190 may be more or less appropriate for certain configurations of cooling unit 100. For example, if tank 110 is extremely large, a separate refrigeration unit 190 may be desirable, while a portable embodiment may benefit from an integrated refrigeration unit 190. Such an integration is only made possible by the efficiencies achieved by implementing the principles as set forth herein, and particularly the use of a reduced-size heat exchanging coil.

By virtue of refrigeration unit 190 and heat exchanging coil 120, in a preferred embodiment, the cooling fluid is cooled to a temperature of between -20 degrees centigrade and -30 degrees centigrade, with a temperature differential throughout the cooling fluid of less than about +/- 0.5 degrees centigrade. In other embodiments, the cooling fluid is cooled to temperatures outside the -20 degrees centigrade to -30 degrees centigrade range in order to control the rate at which a substance is to be frozen. Other embodiments control the circulation rate of the cooling fluid to achieve desired freezing rates. Alternatively, the volume of cooling fluid may be changed in order to facilitate a particular freezing rate. It will be appreciated that various combinations of cooling fluid circulation rate, cooling fluid volume, and cooling fluid temperature can be used to achieve desired freezing rates.

Referring now to FIG. 2, a cross sectional view of the chilling apparatus illustrated in FIG. 1 indicating implementation of cooling systems suitable for freezing relatively large quantities of biological material; an embodiment of cooling system 100 suitable for freezing relatively large quantities of biological material is discussed. Reference numerals in FIG. 2 that are like, similar, or identical to reference numerals in FIG. 1 indicate like, similar, or identical features. Tank 110 contains cooling fluid 140, into which rack 150 may be lowered. Rack 150 is movably coupled to rack support 210, such that rack 150 may be raised or lowered to facilitate the placement of substances into tank 110.

In use, biological material to be frozen is placed in trays 155 of rack 150. Preferably, trays 155 are constructed of wire, mesh, or otherwise, so that cooling fluid 140 may freely circulate over, under, and/or around items placed thereon. Preferably, once the cooling fluid is chilled to a desired temperature, rack support 210 lowers rack 150 into tank 110, in order to submerge trays 155 in cooling fluid 140. Lowering rack 150 may be accomplished manually or using various gear, chain, and/or pulley configurations known to those skilled in the art. Circulators 134 circulate cooling fluid 140 across substances placed in trays 155 to provide quick and controlled freezing. It will be appreciated that other arrangements for immersing biological material into tank 110 may be employed, and that use of an automatic lowering system is not necessarily preferred for use in all circumstances.

Referring now to FIG. 2A, an embodiment of the present invention employing a multi-tiered spiral path conveyor system is discussed. As illustrated, spiral conveyor 200 may be configured to fit inside tank 110 in order to submerge biological material into cooling fluid 140. In use, once the cooling fluid is chilled to a desired temperature, materials to be frozen are fed into an input feed 160 where they are taken onto conveyor belt 170. The material travels from input feed 160, into the cooling fluid 140 on downward spiral 175, out of cooling fluid 140 on upward spiral 176, and out of spiral conveyor at output feed 180. As noted earlier, the cooling fluid 140 is preferably kept at a constant predetermined temperature, and circulated at a rate that ensures rapid, safe freezing of material to be frozen. The time the material spends submerged in cooling fluid 140 can be varied by adjusting the drive unit, 230, or by other suitable means. Ideally, the speed of conveyor belt 170, in combination with the temperature and circulation rate of cooling fluid 140, will be adjusted so that exactly the desired amount of heat will be removed from materials as they travel through tank 110 on the multi-tiered spiral path conveyor system 200.

Referring now to FIG. 3, a method according to one embodiment of the present

invention is illustrated, and designated generally by reference numeral 300. The illustrated method begins at step 310, where cooling fluid is circulated past a heat exchange coil. The heat exchange coil is operably coupled to a refrigeration system as discussed above, and is used to reduce the temperature of the cooling fluid as the cooling fluid is circulated past the heat exchange coil. In step 320, the temperature of the cooling fluid is measured, and the method proceeds to step 330, where it is determined whether the temperature of the cooling fluid is within an optimal temperature range. This optimal cooling fluid temperature range may be different for different applications, however a preferred optimal temperature range for many applications is between -20 degrees centigrade and -30 degrees centigrade.

Should the cooling fluid temperature be determined not to be within an optimal, predetermined temperature range, step 335 is performed. In step 335, the heat exchanging coil is cooled by a refrigeration unit, and the method returns to step 310, in which the cooling fluid is circulated past the heat exchange coil in order to lower the temperature of the cooling fluid. Preferably, steps 310, 320, 330, and 335 are performed continually until the cooling fluid reaches the optimal temperature range.

The temperature of the cooling fluid used to freeze the biological material is an important element of at least one embodiment of the present invention. In order to achieve vitrification using conventional processes, biological material is generally quenched in liquid nitrogen, at a temperature of -196 degrees centigrade. Such a drastic change in temperature over a very short period of time freezes water within cell structures so quickly that ice crystals do not have a chance to form. However, freezing biological material by quenching in liquid nitrogen can cause stress fractures in cellular membranes, thereby limiting the usefulness of quenching in liquid nitrogen for cryopreservation. Since the temperatures used in a preferred embodiment of the present invention are between -20 degrees centigrade and -30 degrees centigrade, stress fractures due to temperature changes are minimized, and vitrification can be achieved with far less

damage to cellular membranes.

While the cooling fluid is being cooled to the proper temperature, biological material to be frozen may be chemically prepared for freezing in step 305. It will be appreciated that materials to be used for pathology do not normally require chemical preparation, and foregoing step 305 by plunging materials to be frozen directly into a cooling fluid is consistent with the teachings set forth herein. As noted earlier, biological material includes, but is not limited to, viable single cells, tissues and organs, nucleic acids, and other biologically active molecules. The biological material does not have to be species specific. Chemically preparing the biological material may include pretreatment of the biological material with agents (stabilizers) that increase cellular viability by removing harmful substances secreted by the cells during growth or cell death. Useful stabilizers include those chemicals and chemical compounds, many of which are known to those skilled in the art, which sequester highly reactive and damaging molecules such as oxygen radicals.

Chemically preparing biological material may also include an acclimation step (not illustrated). During or at some time after pretreatment, the biological material to be preserved may be acclimated to a temperature which is reduced from culturing temperatures, but still above freezing. This may help prepare the biological material for the cryopreservation process by retarding cellular metabolism and reducing the shock of rapid temperature transition. Note well, however, than an acclimation step is not required in order to practice the present invention.

In a preferred embodiment, chemically preparing biological material for freezing includes loading the biological material with a cryoprotectant. Loading generally involves the equilibration of biological material in a solution of one or more cryoprotectants. Substances utilized during loading may be referred to as loading agents. Useful loading agents may include one or more dehydrating agents, permeating and non-

permeating agents, and osmotic agents. Both permeating agents such as DMSO and ethylene glycol, and a combination of permeating and non-permeating osmotic agents such as fructose, sucrose or glucose, and sorbitol, mannitol, or glycerol can be used. It will be appreciated that other suitable cryoprotectants may be employed consistent with
5 the objects of the present invention.

After the cooling fluid reaches a proper temperature, step 315 is performed, in which the chemically prepared biological material is immersed in cooling fluid. As noted earlier, the biological material may be held in a container, or placed directly into the cooling fluid. The method then proceeds to step 337, in which a circulator, such as a
10 submersed motor/impeller assembly or pump, is used to circulate the cooling fluid at the velocity previously discussed, past the immersed biological material. As the cooling fluid passes by the biological material, heat is removed from the material, which is at a higher temperature than the temperature of the cooling fluid, and is transferred to be cooling fluid, which transports the heat away from the biological material to be frozen.
15 According to at least one embodiment of the present invention, a substantially constant circulation of cooling fluid past the biological material to be frozen should be maintained in order to freeze the prepared biological material such that the prepared material is vitrified.

After the cooling fluid is circulated past the biological material to be frozen, step
20 339 is performed. Step 339 adjusts the velocity of the cooling fluid as necessary to account for changes in the cooling fluid viscosity, temperature, and the like. Preferably, the velocity of the cooling fluid is held constant by adjusting the force provided by one or more circulators. Once the biological material has reached the desired frozen state, it is removed as shown in step 340. After the material is removed from the cooling fluid
25 in step 340 by means previously discussed, it may be sectioned and thawed for histological, ultrastructural, and immunohistochemistry examinations, such as fluorescent labeled antibody staining.

The steps illustrated in FIG. 3 are shown and discussed in a sequential order. However, the illustrated method is of a nature wherein some or all of the steps are continuously performed, and may be performed in a different order. For example, at least one embodiment of the present invention uses a single circulating motor to circulate the cooling fluid. In such an embodiment, cooling fluid is circulated past a heat exchanging coil as in step 310, and past the biological material to be preserved in step 337 at the same time. In addition, one embodiment of the present invention measures cooling fluid temperatures, viscosities, and other fluid properties continually, and at multiple locations within the system.

In yet another embodiment, some properties of the cooling fluid are not directly measured. Rather, the change in cooling fluid properties is determined indirectly from the rotational speed of a circulation motor. If the motor is turning at a slower rate, then additional power can be supplied to the motor to return the motor to the desired rotational speed, thereby compensating for the change in cooling fluid properties. In at least one embodiment, a motor is configured to maintain a substantially constant rate of rotation. This substantially constant rate of rotation will result in a substantially constant rate of cooling fluid circulation.

A test of one embodiment of the present invention was performed in which five milliliters (5 ml) of water was frozen in a graduated container. Upon freezing, there was less than one percent increase in total volume, much less than expected with conventional freezing. In another test, ice was frozen in sheets in a conventional freezer, and in a cooling system according to a preferred method of the present invention. After freezing, the ice was examined under dark microscope. As expected, the conventional ice displayed a crystalline pattern, whereas the ice frozen according to the principles of the present invention exhibited no light displacement, indicating little to no ice crystal formation.

Refer now to FIG. 4, in which experimental results comparing various cryopreservation methods are compared. Bar graph 400 compares the number of individual cells damaged by use of four different cryopreservation methods B, C, D, and E against a control group A. No cryopreservation was performed on control group A, method B used a conventional freezer to freeze cells to a temperature of -20 degrees centigrade, method C used an ultralow freezer to freeze cells to a temperature of -80 degrees centigrade, method D used liquid nitrogen to freeze cells to a temperature of -196 degrees centigrade, and method E used a preferred embodiment of the present invention to freeze cells to a temperature of -25 degrees centigrade.

The results of the experiments, shown in bar graph 400, used plant tissue (seedless grapes) which were frozen by the conventional methods previously discussed, as well as by the method as embodied by the present invention, without any form of preparation or cryoprotectant. The frozen plant tissue was then thawed and thin sections were cut and examined, unstained, using phase-contrast microscopy. Plant tissue was employed in the experiments because gross distortion of the tissue by ice crystal formation or water expansion caused by freezing would disrupt the tissue's cell wall structure and could be readily observed. The results, as illustrated in FIG. 4, clearly show the superiority of the method performed according to a preferred embodiment of the present invention. As expected, the control, A, exhibited no cellular damage. Method B, the -20 C freezer, exhibited damage in approximately 45% of the cellular wall structures; method C, the -80 C freezer, exhibited damage in approximately 55% of the cellular wall structures; method D, liquid nitrogen, exhibited damage in approximately 59% of the cellular wall structures. However, the method performed according to a preferred embodiment of the present invention exhibited only about 12.5% cellular damage.

The superiority of the method performed according to a preferred embodiment is also seen in FIG. 5, which illustrates views, as seen through a phase-contrast microscope,

of the morphological appearance of noncryoprotected grape tissue following freeze-thaw cycles of the method of liquid nitrogen and the freezing method according to a preferred embodiment of the present invention. Note in FIG. 5 the altered form and structure of the tissue indicating cellular wall damage is seen to be considerably less in the freeze-thaw method performed according to a preferred embodiment than that seen in the view of tissue freeze-thaw cycled with a method using liquid nitrogen.

Referring now to FIG. 6, views, as seen through a microscope, of the morphological appearance of heart tissue after freezing using standard cryopreparative techniques, and after application of the method according to a preferred embodiment of the present invention is discussed. FIG. 6 illustrates the results of a different experiment which was performed on tissue samples collected post-mortem from mice and canine cadavers. Tissue samples were collected from five organ systems: ovarian, heart, liver, kidney, and lung. Tissues were prepared for conventional histology, cryo-sectioning, or ultrastructural examination using standard freezing techniques, and also following freezing by the method performed according to a preferred embodiment of the present invention. The resulting sections were then evaluated by a trained clinical pathologist. As expected, samples that were never frozen exhibited superior morphology upon histological evaluation. However, the pathologist report states that tissue frozen according to the method of a preferred embodiment of the present invention was at least as well preserved as tissue using standard cryogenic technology, and further that several types of tissue, most notably kidney and muscle (heart) demonstrated marked improvement in tissue integrity when frozen according to the method embodied by the present invention. FIG. 6 clearly indicates that the standard cryopreparative technique has numerous artifacts, such as "accordion folds" 605 seen within the heart muscle sample, as compared to the heart muscle sample which underwent the method as embodied by the present invention.

Refer now to FIG. 7, in which an electron microscope view illustrates the

complex ultrastructural features, such as cellular mitochondria 705, seen after application of the method according to a preferred embodiment of the present invention as compared to a control which was never frozen. The electron microscope views illustrated in FIG. 7 clearly show little if any difference between the tissues frozen by the
5 method according to a preferred embodiment of the present invention and control tissue which had never been frozen. Additionally, tissues frozen by the standard techniques of liquid nitrogen or mechanical freezing (not illustrated) exhibited significantly more damage upon examination than those of tissues frozen by the method according to a preferred embodiment of the present invention.

10 As stated earlier, a major problem with frozen sections created using the current technology is the loss of specific chemical reactions upon freezing. Loss of this activity renders these samples essentially useless for the more modern techniques of immunohistochemistry based upon antibody stain. An experiment which was conducted using a fluorescent labeled antibody (5.1H11, a human NCAM that is muscle specific)
15 demonstrated that primary porcine satellite cells which were previously stained for fluorescence with this antibody continued to fluoresce after freezing when prepared according to the method of a preferred embodiment of the present invention. However, cells frozen in liquid nitrogen failed to fluoresce after thaw. The results of this experiment indicate that the method of a preferred embodiment will allow the newer
20 techniques of cryopathology and immunohistochemistry to be applied in the areas of research and patient care.

Because the present invention can freeze biological material such that the material is vitrified, the formation of stress fractures in cellular membranes is minimized, and chemical activity within the cell is not lost after freezing, various embodiments of the
25 present invention may find application in other medical fields with proper chemical preparation, such as skin grafts, cornea storage, circulatory vessel storage, freezing of transplant tissues, and infertility treatment, as well as in the investigation of molecular

regeneration disease (cancer).

Although an embodiment of the present invention has been shown and described in detail herein, along with certain variants thereof, many other varied embodiments that incorporate the teachings of the invention may be easily constructed by those skilled in
5 the art. Accordingly, the present invention is not intended to be limited to the specific form set forth herein, but on the contrary, it is intended to cover such alternatives, modifications, and equivalents, as can be reasonably included within the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1 1. A method comprising:
2 freezing a biochemically active tissue sample, wherein freezing includes:
3 immersing the tissue sample in cooling fluid;
4 circulating the cooling fluid past the tissue sample at a substantially
5 constant predetermined velocity and temperature to freeze the tissue
6 sample such that the tissue sample is vitrified; and wherein
7 the tissue sample maintains its anatomical structure and remains
8 biochemically active after thaw;
9 thawing the tissue sample; and
10 examining the thawed tissue sample.

1 2. The method as in Claim 1, further comprising sectioning the tissue sample.

1 3. The method as in Claim 1, wherein examining the thawed tissue sample
2 includes histological examination.

1 4. The method as in Claim 1, wherein examining the thawed tissue sample
2 includes ultrastructural examination.

1 5. The method as in Claim 1, wherein examining includes the use of
2 immunohistochemistry examination.

1 6. The method as in Claim 5, wherein immunohistochemistry includes
2 fluorescent labeled antibody staining.

1 7. The method as in Claim 1, wherein more than about 55 percent of the tissue
2 sample exhibits no damage to cellular anatomical structure and remains
3 biochemically active after thaw.

1 8. The method as in Claim 1, wherein more than about 45 percent of the tissue
2 sample exhibits no damage to cellular anatomical structure and remains
3 biochemically active after thaw.

1 9. The method as in Claim 1, wherein more than about 85 percent of the tissue
2 sample maintains its anatomical structure and remains undamaged after thaw.

1 10. The method as in Claim 1, wherein the cooling fluid is maintained at a
2 temperature of between about -20 degrees centigrade and about -30 degrees
3 centigrade.

1 11. The method as in Claim 1, wherein the velocity of the cooling fluid past the
2 tissue sample is about 35 liters per minute per foot of cooling fluid through an
3 area not greater than about 24 inches wide and 48 inches deep.

1 12. The method as in Claim 1, wherein, the cooling fluid is circulated by a
2 motor/impeller assembly immersed in the cooling fluid.

1 13. The method as in Claim 1, further comprising circulating the cooling fluid past
2 a multi-path heat exchanging coil submersed in the cooling fluid, and wherein
3 the heat exchanging coil is capable of removing at least the same amount of
4 heat from the cooling fluid, as the cooling fluid removes from the tissue
5 sample.

1 14. A method for use in preparing a tissue sample for examination, the method
2 comprising:
3 immersing a biologically active tissue sample in cooling fluid; and
4 freezing the tissue sample directly to a temperature higher than about -30
5 degrees centigrade by circulating the cooling fluid past the tissue sample at
6 a substantially constant predetermined velocity and temperature such that
7 the tissue sample is vitrified, the tissue sample maintains its anatomical
8 structure, and the tissue sample remains biochemically active after thaw.

1 15. The method as in Claim 14, further comprising sectioning the tissue sample.

1 16. The method as in Claim 14, further comprising thawing the tissue sample.

1 17. The method as in Claim 14, wherein examination includes histological
2 examination.

1 18. The method as in Claim 14, wherein examination includes ultrastructural
2 examination.

1 19. The method as in Claim 14, wherein examination includes the use of
2 immunohistochemistry examination.

1 20. The method as in Claim 19, wherein immunohistochemistry includes
2 fluorescent labeled antibody staining.

1 21. The method as in Claim 14, wherein more than about 40 percent of the tissue
2 sample maintains its anatomical structure and remains biochemically active
3 after thaw.

1 22. The method as in Claim 14, wherein more than about 80 percent of the tissue
2 sample maintains its anatomical structure and remains biochemically active
3 after thaw.

1 23. The method as in Claim 14, wherein more than about 85 percent of the tissue
2 sample maintains its anatomical structure and remains undamaged after thaw.

1 24. The method as in Claim 14, wherein the cooling fluid is maintained at a
2 temperature of between about -20 degrees centigrade and about -30 degrees
3 centigrade.

1 25. The method as in Claim 14, wherein the velocity of the cooling fluid past the
2 tissue sample is about 35 liters per minute per foot of cooling fluid through an
3 area not greater than about 24 inches wide and 48 inches deep.

1 26. The method as in Claim 14, wherein, the cooling fluid is circulated by a
2 motor/impeller assembly immersed in the cooling fluid.

1 27. The method as in Claim 14, further comprising circulating the cooling fluid
2 past a multi-path heat exchanging coil submersed in the cooling fluid, and
3 wherein the heat exchanging coil is capable of removing at least the same
4 amount of heat from the cooling fluid, as the cooling fluid removes from the
5 tissue sample.

1 28. A system for use in preparing a tissue sample for examination, the system
2 comprising:
3 a cooling fluid reservoir configured to receive a biochemically active tissue
4 sample for immersion in cooling fluid;
5 one or more cooling fluid circulators configured to circulate said cooling fluid;
6 a heat exchanging coil for removing heat from said cooling fluid;
7 a refrigeration unit configured to remove heat from said heat exchanging coil;
8 and wherein
9 said cooling fluid reservoir, said one or more circulators, and said refrigeration
10 unit cooperate to freeze the tissue sample directly to a temperature higher
11 than about -30 degrees centigrade by circulating the cooling fluid past the
12 tissue sample at a substantially constant predetermined velocity and
13 temperature such that the tissue sample is vitrified, the tissue sample
14 maintains its anatomical structure, and the tissue sample remains
15 biochemically active after thaw.

1 29. The system as in Claim 28, wherein examination includes histological
2 examination.

1 30. The system as in Claim 28, wherein examination includes ultrastructural
2 examination.

1 31. The system as in Claim 28, wherein examination includes the use of
2 immunohistochemistry examination.

1 32. The system as in Claim 31, wherein immunohistochemistry includes
2 fluorescent labeled antibody staining.

1 33. The system as in Claim 28, wherein more than about 40 percent of the tissue

2 sample maintains its anatomical structure and remains biochemically active
3 after thaw.

1 34. The system as in Claim 28, wherein more than about 80 percent of the tissue
2 sample maintains its anatomical structure and remains biochemically active
3 after thaw.

1 35. The system as in Claim 28, wherein more than about 85 percent of the tissue
2 sample maintains its anatomical structure and remains undamaged.

1 36. The system as in Claim 28, wherein the cooling fluid is maintained at a
2 temperature of between about -20 degrees centigrade and about -30 degrees
3 centigrade.

1 37. The system as in Claim 28, wherein the velocity of the cooling fluid past the
2 tissue sample is about 35 liters per minute per foot of cooling fluid through an
3 area not greater than about 24 inches wide and 48 inches deep.

1 38. The system as in Claim 28, wherein, the circulator is a motor/impeller
2 assembly immersed in the cooling fluid.

1 39. The system as in Claim 28, wherein the cooling fluid is circulated past a multi-
2 path heat exchanging coil submersed in the cooling fluid, and wherein the heat
3 exchanging coil is capable of removing at least the same amount of heat from
4 the cooling fluid, as the cooling fluid removes from the tissue sample.

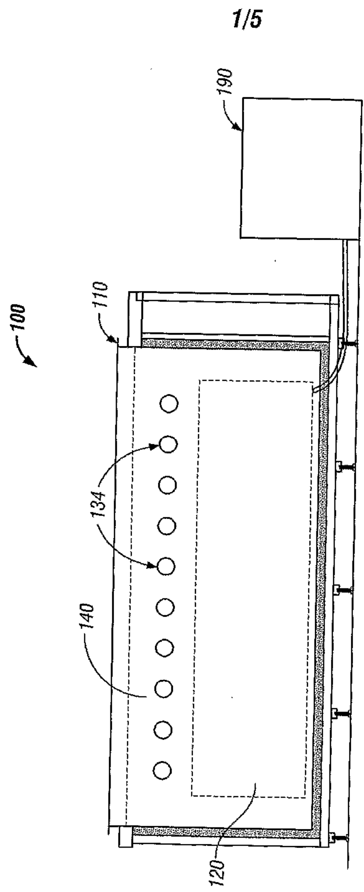

FIG. 1

2/5

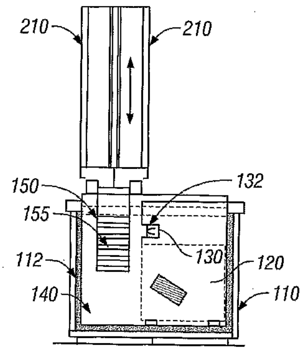

FIG. 2

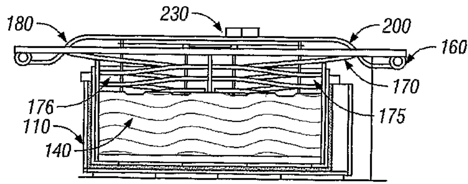

FIG. 2A

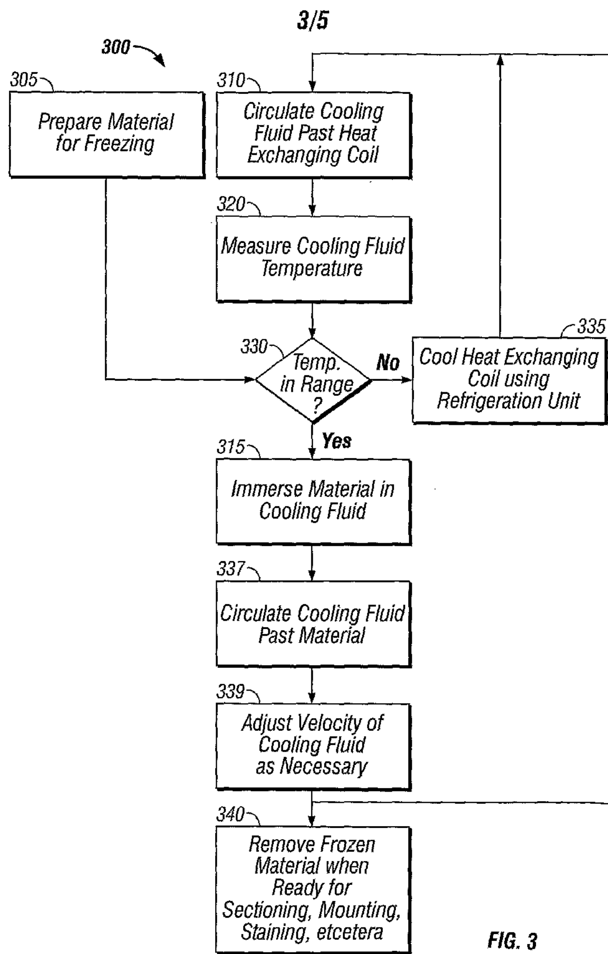

FIG. 3

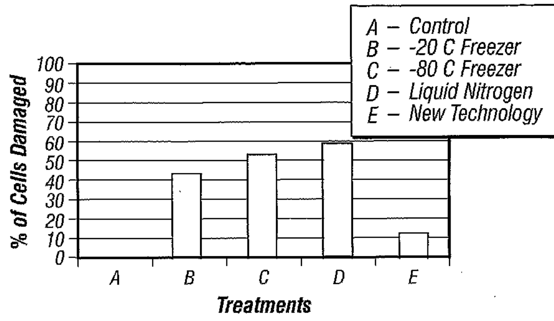

FIG. 4

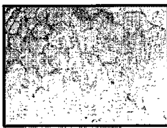

FIG. 5A

FIG. 5B

FIG. 5C

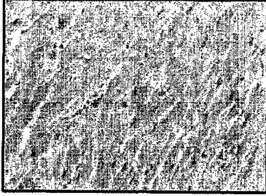

FIG. 6A

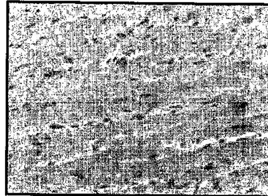

FIG. 6B

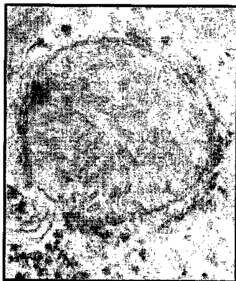

FIG. 7A

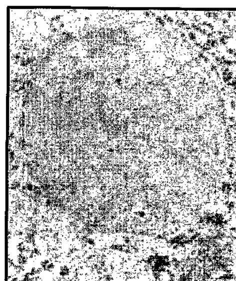

FIG. 7B

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/49595

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N1/42 A01N1/00 F25D17/02 F25D31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N A01N F25D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 01 95716 A (CASSELL ALLAN J ;WOOD BRIAN (AU); SUPACHILL INTERNAT PTY LTD (AU);) 20 December 2001 (2001-12-20) page 8, line 8 -page 11, line 8	1-39
X	US 5 191 773 A (CASSELL ALLAN J) 9 March 1993 (1993-03-09) column 3, line 9 - line 56; figures 1-3 column 4, line 44 - line 50; figure 6 column 5, line 65 -column 6, line 7	28
X	EP 0 275 114 A (MURRAY PIETER WYNAND LE ROUX) 20 July 1988 (1988-07-20) column 1, line 17 - line 30 column 5, line 30 - line 51; figure 1 column 6, line 14 - line 20; figure 1	1-9

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 January 2003	31/01/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hocquet, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/49595

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 146 418 A (REICHERT OPTISCHE WERKE AG) 17 April 1985 (1985-04-17) page 1, line 27 - line 30 page 1, line 76 - line 78 page 2, line 100 - line 108 page 3, line 16 - line 76; figures	1-9
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摘要(译)

通过将材料浸入冷却剂罐中并使冷却剂以基本恒定的预定速率循环通过材料以冷冻材料来冷冻保存活的生物材料。在冷冻之前，将材料直接浸入冷却剂中或在没有处理的情况下进行化学处理。本发明的方法中，冷冻的足够快的生物材料，以避免冰晶的细胞结构内形成，并保持其解剖结构有关样品，留下一个生化活性冷冻后。冷却剂的温度优选在-20°C和-30°C之间，这是足够温暖的温度，以最小化应力破裂的发生和由于热变化在细胞膜中形成伪影。与通过病理和组织技术中使用的其他冷冻保存冷冻的细胞相比，使用本发明的方法冷冻的细胞在细胞和细胞之间具有显着更小的损伤。由于本发明可以冷冻生物材料以使材料玻璃化，因此细胞中的生物化学活性在冷冻后不会消失，并且本发明的各种实施方案涉及研究和患者护理领域。，它可以用于制备生物材料的装置，用于冷组织学和免疫组织化学的新方法。